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Sex-chromosome evolution of Palearctic tree frogs in space and time

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Sex-chromosome evolution of Palearctic tree frogs in space and time

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Prof. Vladimir Katanaev

TABLE OF CONTENTS

SUMMARY

Sexual reproduction is nearly universal in eukaryotes and genetic determination of sex prevails among animals. The astonishing diversity of sex-determining systems and sex chromosomes is yet bewildering. Some taxonomic groups possess conserved and dimorphic sex chromosomes, involving a functional copy (e.g. mammals' X, birds' Z) and a degenerated copy (mammals' Y, birds' W), implying that sex-chromosomes are expected to decay. In contrast, others like amphibians, reptiles and fishes yet maintained undifferentiated sex chromosomes. Why such different evolutionary trajectories? In this thesis, we empirically test and characterize the main hypotheses proposed to prevent the genetic decay of sex chromosomes, namely occasional X-Y recombination and frequent sex-chromosome transitions, using the Palearctic radiation of *Hyla* tree frogs as a model system. We take a phylogeographic and phylogenetic approach to relate sex-chromosome recombination, differentiation, and transitions in a spatial and temporal framework. By reconstructing the recent evolutionary history of the widespread European tree frog *H. arborea*, we showed that sex chromosomes can recombine in males, preventing their differentiation, a situation that potentially evolves rapidly. At the scale of the entire radiation, X-Y recombination combines with frequent transitions to prevent sex-chromosome degeneration in *Hyla*: we traced several turnovers of sex-determining system within the last 10My. These rapid changes seem less random than usually assumed: we gathered evidences that one chromosome pair is a sex expert, carrying genes with key role in animal sex determination, and which probably specialized through frequent reuse as a sex chromosome in *Hyla* and other amphibians. Finally, we took advantage of secondary contact zones between closely-related *Hyla* lineages to evaluate the consequences of sex chromosome homomorphy on the genetics of speciation. In comparison with other systems, the evolution of sex chromosomes in *Hyla* emphasized the existence of consistent evolutionary patterns within the chaotic diversity of flexibility of cold-blooded vertebrates' sex-determining systems, and provides insights into the evolution of recombination. Beyond sex-chromosome evolution, this work also significantly contributed to speciation, phylogeography and applied conservation research.

RÉSUMÉ

La reproduction sexuée est quasi-universelle chez les eucaryotes et le sexe est le plus souvent déterminé génétiquement au sein du règne animal. L'incroyable diversité des systèmes de reproduction et des chromosomes sexuels est particulièrement étonnante. Certains groupes taxonomiques possèdent des chromosomes sexuels dimorphiques et très conservés, avec une copie entièrement fonctionnelle (ex : le X des mammifères, le Z des oiseaux) et une copie dégénérée (ex : le Y des mammifères, le W des oiseaux), suggérant que les chromosomes sexuels sont voués à se détériorer. Cependant les chromosomes sexuels d'autres groupes tels que les amphibiens, les reptiles et les poissons sont pour la plupart indifférenciés. Comment expliquer des trajectoires évolutives si différentes? Au cours de cette thèse, nous avons étudié empiriquement les processus évolutifs pouvant maintenir les chromosomes sexuels intacts, à savoir la recombinaison X-Y occasionnel ainsi que les substitutions fréquentes de chromosomes sexuels, en utilisant les rainettes Paléarctiques du genre *Hyla* comme modèle d'étude. Nous avons adopté une approche phylogéographique et phylogénétique pour appréhender les événements de recombinaison, de différenciation et de transitions de chromosomes sexuels dans un contexte spatio-temporel. En retraçant l'histoire évolutive récente de la rainette verte *H. arborea*, nous avons mis en évidence que les chromosomes sexuels pouvaient recombiner chez les mâles, empêchant ainsi leur différenciation, et que ce processus avait le potentiel d'évoluer très rapidement. A l'échelle plus globale de la radiation, il apparait que les phénomènes de recombinaison X-Y soient également accompagnés de substitutions de chromosomes sexuels, et participent de concert au maintien de chromosomes sexuels intacts dans les populations: le système de détermination du sexe des rainettes a changé plusieurs fois au cours des 10 derniers millions d'années. Ces transitions fréquentes ne semblent pas aléatoires: nous avons identifié une paire de chromosomes qui présente des caractéristiques présageant d'une spécialisation dans le déterminisme du sexe (notamment car elle possède des gènes importants pour cette fonction), et qui a été réutilisée plusieurs fois comme tel chez les rainettes ainsi que d'autres amphibiens. Enfin, nous avons étudié l'hybridation entre différentes espèces dans leurs zones de contact, afin d'évaluer si l'absence de différenciation entre X et Y jouaient un rôle dans les processus génétiques de spéciation. Outre son intérêt pour la compréhension de l'évolution des chromosomes sexuels, ce travail contribue de manière significative à d'autres domaines de recherche tels que la spéciation, la phylogéographie, ainsi que la biologie de la conservation.

INTRODUCTION

Sexual reproduction is one of the best success-story of evolution, being nearly universal among eukaryotes. By mixing genomes from different individuals, through meiosis and fusion of gametes, sex allows to form new beneficial genetic combinations, boosting the evolutionary potential of organisms (Otto 2009). Despite its complexity, meiotic sex has a very early origin and was already practiced in some of the most basal lineages, at least one billion years ago (Ramesh et al. 2005). In multicellular organisms, sexual reproduction has led to the evolution of separate sexual functions, carried by separate sexes (i.e. male and female) or simultaneously by single individuals (i.e. hermaphrodites). The developmental pathways governing sexual differentiation are remarkably conserved among lineages (Raymond et al. 1998, Schartl 2004, Graves & Peichel 2010), but can be initiated in a diversity of ways, involving genetic and environmental factors (reviewed by Charlesworth 1996, Bachtrog et al. 2014, Beukeboom & Perrin 2014). Genetic sex determination (GSD), which is prevalent in eukaryotes, usually involves sex chromosomes with male (XY, as in mammals) or female (ZW, as in birds) heterogamety, multiple factors (e.g. XYZW, as in some fishes), as well as others mechanisms, like haplo-diploidy (unfertilized haploid eggs develop as males and fertilized diploid eggs develop as females, as in hymenopters and most acarids) or elimination of the paternal genome (as in most scale insects). In parallel, environmental sex-determination (ESD) is frequent in some groups (e. g. fishes, reptiles), where sexual development of the embryo can depend on temperature, but also photoperiod, growing conditions and social context (Beukeboom & Perrin 2014). Environmental and genetic factors may interact to determine sex in an additive way, as both contribute to reach (or not) an hormonal threshold, fating the sex of the individual in a dose-sensitive fashion (Bulmer & Bull 1982).

CLASSICAL MODEL OF SEX-CHROMOSOME EVOLUTION

The prevalence of sex-chromosomes across the tree of sex has attracted evolutionary biologists early on, and the evolution of sex chromosomes has received much interest over the last century. Research on model organisms, including mammals, birds, and *Drosophila* laid the ground work to characterize the main evolutionary steps (reviewed by Charlesworth & Charlesworth 2000). Müller (1914) first proposed that the human X and Y evolved from an homologous pair of autosomes. Decades later, comparative genomics confirmed his theory, revealing that the sex chromosomes of some lineages have autosomal orthologues in others. For instance, the chicken Z chromosome largely maps to human chromosomes 5, 9 and 18 (Nanda et al. 2002), and the X-added region of therian mammals is homologous to chicken chromosomes 1 and 4 (Kohn et al. 2004). Earlier on, comparative cytogenetic analyses across families of snakes reached similar conclusions (Ohno 1967).

A first step in the evolution of sex chromosome from an autosome is taken with the appearance of a sex-determination function (SD) by the mutation, translocation, or duplication of gene(s) involved in the sex-determining pathway, giving rise to proto-sex chromosomes (Rice 1996; Figure 1a). Note that two linked functions are required when evolving from hermaphrodism or ESD, one silencing male fertility and one silencing female fertility (Charlesworth 1996, Bachtrog et al. 2011). Theory predicts that recombination at the sex-determining region will quickly be selected against in the heterogametic sex, in order to maintain strong linkage between co-adapted alleles of different genes involved in sex-determination and differentiation, and neighboring sex-antagonistic (SA) loci (Bull 1983, Rice 1987, 1996; Figure 1b). For instance, in XY systems this allows male- and female-beneficial alleles to be exclusively inherited by sons and daughters respectively. The process is self-reinforcing: tight linkage will favor the accumulation of new SA genes, which will in turn select for even tighter linkage (Rice 1987).

However, this arrest of recombination has far-reaching consequences on the evolutionary faith of the non-recombining gametolog (thereafter Y). First, by being isolated from the X and carried by only one sex (male), the Y endures a drastic drop of its effective size, down to ¼ of an autosome (i.e. for four copies of autosomes, there are three copies of X and one copy of Y in natural populations). Müller conceptualized this enhanced drift as a ratchet, the diversity of the Y being irremediably shrunk down click after click (Müller 1914). Second, because it acts on the entire chromosome as a whole, selection becomes inefficient on the Y. Due to linkage disequilibrium, slightly deleterious alleles segregate along with advantageous combinations at SA genes.

Reciprocally, selection against "bad" SA combinations causes the loss of "good" alleles at other loci. These selective sweeps, background selection and Müller's ratchet combine to drive the non-recombining segment into degeneration, by fixing deleterious mutations and repetitive elements and eventually causing the loss of gene functions (Charlesworth & Charlesworth 2000; Figure 1c).

At the same time, chromosomal regions at the border of the non-recombining segment become hotspots for the accumulation of new SA genes (Rice 1987). Like a chain reaction, the non-recombining segment will thus tend to expand to these neighboring regions (Figure 1c). Such successive events of recombination arrest recruit new chromosomal portions into the non-recombining segment, yielding evolutionary strata i.e. discrete genomic regions differing by stepwise increased amount of divergence. For instance, comparing X and Y sequence divergences allowed to identified four evolutionary strata in the mammalian sex chromosomes (Lahn & Page 1999, Hughes et al. 2012), and similar patterns were found in other XY and ZW systems (e.g. Handley et al. 2004, Nicolas et al. 2005).

Without resuming X-Y recombination (see next section), the genetic decay of the non-recombining gametolog may become irreversible. Structural changes through accumulation of repetitive sequences, transposons as well as inversions and large deletions dramatically alter the Y chromosome, and ultimately lead to sex-chromosome heteromorphy (Charlesworth & Charlesworth 2000, Figure1d). At this stage, X and Y are not anymore homologous and X-Y recombination is then physically impossible, even in sex-reversed females. The process can go even further and the Y was actually lost in several lineages, forming XX/X0 systems (Waters et al. 2007).

This widely accepted model accounts for the high sex-chromosome differentiation found in mammals, birds, as well as some insects (e.g. *Drosophila*; Bachtrog 2004, Kaiser & Bachtrog 2010) and plants (e.g. *Silena*, Bergero & Charlesworth 2011). In mammals, ~170 My of evolution since the appearance of the masculinizing gene *SRY* has led the Y chromosome to be highly-deprecated, largely heterochromatic and harboring less than 50 functional genes (>1500 on the X) (Livernois et al. 2012). Similar features are shared by the female-specific degenerated W of birds (Handley et al. 2004). Note that to accommodate for such male-female dimorphism, these groups have evolved dosage compensation: in mammals this is done by female X-inactivation, whereas in *Drosophila* the males (XY) double the expression of their X chromosome; in birds sex-linked gene expression is equalized by subtle gene-by-gene regulations (Mank 2009). The evolution of dosage compensation may actually accelerate the degeneration process by further relaxing selection on the Y (Charlesworth 1978, Engelstädter 2008).

More generally, this model has emphasized the dramatic view that sex chromosomes are "born to be destroyed" (Steinmann & Steinmann 2005). The speed of degeneration, however, might greatly vary depending on organisms. In plants, genetic decay may be slower than in animals because purifying selection is still efficient during the haploid gametic phase, i.e. pollen (Chibilana & Filatov 2011). In mammals, the faith of the male Y is under much debate. It is predicted to ultimately disappear (Graves 2005, 2006; as already in some rodents), but seems to have remained stable over

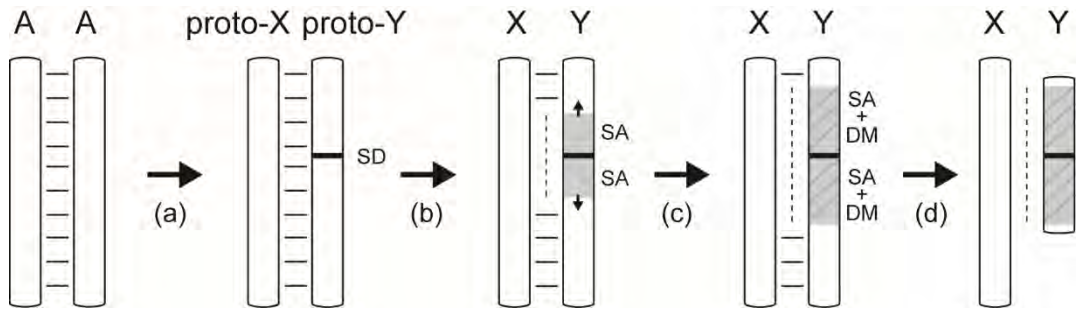

FIGURE 1: Classical model of sex chromosome evolution leading to sex chromosome heteromorphy (Charlesworth & Charlesworth 2000). SD: sex-determining locus. SA: sex-antagonistic loci, DM: deleterious mutations. Lines indicate X-Y recombination (horizontal) or recombination arrest (vertical). Steps are described in the text.

the past 25 My, most likely as it retained housekeeping genes with pleiotropic effects on maleness (Bellot et al. 2014, Cortez et al. 2014).

ALTERNATIVE MODELS AND IMPLICATIONS

The above model accounts very well for the extremely conserved mammalian and avian sex-determining systems. All therian mammals share the same ancestral pair of sex chromosomes, nowadays heteromorphic, harboring the sex-determining gene *SRY* (Livernois et al. 2012). In birds, sex is universally controlled by the expression level of the Z-linked *DMRT1*, present twice in ZZ males and once in ZW females (Smith et al. 2009). Birds ZW chromosomes are also widely heteromorphic (Handley et al. 2004), although undifferentiated sex chromosomes are found in a few basal species (ratites, e.g. emu), which evolved sex-antagonistic functions through sex-biased gene expression rather than sex-linked mutations (Vicoso et al. 2013), a rare mechanism considered difficult to evolve (Rice 1996).

In striking contrast, other groups of vertebrates feature an astonishing diversity of sex-determining systems and mostly undifferentiated sex chromosomes, challenging the classical model. In amphibians, reptiles and fishes, sex can be determined by one or several genes/chromosomes, involving male or female heterogamety (XY, ZW), by the environment (especially temperature) or interactions between different systems. Sex chromosome differentiation is very rare: in amphibians, less than 4% of examined species show heteromorphic sex chromosomes (Schmid et al. 1991, Eggert 2004); in fish, it is about 10% (Devlin & Nagahama 2002); it is slightly higher in reptiles, for instance 20% in lizards (Ezaz et al. 2009a). As a consequence, these sex chromosomes are often interpreted as primitive, i.e. on the first steps of differentiation, but it is rather unlikely that all cold-blooded vertebrates recently evolved new GSD systems. Given that this prevalent homomorphy does not fit the predictions made for sex chromosome evolution, deciphering the alternative mechanisms involved has become an attractive question. As described below the last decade of research has seen the conceptualization of two major hypotheses. The main idea of this thesis was to test and characterize these models empirically in natural populations.

TURNOVERS OF SEX CHROMOSOMES

As noted by Schartl (2004), transitions of sex-determining systems are frequent in groups with homomorphic sex chromosomes. In cold-blooded vertebrates, different pairs of sex-chromosomes can be found between different lineages (Tanaka et al. 2007, Volff et al. 2007, Ezaz et al. 2009b, Mank & Avise 2009, Ross et al. 2009, Kitano & Peichel 2011, Kikuchi & Hamaguchi 2013, Malcom et al. 2014), and even between different populations of the same species (Miura 2007). For instance, Hillis & Green (1990) identified at least seven heterogametic transitions (i.e. from XY to ZW and vice versa) during amphibian evolutionary history. Such turnovers may thus happen frequently enough so no degenerated chromosome pair are observed (Figure 2). As illustrated by Volff et al. (2007), in these groups SD genes can thus be viewed as "ephemeral dictators" enduring frequent

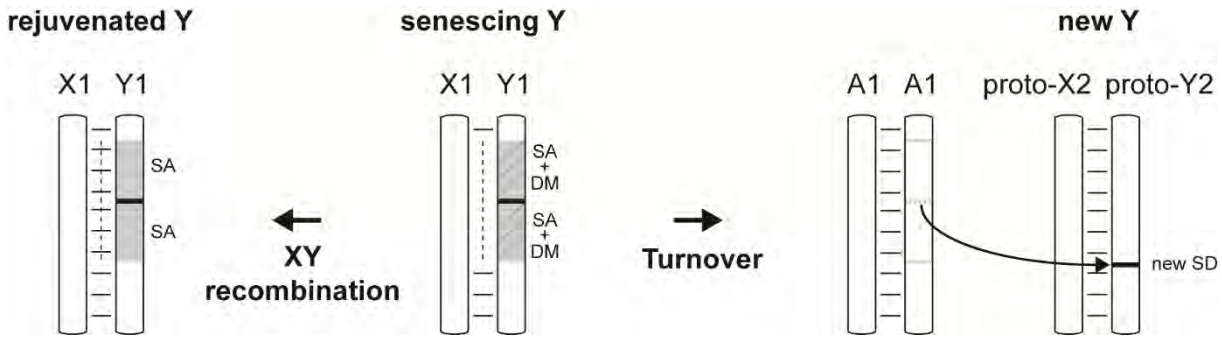

FIGURE 2: Alternative mechanisms preventing sex chromosome degeneration: occasional X-Y recombination (left) and transition towards another chromosome pair (right).

"evolutionary putsches". These putsches occur as the SD gene is translocated or overridden by a new SD on a different chromosome pair.

Theoretical work has delineated several potential causes to these transitions. Strong sex-antagonistic selection on an autosomal gene can favor the spread of a new SD gene linked to it (van Doorn & Kirkpatrick 2007), and causing heterogametic transitions (van Doorn & Kirkpatrick 2010). Sex chromosome turnovers may also be triggered by the genetic load of deleterious mutations accumulated on the non-recombining Y (Blaser et al. 2013). In non-purely GSD systems where extreme environmental conditions may skew sex-ratios in populations, sex-ratio selection can set off new pairs of sex chromosomes reestablishing even male-female proportions (Grossen et al. 2011).

Moreover, turnovers may be biased towards a limited set of chromosome pairs that carry important genes with upstream functions in the sex-determining cascade (Graves & Peichel 2010, O'Meally et al. 2012). For instance, only five chromosomes out of 13 are co-opted as sex chromosomes among 17 species/populations of Ranid frogs (Miura 2007). This pattern extends to single genes: the transcription co-factor *DMRT1*, which determines sex in birds (Smith et al. 2009), has orthologues and paralogues with key roles for sex-differentiation in a variety of organisms (reviewed by Matson & Zarkower 2012), including dipters (*doublesex* in *Drosophila*), nematods (*mab3* in *Caenorhabditis elegans*), fishes (*DM-Y* in *Oryzias latipes*) and amphibians (*DM-W* in *Xenopus laevis*).

Although turnovers received clear supports from several systems, what we know regarding their dynamics and underlying causes mostly stem from theoretical work. Here we address the matter empirically in amphibians. In this context of rapid change, we outline homologies of sex-chromosomes (**Appendix**) and sex-determining genes (**Chapter V**), and estimate the timing and potential bias of turnovers in a well-resolved radiation (**Chapter VI**).

THE EVOLUTION OF X-Y RECOMBINATION

Even if turnovers are indeed frequent in some groups, it is highly unlikely that they solely account for the integrity of sex chromosomes among all animals and plants. Instead of replacing senescing Y chromosomes by new chromosome pairs, another alternative is to maintain non-zero rates of X-Y recombination at the same ancestral pair, enabling to purge and rejuvenate the Y (Figure 2). This implies that young sex chromosomes may often harbor old sex-determining genes (i.e. "old wine in a new bottle", Perrin 2009). In theory, such amount of X-Y recombination should be a balance between the positive effects of sex-antagonistic genes (which prevent recombination) *versus* the negative effects of accumulating deleterious mutations (which favor some recombination), and extremely low rates seem sufficient to maintain this equilibrium (Grossen et al. 2012). Note that other forms of selection, involving overdominance in males may also promote recombination between sex chromosome (Otto 2014).

Perrin (2009) proposed that ectothermic species like cold-blooded vertebrates could generate X-Y recombinants via rare sex-reversal events (the "fountain-of-youth" hypothesis), based on two grounds: the fact that environmental (e.g. temperature-induced) sex-reversal is possible in these species, and assuming that recombination rates depends on phenotypic rather than genotypic sex (Matsuba et al. 2010). Then, an XY individual, developing as a female due to extreme environmental conditions might recombine as a regular XX female and yield new Y haplotypes. At this point, natural selection can favor the spread of the fittest new Ys in populations, i.e. those free of deleterious mutations and that retained advantageous sex-antagonistic combinations. Instead of sex-reversal, X-Y recombination might also be obtained by occasional male recombination, which however involve different proximate mechanism and dynamics i.e. continuously low rates (opposed to rare bursts of recombination). This largely unexplored hypothesis is receiving increasing support (Stöck et al. 2011, 2013a; this thesis), raising interesting theoretical and empirical perspectives regarding the evolution of recombination and sex-antagonistic genes, as well as opportunities to map sex-determining loci by screening Y genomic regions protected from recombination, as done in **Chapter V**.

A large part of the work presented here is dedicated to understand how evolutionary stable rates of X-Y recombination evolve in the wild, notably by dissecting X-Y recombination variation between (**Chapter III**) and within populations (**Chapter VII**), as well as their consequences for the dynamics of sex-chromosome differentiation (**Chapter II** and **III**). Finally, we address how both sex-chromosome recombination and turnovers may co-occur and together contribute to the prevalence of homomorphic sex chromosomes in a species group (**Chapter VI**).

HOMOMORPHIC SEX CHROMOSOMES AND SPECIATION

The lack of differentiation at sex chromosomes can have important implications for the genetics of speciation. Sex chromosomes take a core place in reproductive, especially post-zygotic isolation

between incipient species (Qvarnström & Bailey 2009). It is best illustrated by hybrid zone analyses in several mammalian and avian systems, where sex-linked loci struggle to pass interspecific reproductive barriers (e.g. Payseur et al. 2004, Carling & Brumfield 2008).

As recently reviewed (Pesgraves 2008, Qvarnström & Bailey 2009, Schilthuizen et al. 2011), several mechanisms prevail to account for this pattern. First, assuming degeneration of the Y, incompatibilities in hybrid genomes, which usually involve partly recessive alleles, should cause more problems when X-linked than autosomal, because of hemizygous exposure in the heterogametic sex (the "dominance" theory, Müller 1940, Turelli & Orr 1995). By increasing the efficiency of purifying selection, dominance effects are also expected to accelerate genetic changes on X chromosomes, which may thus accumulate more divergence than the rest of the genome (the faster-X effect, Charlesworth et al. 1987). Faster evolution of male-expressed genes (e.g. through sexual selection) is expected to have an effect on sex-specific hybrid fitness, but it is unclear whether these genes non-randomly build up on sex chromosomes (Wu & Davies 1993, Wu et al. 1996). Sex-chromosomes may also be affected by incompatibilities between X and Y alleles, X-Y epistatis being important for proper meiotic segregation of sex chromosomes (McDermott & Noor 2010) and differentiation of the heterogametic sex. Finally, sex chromosomes may be involved in other types of reproductive barriers, like pre-mating isolation (Saetre et al. 2003, Saether et al. 2007, Pryke 2010), also it remains controversial (Qvarnström & Bailey 2009). These different mechanisms are reflected by two main empirical observations of sex-specific hybrid fitness, namely Haldane's rule (lower hybrid fitness for the heterogametic sex, Haldane's 1922, Schilthuizen et al. 2011) and the large-X effect (disproportional effect of X-chromosomes on hybrid sterility, Turelli & Moyle 2007, Masly & Pesgraves 2007).

Evidence from these processes almost exclusively arise from species with dimorphic sex chromosomes, where sex-linked dominance effects confound with other mechanisms. In species with undifferentiated sex chromosomes, however, the context is different as the X is not hemizygous and dominance effects should be negligible. These organisms thus offer opportunities to test for the role of sex-chromosome differentiation in reproductive isolation, and gauge the relative contributions of the different mechanisms, without the confounding effects of dominance. We perform such study in **Chapter X**, contrasting sex-linked *versus* autosomal introgression patterns across hybrid zones of tree frogs, a first of this kind in an animal system with homomorphic sex chromosomes.

PHYLOGEOGRAPHIC VIEW OF SEX-CHROMOSOME EVOLUTION

Multi-level molecular ecology surveys of individuals, populations and species are powerful ways to make sense of patterns of sex-chromosome differentiation and their underlying mechanisms in a natural context, but require well-resolved phylogenetic and phylogeographic frameworks. Biogeographic processes obviously overlay with intrinsic sex chromosome dynamics to shape patterns of divergences and diversity at sex-linked markers.

In the northern hemisphere, the climatic oscillations experienced by our planet over the last few million years induced Quaternary glaciations, a series of long cold and dry glacials interspersed with short warm and wet interglacials (Hewitt 2000). For instance, the last glacial cycle a.k.a. the Würm, spanned from 110'000 to 21'000 years ago (Figure 3), after which Earth climate quickly warmed up again. The repeated shifts between glacial and interglacial periods had tremendous consequences on the distribution and genetic diversity of animal and plant biota (reviewed by Hewitt 2000 and Schmitt 2007). In Europe, where northern habitats became unsuitable during ice ages, most species were restricted to so-called glacial refugia, located across southern Mediterranean Peninsula as well as eastern continental lowlands (Figure 3). Disconnection of these separate populations resulted in genetic divergences, nowadays forming distinct lineages in many organisms. In addition, refugial areas feature high amounts of genetic diversity, both because of the long-term demographic stability of populations (i.e. they survived several ice-ages) and because their topographic complexity made them hotspots for regional allopatric diversifications (i.e. refugia within refugia, Gómez & Lunt 2007). In contrast, northern populations originated from recent (i.e. post-glacial) recolonization, during which much diversity was lost due to expansion-associated drift: as a result, only a subset of the refugial diversity is found in post-glacial populations. Although location of refugia and post-glacial routes of recolonization varies given historical distributions, ecological requirements and life-history traits of species, comparative phylogeographies delineated major biogeographic paradigms, pinpointing hotspots of diversity and divergences, emphasizing the role of major mountain ranges as barriers to recolonization, and

identifying secondary contact zones (Hewitt 2000, 2011, Schmitt 2007).

Studying sex-chromosome evolution at the intraspecific level thus demands knowledge of the basal amounts of diversity and divergences, their distributions, along with reconstruction of the demographic events stemming from refugial maintenance and post-glacial expansions. **Chapter I** provides such analyses for the widespread European tree frog (*Hyla arborea*), used as a framework for subsequent phylogeographic surveys of sex-linked markers (**Chapter II** and **III**). Multi-species comparisons also necessitate prerequisites on phylogenetic relationships and divergence times (**Chapter V**, **VI**), as well as location of hybrid zones (**Chapter X**), which we assessed beforehand in **Chapter IV**.

MODEL SYSTEM: PALEARCTIC TREE FROGS

Hylid tree frogs or "true" tree frogs constitute one of the richest amphibian family worldwide, widespread over temperate and tropical parts of the Nearctic, Neotropic, Palearctic and Australasian ecozones (Wiens et al. 2010). Palearctic species originated from the Nearctic, through several successive colonization waves via northern landbridges which regularly connected North-America to Eastern-Asia over the last 60 My (Smith et al. 2005). The Western-Palearctic radiation diversified within the last 10 My, giving rise to at least ten lineages (some of them still to be described taxonomically) distributed around the Mediterranean Basin (Stöck et al. 2008), including the type species *Hyla arborea*.

European *Hyla* tree frogs are small and slender (3-5 cm long), mainly of uniform green coloration (with variation from grey to brown), and equipped with characteristic toe discs, used for climbing. Adults are sexually dimorphic, with males possessing a dark vocal sac on the throat, absent in females. Tree frogs are lek-breeding species: males gather at suitable water bodies (shallow and sunny temporary ponds) during the breeding season (February-June), and nightly call in chorus to attract females. Male and female mate by pairing together in amplexus (Figure 4), and the female lay ~200-400 eggs on the aquatic vegetation, externally fertilized by the male. Eggs hatch after approximately one week, depending on water temperature, and tadpoles continue growing up to metamorphosis (two-three months after hatching). Juveniles then become sexually-mature after 1-2 years (males) or 2-3 years (females) and can live up to 7-10 years in the wild (Arnold & Ovenden 2002). Because of their noisy

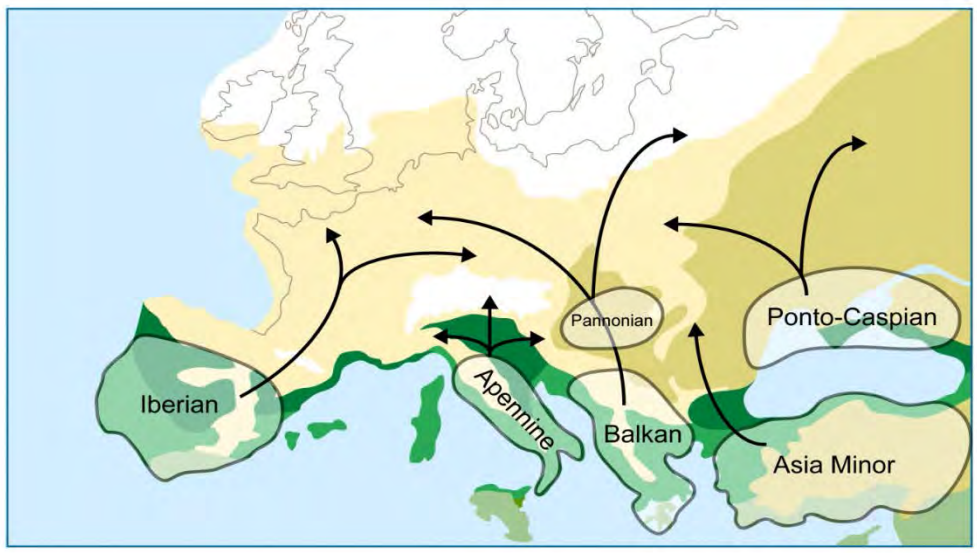

FIGURE 3: Europe during the last glacial maximum (21'000 years ago): glacial vegetation (layout from Ruddiman 2008), mainland refugia and major routes of post-glacial recolonization.

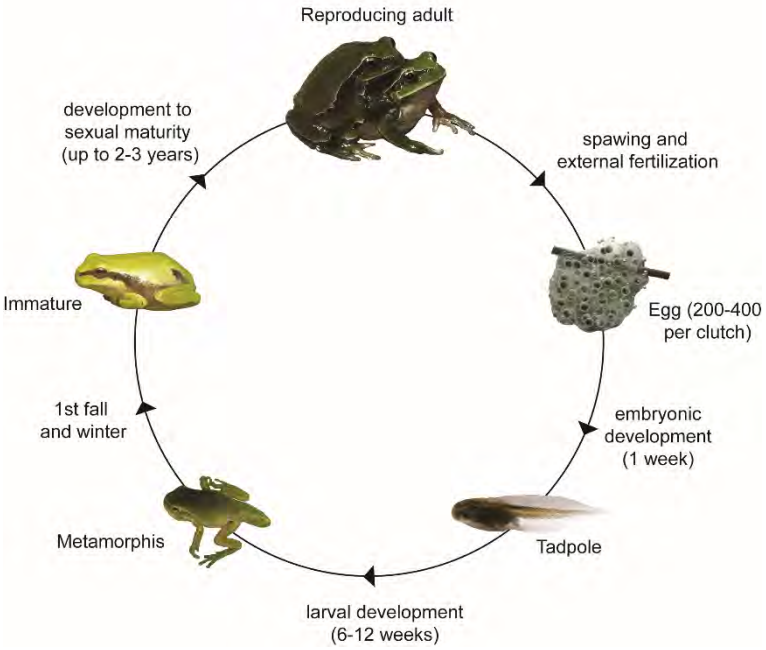

FIGURE 4: Life cycle of European tree frogs. For population genetics analyses, adults are caught by night during the breeding season (February-June) and DNA is collected with non-invasive buccal swabs. For sibship analyses, breeding pairs (usually captured in amplexus, top picture) are kept in tanks until spawning, and young tadpoles are sampled (ethanol-fixed) after hatching, ~1 week later. For phenotypic sexing (identification of gonads), offspring are raised in the lab until several weeks after metamorphosis.

calls (easily detectable for census) and local abundances, tree frogs are popular umbrella species and have received interest in various fields of ecological and evolutionary research, such as metapopulation dynamics (e.g. Andersen et al. 2006), mating systems (e.g. Broquet et al. 2009) and landscape genetics (e.g. Dubey et al. 2009). Like most amphibians, *Hyla* populations are threatened in many regions and countries and benefit from the conservation perspectives of population genetics and phylogeographic studies (c.f. **Chapter I**).

Over the last decade, the European tree frog *Hyla arborea* has also become a model system for studying sex-chromosome evolution. All Eurasian species have homomorphic sex chromosomes (Anderson 1991). Discovery of sex-linked anonymous microsatellite markers revealed male heterogamety (XY; Berset-Brändli et al. 2006), and sibship analyses in Swiss populations evidenced recombination arrest between X and Y (Berset-Brändli et al. 2008), in line with the impoverished effective size of the latter (Berset-Brändli et al. 2007). Although seemingly nascent, comparative linkage mapping showed that these XY sex chromosomes were shared by the closely related taxa *H. molleri*, *H. intermedia* and *H. orientalis*, where male recombination is also suppressed (Stöck et al. 2011, 2013b). However, despite this apparent recombination arrest, patterns of X-Y similarities between *H. arborea*, *H. intermedia* and *H. molleri* still indicate some gene exchange between X and Y since the splits of these species 5-7 Mya, supporting the occasional recombination model, but raising a series of questions regarding the proximate mechanisms and their extent across the radiation (Stöck et al. 2011, Guerrero et al. 2012).

CHAPTERS OVERVIEW

In the first part of this work (**Chapter I-III**), we solve this paradox by exposing how sex-chromosome recombination evolved in the widespread *H. arborea*, through intraspecific phylogeographic analyses. We set an autosomal and mitochondrial phylogeographic framework (**Chapter I**), contrast range-wide sex-chromosome sequence similarities (**Chapter II**) and trace the evolution of X-Y recombination and differentiation combining sibship analyses with fine-scale phylogeographic reconstruction of X and Y haplotypes, a first of its kind in a species with homomorphic sex chromosomes (**Chapter III**).

In the second part (**Chapter IV-VII**), we conduct multi-species analyses to understand how sex-determination and sex-chromosomes evolved in the radiation. We clear up distributions and divergences of Western-Palearctic lineages in a phylogenetic framework (**Chapter IV**), screen for patterns of X-Y divergence to isolate the sex-determining gene of the youngest species (**Chapter V**), test for turnovers *versus* X-Y recombination models over ~40My of *Hyla* sex-chromosome evolution (**Chapter VI**), and dissect individual variation of X-Y recombination across diverged species (**Chapter VII**).

The third part (**Chapter VIII-X**) is dedicated to document the influence of the tree frogs' homomorphic sex chromosomes on reproductive isolation. We develop (**Chapter VIII**) and map (**Chapter IX**) cross-amplifying and species diagnostic genetic markers, and contrast introgression at sex-linked *versus* autosomal loci across natural hybrid zones (**Chapter X**).

PART 1

EVOLUTION OF SEX CHROMOSOMES IN *HYLA ARBOREA*

CHAPTER I

CONSERVATION PHYLOGEOGRAPHY: DOES HISTORICAL DIVERSITY CONTRIBUTE TO REGIONAL VULNERABILITY IN EUROPEAN TREE FROGS (*HYLA ARBOREA*)?

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Conservation phylogeography: does historical diversity contribute to regional vulnerability in European tree frogs (*Hyla arborea*)?

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Abstract

Documenting and preserving the genetic diversity of populations, which conditions their long-term survival, have become a major issue in conservation biology. The loss of diversity often documented in declining populations is usually assumed to result from human disturbances; however, historical biogeographic events, otherwise known to strongly impact diversity, are rarely considered in this context. We apply a multilocus phylogeographic study to investigate the late-Quaternary history of a tree frog (*Hyla arborea*) with declining populations in the northern and western part of its distribution range. Mitochondrial and nuclear polymorphisms reveal high genetic diversity in the Balkan Peninsula, with a spatial structure moulded by the last glaciations. While two of the main refugial lineages remained limited to the Balkans (Adriatic coast, southern Balkans), a third one expanded to recolonize Northern and Western Europe, loosing much of its diversity in the process. Our findings show that mobile and *a priori* homogeneous taxa may also display substructure within glacial refugia ('refugia within refugia') and emphasize the importance of the Balkans as a major European biodiversity centre. Moreover, the distribution of diversity roughly coincides with regional conservation situations, consistent with the idea that historically impoverished genetic diversity may interact with anthropogenic disturbances, and increase the vulnerability of populations. Phylogeographic models seem important to fully appreciate the risks of local declines and inform conservation strategies.

Keywords: biodiversity, conservation genetics, *Hyla arborea*, phylogeography, Quaternary refugia, red list status

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Introduction

Documenting and maintaining the genetic diversity of populations are becoming a central issue in conservation biology (Beebee 2005; Hughes *et al.* 2008). A link between diversity and viability has been established across many taxonomic groups (e.g. Oostermeijer *et al.* 1995; Rowe *et al.* 1999; Luijten *et al.* 2000; Hansson & Westerberg 2002; Reed & Frankham 2003), and genetic erosion is thought to be a major player of extinction vortices (e.g. Newman & Pilson 1997; Saccheri *et al.* 1998; Westemeier *et al.* 1998; Rowe & Beebee 2003; Frankham 2005). In addition, intraspecific variability

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represents an adaptive potential to face sudden environmental changes and conditions the capacity of populations to colonize and survive in suboptimal habitats (e.g. Meagher 1999; Frankham 2005; Rowe & Beebee 2005). As a consequence, conservation measures are designed to maximize the amount of protected diversity, especially through the management of evolutionary significant units (ESU, 'phylogeographic' approach, Moritz 1994) or evolutionary populations ('demographic' approach, Waples & Gaggiotti 2006).

Anthropogenic factors, such as land use or climate change, are often proposed as the main triggers for the decrease in biodiversity worldwide (e.g. Vitousek *et al.* 1997; Epps *et al.* 2005). Especially, genetic impoverishment is usually assumed to result from population disconnection in fragmented landscapes (van Dongen *et al.* 1998; Buza *et al.* 2000; Madsen *et al.* 2000). However, Quaternary climatic oscillations have already strongly influenced the distribution of genetic variation (Hewitt 2000). Formerly, glaciated regions are expected to feature shallow genetic structure, with poor intraspecific variation, as a result of multiple bottlenecks during post-glacial expansions from a single or a few source population(s) (Hewitt 2000). In contrast, refugial areas that survived several northern glaciations constitute hotspots of diversity (Petit *et al.* 2003), because of both a higher demographic stability and more pronounced structure due to allopatric differentiation ('refugia within refugia', Gómez & Lunt 2007). As a consequence, ancient and genetically rich southern populations are expected to be in better condition to withstand anthropogenic factors than are recently expanded and genetically impoverished northern populations (Schmitt 2007), a prediction that has rarely been tested (Schmitt & Hewitt 2004). Species with wide distributions and regional information on their conservation status are best suited to address this fundamental question.

The European tree frog (*Hyla arborea*) recently expanded from the Balkan Peninsula, from which it recolonized most parts of Central and North-Western Europe (Stöck *et al.* 2012). Interestingly, its conservation situation across the range is much contrasted: while the species is not considered threatened in south-eastern Europe, it is mostly declining and regionally endangered in northern and western ranges (Fig. S1 and Table S1, Supporting information). A recent study by Luquet *et al.* (2011) evidenced a positive correlation between heterozygosity and fitness in tree frogs, highlighting the importance of genetic diversity for population viability. In line, several population genetics surveys independently documented low levels of variability in Western Europe, where *H. arborea* is the most vulnerable (Edenhamn *et al.* 2000; Andersen *et al.* 2004; Arens *et al.* 2006; Dubey *et al.* 2009). Although this was attributed to population bottlenecks associated with landscape management, these studies did not recover signs of disconnection within metapopulations. Alternatively, we propose that historical expansions from southern refugia could account for this reduced diversity and potentially increase the susceptibility of Western European populations to the current anthropogenic pressures responsible for their severe decline (especially industrial agriculture, Brühl *et al.* 2013).

From previous phylogenetic and phylogeographic studies (Stöck *et al.* 2008b, 2012), *H. arborea* forms a genetically poor monophyletic clade with little variation across its range, suggesting one uniform glacial refugium with a global post-glacial expansion. Nevertheless, *cytochrome b* networks identified a few slightly diverged haplotypes in some of the southern localities (Stöck *et al.* 2012; see also Stöck *et al.* 2008b for nuclear data), potentially indicative of cryptic structure. In this study, we use a higher-resolution framework, with faster-evolving molecular markers and denser sampling, (i) to test whether the vulnerable condition of Western European *H. arborea* populations is consistent with a biogeographic loss of variability and (ii) to search for possible cryptic structures associated with refugia and re-expansions during and after the last glaciations, asking in particular whether recently diverged populations are relevant for defining conservation units.

Methods

DNA extraction

A total of 779 specimens from 65 localities (considered as separated populations) covering the entire distribution range of *H. arborea* were included in this study (Table S2 and S3, Supporting information). Genomic DNA was extracted from ethanol-preserved tissues (tadpole tail-tips, adult vouchers) and noninvasive buccal swabs (live adults; Broquet *et al.* 2007) with the Qiagen DNeasy Tissue kit or the Qiagen BioSprint robotic workstation.

Sequence data

Two mitochondrial and one nuclear marker were sequenced in representative subsets of samples. Detailed protocols and primer information are provided in Data S1 and Table S4 (Supporting information). Following Stöck *et al.* (2008b, 2012), we first amplified the mitochondrial gene *cytochrome b* (957 bp) in 211 new samples, complemented by 27 *cyt b* sequences from Stöck *et al.* (2012). Second, we developed new primers (adapted from Goebel *et al.* 1999) to sequence 590 bp from the 5′ hypervariable region of the mitochondrial

D-loop (Table S4, Supporting information) in these 238 individuals. In addition, we sequenced the *D-loop* in two *H. orientalis* samples for which *cyt b* was already available. Mitochondrial sequences could then be concatenated (238 *H. arborea* samples representing 59 localities, + two individuals of *H. orientalis*). Third, based on the alignment of sequences available on GenBank, new primers were designed to amplify a portion of the nuclear gene *rag-1* (737 bp; Table S4, Supporting information) in 100 individuals. This was complemented by eight published sequences (Stöck *et al.* 2008b, 2012), for a total of 108 *H. arborea* (55 localities), and we also included *rag-1* sequences from closely related hylid species. All sequences were visualized on an ABI-3730 sequencer (AppliedBiosystems, Inc.) and aligned using Seaview 4.2 (Gouy *et al.* 2010). For *rag-1*, haplotypes of heterozygous individuals were reconstructed with the phase algorithm implemented in DnaSP 5 (Librado & Rozas 2009), using a recombination model with no assumption about rate variation and an initial estimate of 0.0004. The MCMC chain was run for 1000 iterations with a burnin of 100 and a thinning interval of 1, and output probability thresholds were set to 0.9.

Microsatellite data

Seventeen previously published autosomal microsatellites (Arens *et al.* 2000; Berset-Brändli *et al.* 2008a,b) were used in this study. We also included three new autosomal microsatellite loci recently developed on the basis of transcriptomic sequences from *H. arborea* (Brelsford *et al.* 2013) and took advantage of this transcriptome to develop ten additional polymorphic microsatellites, following the exact same methodology. Altogether, 750 individuals from 53 populations were genotyped for these 30 markers (see Data S1 and Table S4, Supporting information for information on protocols and primers). In most cases, PCRs were performed in multiplex. Amplicons were subsequently analysed on an ABI-3100 sequencer and allele sizes scored using the size standards Rox-350 or Rox-500 (GeneMapper 4.0; AppliedBiosystems, Inc.). We checked for genotyping errors due to stuttering, allelic dropout and null alleles with Micro-Checker 2.2.3 (van Oosterhout *et al.* 2004) and performed corrections when necessary.

Phylogenetic analyses, molecular dating and Bayesian phylogeographic reconstruction

Maximum-likelihood phylogenetic reconstructions (PhyML 3.0, Guindon & Gascuel 2003) were performed on a concatenated data set of the two mitochondrial markers (1547 bp) and separately on the *rag-1* data set (737 bp). In both cases, we used a HKY+G+I model (jModelTest 0.1.1, Posada 2008) and tested the robustness of topologies by 1000 bootstrap replicates. We estimated the divergence time between major haplogroups from our *cyt b* data set in BEAST 1.6.2 (Drummond & Rambaut 2007), using a strict molecular clock (ucld.stdev parameter < 1 with a frequency histogram abutting 0 when testing with a relaxed clock, BEAST manual version July 2007) and a coalescent prior (appropriate for intraspecific radiations). To decide which one to use, we performed short runs (1 chain of 5 million iterations) with the different coalescent priors available in BEAST and choose the one with the highest likelihood (coalescent: exponential growth). We used a HKY+G+I model of sequence evolution (jModelTest), and the tree was calibrated to the splits of *H. meridionalis*, *H. savignyi*/*H. felix arabica*, *H. arborea* and *H. orientalis*/*H. molleri* (respectively, approximately 10, 6.2, 6.1, and 3.7 Mya, based on previous work, Smith *et al.* 2005; Stöck *et al.* 2012; using sequences available on GenBank, see Table S3, Supporting information), with normally distributed priors. We ran three independent chains of 30 million iterations each and used Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) to check for convergence and combine the results.

We reconstructed the phylogeographic history of *H. arborea* by a Bayesian phylogeographic analysis of our mtDNA data set using spatial continuous diffusion models (Lemey *et al.* 2010) in BEAST 1.7.5 (Drummond *et al.* 2012), following the methodology recommended by Suchard & Lemey (2013). Four different models were ran (homogeneous Brownian diffusion across branches, branch-specific diffusion rates (relaxed-random walks, RRWs): Gamma RRW, Cauchy RRW and Lognormal RRW) during 100 million iterations (sampling every 10 000), with a strict molecular clock (see above) and the Bayesian skyline plot as a flexible demographic prior. To perform model selection, we computed marginal likelihoods with 100 power posteriors along the path between prior and posterior (each of 100 000 iterations following 10 000 of burn-in, sampling every 1000) and estimated log marginal likelihoods using path and stepping stone sampling (shown to outperform other estimators, Baele *et al.* 2012, 2013). For the best-fitting diffusion model, the maximum clade credibility (MCC) tree was computed and annotated using the BEAST module TreeAnnotator 1.7.5. We then used SPREAD 1.0.4 (available: http://www.phylogeography.org/SPREAD.html) to project the MCC phylogeny in a spatial framework and summarized the full posterior distribution of trees to calculate the 80% highest posterior density (HPD) of node locations. Final results were visualized in Google Earth (http://earth.google.com/).

Analyses of genetic structure

For sequence data (concatenated mtDNA, *rag-1*), we built statistical parsimony networks with TCS 1.21 (Clement *et al.* 2000) which connects haplotypes under a parsimony limit (set to 95%). For both data sets, the main haplogroups were defined with the individual-based, spatially explicit BAPS model (version 6.0, Corander *et al.* 2008) for clustering DNA sequence data (Cheng *et al.* 2013). This model combines sample locations with likelihood of the genetic data and is particularly efficient with large data sets (Cheng *et al.* 2013). We performed analyses using default parameters and following the software manual (version 21.12.2012). Several runs were repeated to ensure convergence and consistency of the results.

For microsatellites, we conducted individual-based assignment with the Bayesian algorithm of STRUCTURE 2.3.3 (Pritchard *et al.* 2000), using an admixture ancestry model with sampling locations as prior, and correlated allele frequencies between populations (as recommended for subtle population structure). Ten replicates (each consisting of 10^5 iterations following a burn-in of 10^4) were performed for every number of clusters (K) between 1 and 11, from which we computed the corresponding ΔK ad hoc statistics (Evanno *et al.* 2005) with STRUCTURE HARVESTER 0.6.92 (Earl & VonHoldt 2012, http://taylor0.biology.ucla.edu/struct_harvest). Replicates were combined with CLUMPP (full search algorithm, Jakobsson & Rosenberg 2007) and graphs of assignment probabilities built using DISTRUCT 1.1 (Rosenberg 2004). We conducted hierarchical analyses, by rerunning STRUCTURE within each of the major clusters, including populations assigned with a probability of at least 80%. In addition, population differentiation was inferred by a principal component analysis (PCA) on allelic frequencies with PCAGEN 1.2 (http://www2.unil.ch/popgen/softwares/pcagen.htm), which evaluates the significance of axes by permutations. To estimate the level of isolation by distance, we also performed Mantel tests of genetic (F_{ST}) versus geographic distances (ARLEQUIN 3.5, Excoffier *et al.* 2005) across populations (where $n \geq 6$ individuals) assigned to the main groups defined by STRUCTURE (with a probability of at least 80%).

Analyses of genetic diversity

For mtDNA and *rag-1*, we computed (ARLEQUIN) the haplotype (Hd) and nucleotide diversity (π) within populations where $n \geq 4$ sequences. For microsatellites where no null allele was detected, we assessed allelic richness and heterozygosity for populations where $n \geq 6$ genotyped samples with FSTAT, which performs a rarefaction procedure to a common sample per locus (Goudet 1995).

Demographic analyses

The demographic fluctuations of the main identified haplogroups were inferred from our sequence data sets (*cyt b*, *D-loop*, *rag-1*) by three separate approaches. First, we performed Bayesian coalescent-based analyses to evaluate the historical demographic fluctuations within each of the main haplogroups using the Extended Bayesian Skyline Plot (EBSP, Heled & Drummond 2008) implemented in BEAST 1.6.2. The EBSP can combine several sequence sets (in our case: *cyt b*, *D-loop*, *rag-1*) and fits different demographic scenarios by allowing changes in population size overtime. For all three markers, we used HKY+G+I models (JMODELTEST). The clock rate (μ) of the *D-loop* and *rag-1* was estimated from *cyt b*, where μ was fixed to the value previously obtained from the molecular calibration. Other parameters were either left as default or optimized for the EBSP (following Heled 2010), and chains were run for 60 million iterations. We used TRACER to assess burn-in and effective sample sizes (ESS) of parameters.

Second, we performed analyses of mismatch distributions, by comparing observed pairwise number of differences to distributions simulated under models of demographic (Schneider & Excoffier 1999) and range expansions (Excoffier 2004), as implemented in ARLEQUIN. These models estimate the parameters of population expansion using a generalized least-square approach and compute their confidence intervals by bootstrapping (10 000 replicates in our case). Tests of goodness-of-fit (sum of squared deviation and Harpending's raggedness index) were computed to measure departures between observed and simulated distributions (ARLEQUIN). For each mitochondrial haplogroup, we estimated the time since expansion from the parameter τ ($\tau = 2\mu t$, where μ is the mutation rate, and t the time since expansion) and given the clock rates previously estimated by BEAST.

Finally, for mtDNA (concatenated *cyt b*/*D-loop*) and *rag*-1, we computed (DNASP 5) the following tests of selective neutrality within each of the main haplogroups: Fu's Fs, Tajima's D and Ramos-Onsins and Rozas's R^2, which has more statistical power for small sample sizes (Ramos-Onsins & Rozas 2002). Their significances were tested by coalescent analyses (10 000 replicates).

Intergroups gene flow

To measure the level of gene flow between the main clusters, we calculated the demographic parameters θ

($\theta = x N_e \mu$, where N_e is the effective size, μ the mutation rate and x a multiplier depending on the ploidy and the inheritance of the data: $x = 1$, 2 or 4 for mtDNA, haploid or diploid data, respectively) and M ($M = m/\mu$, with m the immigration rate) from sequences (combining *cyt b*, *D-loop*, *rag-1*), and microsatellite data sets. From the product of θ and M, we can calculate the number of immigrant genes per generation (xN_em) from one cluster to another. Population boundaries can be defined when $xN_em < 1$ but values up to 25 can still reflect departure from panmixia (Waples & Gaggiotti 2006). We delimited our candidate groups according to the main distribution of haplogroups and microsatellite clusters, and estimated θ and M with MIGRATE 3.5.1 (full migration rates matrix, Beerli & Felsenstein 2001) using the Bayesian inference search strategy (Beerli 2006). We first performed preliminary runs to optimize the prior distributions and burn-in periods. Relative mutation rates among loci were directly inferred from the data (through measures of Watterson's θ; microsatellites) or calculated from the BEAST estimates (sequences). For microsatellites, we used the Brownian motion approximation to the ladder model, which gives very similar results to the stepwise-mutation model but with much faster parameter estimation (MIGRATE manual, version 16.05.2012). In the final analyses, we ran one MCMC chain per locus (50 000 recorded genealogies among 5 000 000 visited), with burn-ins of 500 000 (combined sequence data set) or 20 000 steps (microsatellite data set). We monitored the effective sample sizes (ESS) of each parameter (including the likelihood) to make sure that the chains ran long enough (ESS > 1000, MIGRATE manual). Each data set was re-analysed several times to ensure the consistency of the results.

Results

Phylogenetic analyses, molecular dating and Bayesian phylogeographic reconstruction

Phylogenetic analyses of mtDNA (112 haplotypes, 1547 bp) revealed a supported clade restricted to the north-eastern Adriatic coast (H1–H7), and a paraphyletic group distributed across the rest of the range (Fig. S2, Supporting information). The *rag-1* phylogeny (27 haplotypes) displayed a large polytomy involving our *H. arborea* sequences and several close relatives (*H. molleri*, *H. intermedia* and *H. orientalis*) with incomplete lineage sorting (Fig. S3, Supporting information). From *cyt b* (estimated clock rate = 0.036 substitution/My), we dated the divergence of the mitochondrial Adriatic isolate to be approximately 180 kya (95% CI = 70–300; approximately two ice-ages). The time to the most recent common ancestor of the main clade roughly corresponds to the end of the last interglacial (90 kya, 95% CI = 40–160). All chains yielded nearly identical estimates, suggesting convergence. Because of its unresolved phylogeny, we did not date divergence times between *rag-1* haplogroups.

The pattern and timing of dispersal of *H. arborea* mitochondrial haplogroups were best inferred from the Lognormal RRW diffusion model (Table S5, Supporting information). The analysis estimated the location of ancestor sequences on the Adriatic coast, from which the southern Balkans were early colonized and then diversified, particularly south-east and north-west of the Pindus mountain range (Data S2). From the latter, Central and Western Europe were recently invaded by a first wave of colonization and frogs later expanded to northern areas. At the same time, the Hellenic peninsula and then Crete were fully colonized from central Greece. The analysis also depicted recent southward migrations, from Central Europe to the Balkans.

Genetic structure and diversity

The spatial clustering method of BAPS defined three major groups from our mtDNA data set (optimal partition, log(likelihood) = −2622.5; Fig. 1): the Adriatic isolate (H1-7, haplogroup 1), sequences from southern and eastern Greece (H79-H112, haplogroup 2), and a widespread haplogroup distributed throughout the range (H8-78, haplogroup 3), with some geographic association of haplotypes (H31-45: only North and Western Europe; H54-59: only north-eastern Adriatic coast; H60-H78: only southern Balkans). Haplotype diversity (Hd) was greatly variable throughout the range (Table S2, Supporting information), but nucleotide diversity (π) shows some geographic differences, being higher in the southern and western Balkans (Fig. 2b). Accordingly, the architecture of our mtDNA network (Fig. 1) was more complex for haplotypes occurring in the southern Balkans and on Crete (H60-H78, H79-H93), contrasting with the starlike shapes of haplotypes sampled further north (e.g. H94-H112, H54-H59, H8-H30, H31-H46).

Clustering analyses (BAPS) of the nuclear *rag-1* (optimal partition: 4 groups, log(likelihood) = −731.1; Fig. 3) also distinguished western Balkans (H1-3, haplogroup 1) from southern Balkans (H4-H10, H13-H17, haplogroup 2) and the rest of the range (H18-27, haplogroup 3). A fourth cluster includes two haplotypes (H11-12, haplogroup 4) occurring in the most eastern populations from Romania (loc. 41), Serbia (loc. 21–22) and Greece (loc. 11), at the contact zone with *H. orientalis* and clustering with alleles from this latter species. Because this haplogroup probably originated from introgression events (no similar

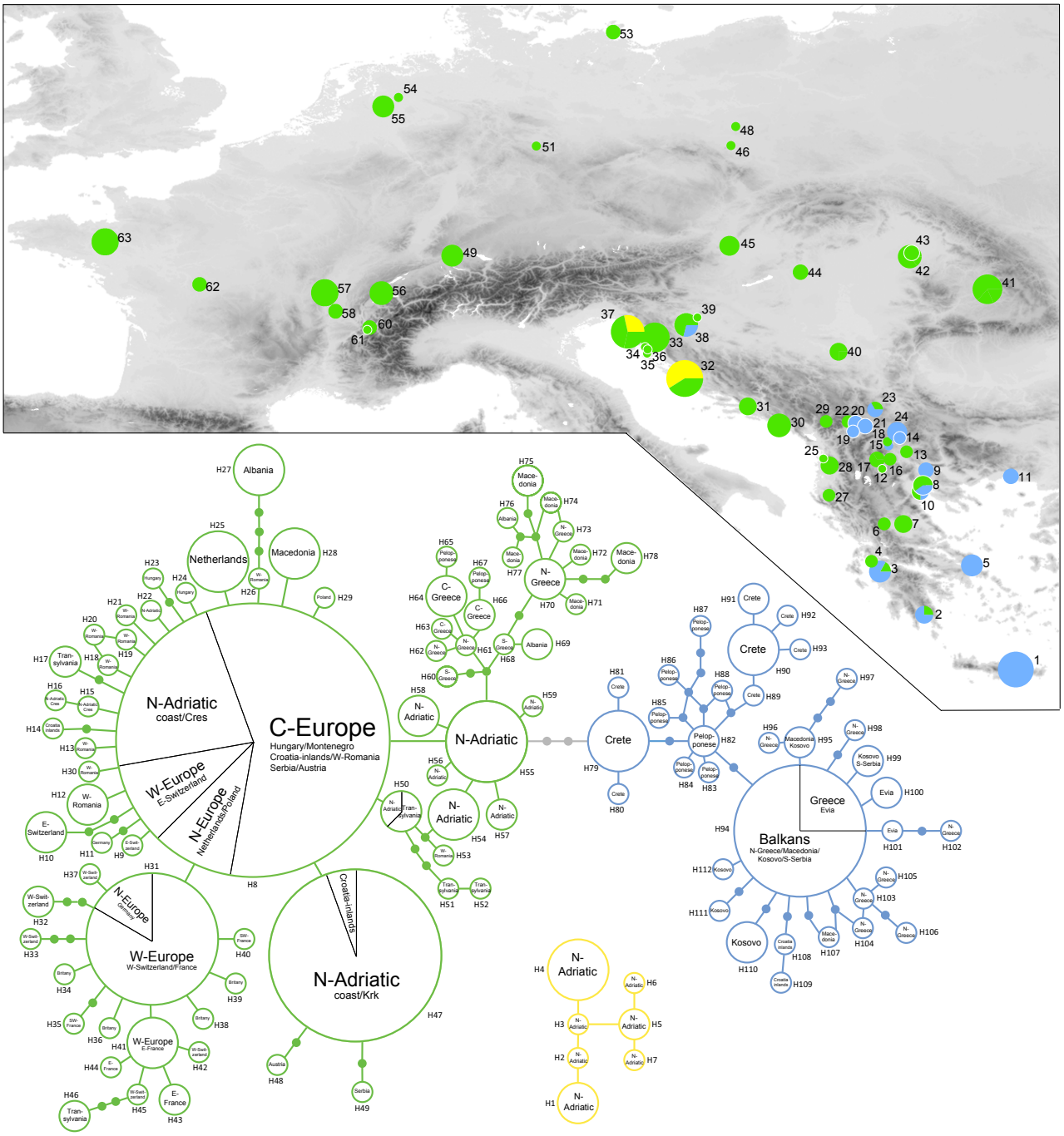

Fig. 1 Parsimony-based haplotype network of mitochondrial sequences (concatenated *cyt b* + *D-loop*) and distribution of the haplogroups defined by BAPS (yellow: haplogroup 1, blue: haplogroup 2, green: haplogroup 3). The following abbreviations are used: N: north; S: south; E: east; W: west; C: central. 'Macedonia' corresponds to the Former Yugoslav Republic of Macedonia.

cluster was found from the other markers), and also based on a very limited sample size, it was not included in the demographic analyses. The distribution of the *rag-1* diversity is similar as for mtDNA, where the most southern haplogroup (haplogroup 2) is also the richest (Table S2, Supporting information, Fig. 2c) and the most complex (Fig. 3). In both BAPS analyses, permuting sequences between groups resulted in substantial decreases in log(likelihood) (on average −42.6 for mtDNA, ranging from −9.4 to −79.1; on average −21.4 for *rag-1*, ranging from −1.4 to −42.6), suggesting robust assignments.

Individual-based clustering of microsatellite genotypes with STRUCTURE suggested two major groups

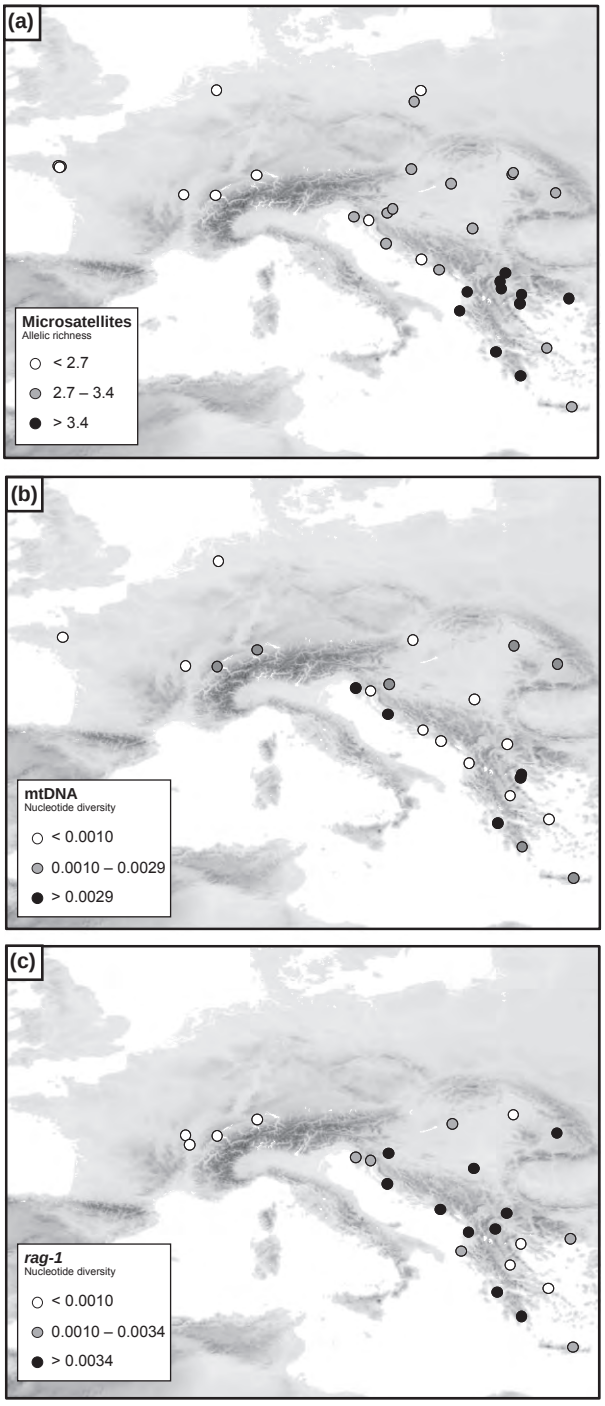

Fig. 2 Distribution of microsatellite allelic richness (scaled to 5 individuals; a) and mitochondrial (concatenated *cyt b* + *D-loop*; b) and *rag-1* nucleotide diversity (c). Classes were built from natural breaks (Jenks) in ArcGIS 9.3 (ESRI). Details are available in Table S2 (Supporting information).

(K = 2, highest ΔK = 704.4; Fig. S4, Supporting information) contrasting southern (loc. 1–7) and central/north-western (loc. 34–65) populations (Fig. 4). Populations parapatric to these two groups featured intermediate probabilities of assignment (loc. 8–33). We could detect fine substructure within each cluster (Fig. 4). In southern Greece (K = 2, highest ΔK = 601.2; Fig. S4, Supporting information), Structure distinguished Crete (loc.1) from Thessaly and Evia Island (loc. 4–7) with admixed populations in the Peloponnese Peninsula (loc. 2–3). In the rest of the range (K = 2, highest ΔK = 755.1; Fig. S4, Supporting information), populations from France and western Switzerland (loc. 56–65) were differentiated from Central Europe (loc. 37–45). The populational PCA (Fig. S5, Supporting information) also contrasted Southern from Central Europe (N-S gradient, first axis) and depicted differentiation between eastern and western populations from the continental part of the range (E-W gradient, second axis). Both axes were significant. As null alleles were detected for several markers, especially in southern populations, we reran our analyses by (i) discarding the corresponding loci and (ii) considering null alleles as missing data. In both cases, results from Structure and the PCA remained unchanged. Finally, we found striking differences in microsatellite variability (allelic richness and heterozygosity) between the Balkans, Central and North-Western Europe, which are strongly decreasing with distances from southern areas (Table S2, Supporting information, Fig. 2a). Southern insular populations (Crete: loc.1, Evia: loc. 5) were also genetically poorer than neighbouring mainland areas (Table S2, Supporting information, Fig. 2a).

The correlation between pairwise F_{ST} values and geographic distances was significant for the central/north-western microsatellite cluster (loc. 34–65; correlation coefficient = 0.74; regression coefficient = 0.000158), but not for the southern group (loc. 1–7).

Demographic analyses

The extended Bayesian skyline plot reconstructed a recent increase in population size for haplogroups 2 and 3 since the last glacial maximum (Fig. 5). Interestingly, the expansion was much stronger (100-fold increase) for haplogroup 3 than haplogroup 2 (10-fold increase) and indicated that the latter maintained a higher effective size during the last glaciation. In contrast, the 95% posterior distribution of the number of changes in population size (demographic.populationSizeChanges) does include 0 for haplogroup 1 (which is not the case for haplogroups 2 and 3). The unimodal distributions of pairwise nucleotide differences within the three main mtDNA and *rag-1* haplogroups suggest past population expansions (Fig. 5). Based on the sum of squared differences and raggedness index, the fits to the spatial expansion model could never be rejected for

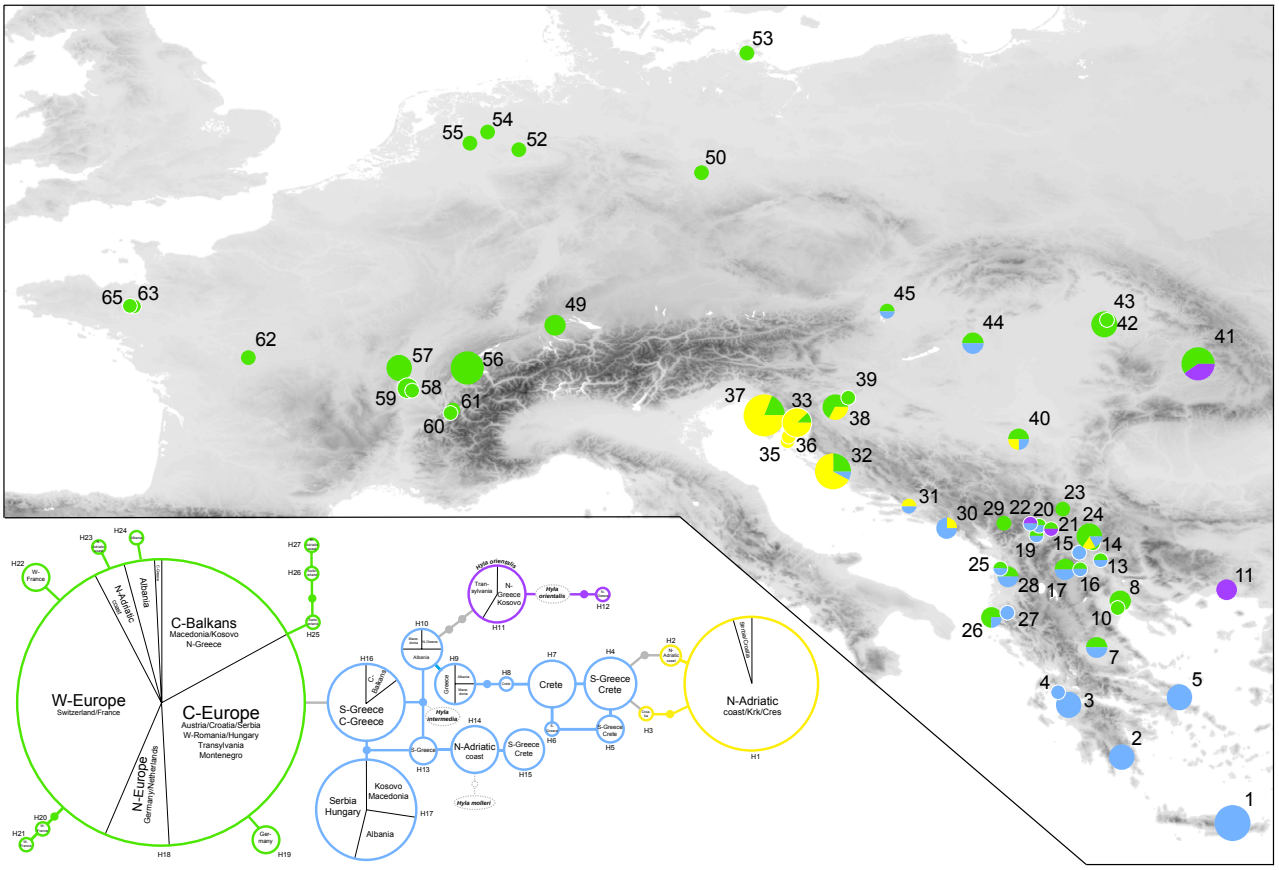

Fig. 3 Parsimony-based haplotype network of *rag-1* nuclear sequences and distribution of the haplogroups defined by BAPS (yellow: haplogroup 1, blue: haplogroup 2, green: haplogroup 3, purple: haplogroup 4). Haplotypes sampled in closely related species are displayed. Abbreviations are the same as in Fig. 1.

any haplogroups of the two markers (Table 1). The mismatch distribution simulated under the model of demographic expansion was significantly different from observed distributions only for the *rag-1* southern haplogroup 2 (Table 1). Calculated from the parameter τ, the time since expansion pointed to the late-Pleistocene for all mitochondrial haplogroups (Table 1). Accordingly, tests for departure from neutrality were significant for the main mitochondrial haplogroups 2 and 3 but not for haplogroup 1 (Table 1). For *rag-1*, departure from neutral values could only be ascertained for haplogroup 3 (Table 1).

Intergroups gene flow

Because the distributions of nuclear and mitochondrial haplogroups/clusters defined by BAPS and STRUCTURE were congruent, but not strictly identical across markers (with many admixed localities), it was difficult to define precise spatial boundaries between the three main genetic groups. Therefore, we delimited candidate populations according to the geographic regions where these haplogroups principally occurred: southern Balkans (loc. 1–31), northern Adriatic (loc. 32–37) and Central/North-Western Europe (loc. 38–65). Multiple runs of MIGRATE performed on this framework led to similar estimates, and all parameters had unimodal posterior distributions, suggesting that single optima were reached. Because the posterior distribution abutted 0 for most parameters, we preferred the modes to the medians for calculating the demographic estimator $x\mathrm{N_em}$ ($= \theta \times \mathrm{M}$). Migration estimates ($x\mathrm{N_em}$) ranged from 0.6 to 8.5 for sequence data and from 6.2 to 19.2 for microsatellites (Fig. S6, Supporting information), which is below the cut-off values for departures from panmixia ($x\mathrm{N_em} < 25$). The estimated gene flows were mostly asymmetric, with the expanding groups (southern Balkans, Central/North-Western Europe) providing migrants to the Adriatic populations (Fig. S6, Supporting information). Table S6 (Supporting information) displays the parameters estimated by MIGRATE and their 95% posterior distribution.

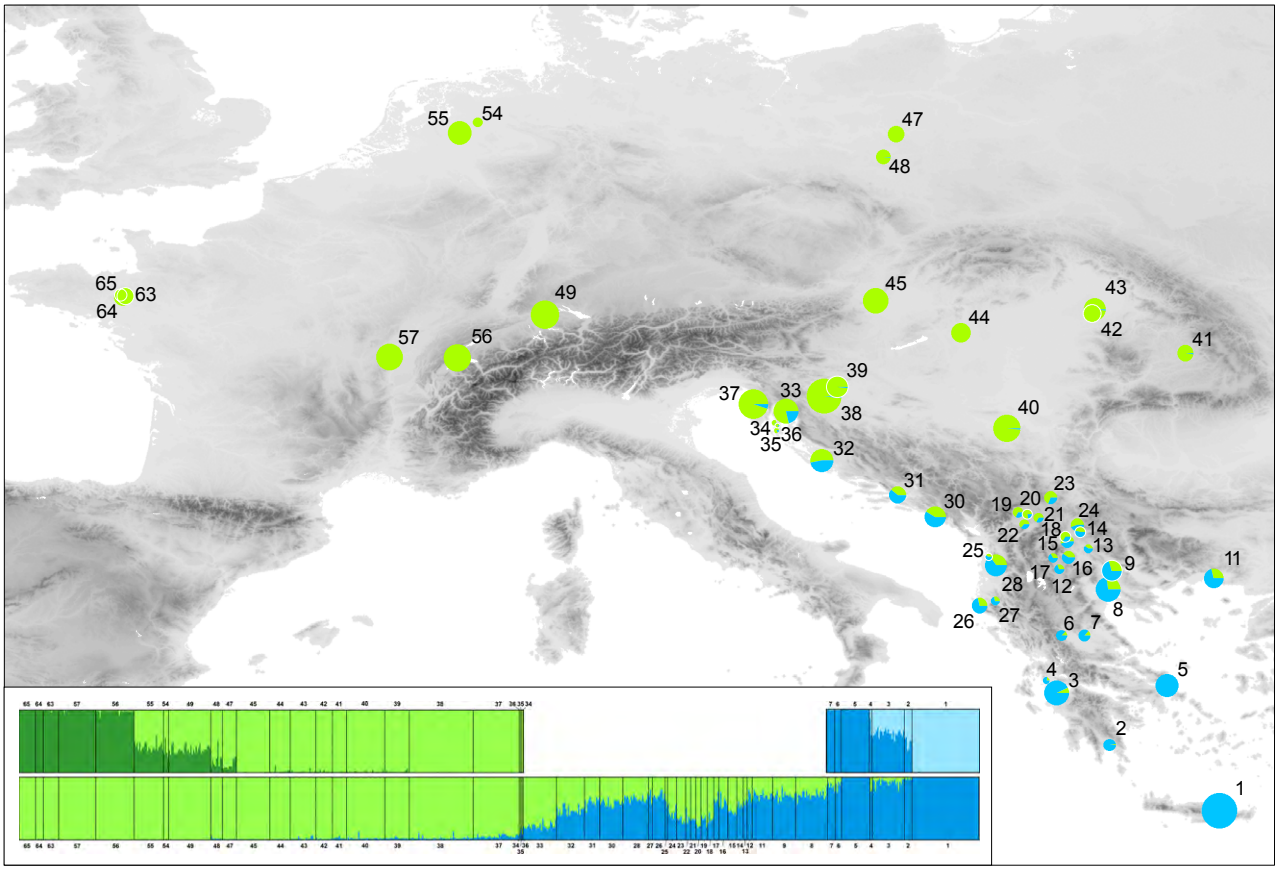

Fig. 4 Genotyped-based assignments of *Hyla arborea* individuals, based on Bayesian clustering analyses of 30 microsatellites (STRUCTURE). Barplots represents the assignment probability of each individual. The lower chart includes all samples ($K = 2$, see main text and Fig. S4, Supporting information). The upper charts correspond to hierarchical analyses on each of the two main clusters (upper left: Central/North-Western Europe, $K = 2$; upper right: southern Greece, $K = 2$). The map shows the mean assignment probability of every locality to each of the main clusters.

Discussion

Our study provides a fine-scale reconstruction of the phylogeography of the widespread European tree frog *H. arborea*. Our dense sampling and multilocus approach allowed us to uncover hidden diversity, and were especially suitable to test assumptions regarding the distribution of genetic variation and its implications for conservation. First, cryptic structure could be documented across the Balkan Peninsula, allowing us to check whether the recently diverged groups match the commonly used criteria for ESUs and evolutionary populations. Second, we could accurately assess the geographic patterns of diversity and map them to the regional levels of vulnerability.

Refugia within refugia: insights into the Balkans biogeography

One aim of our study was to test whether southern areas exhibit cryptic structuring, as previously suspected (Stöck *et al.* 2008b, 2012) and expected under the 'refugia within refugia' paradigm (Gómez & Lunt 2007). Indeed, although there were a few discrepancies on the precise boundaries, our mitochondrial and nuclear data sets were largely congruent in support of a late-Pleistocene diversification. This involved several major genetic groups in southern areas (western Adriatic coast; southern Balkans), of which one recently expanded across the rest of the range, from the Balkans to Western Europe. The only exception came from the Adriatic group, which does not significantly stand out from our microsatellite data set, maybe because of recent backcrossing by southern and Central European immigrants (see below). In addition, it is unclear whether the fourth *rag-1* haplogroup found in the most eastern populations stem from an ancestral polymorphism or (more probably) from introgressive hybridization with the proximate *H. orientalis* (Stöck *et al.* 2012), which displays closely related haplotypes (Fig. 3). We further detected subtle structure from our microsatellite data set (consistent with spatial association of mtDNA

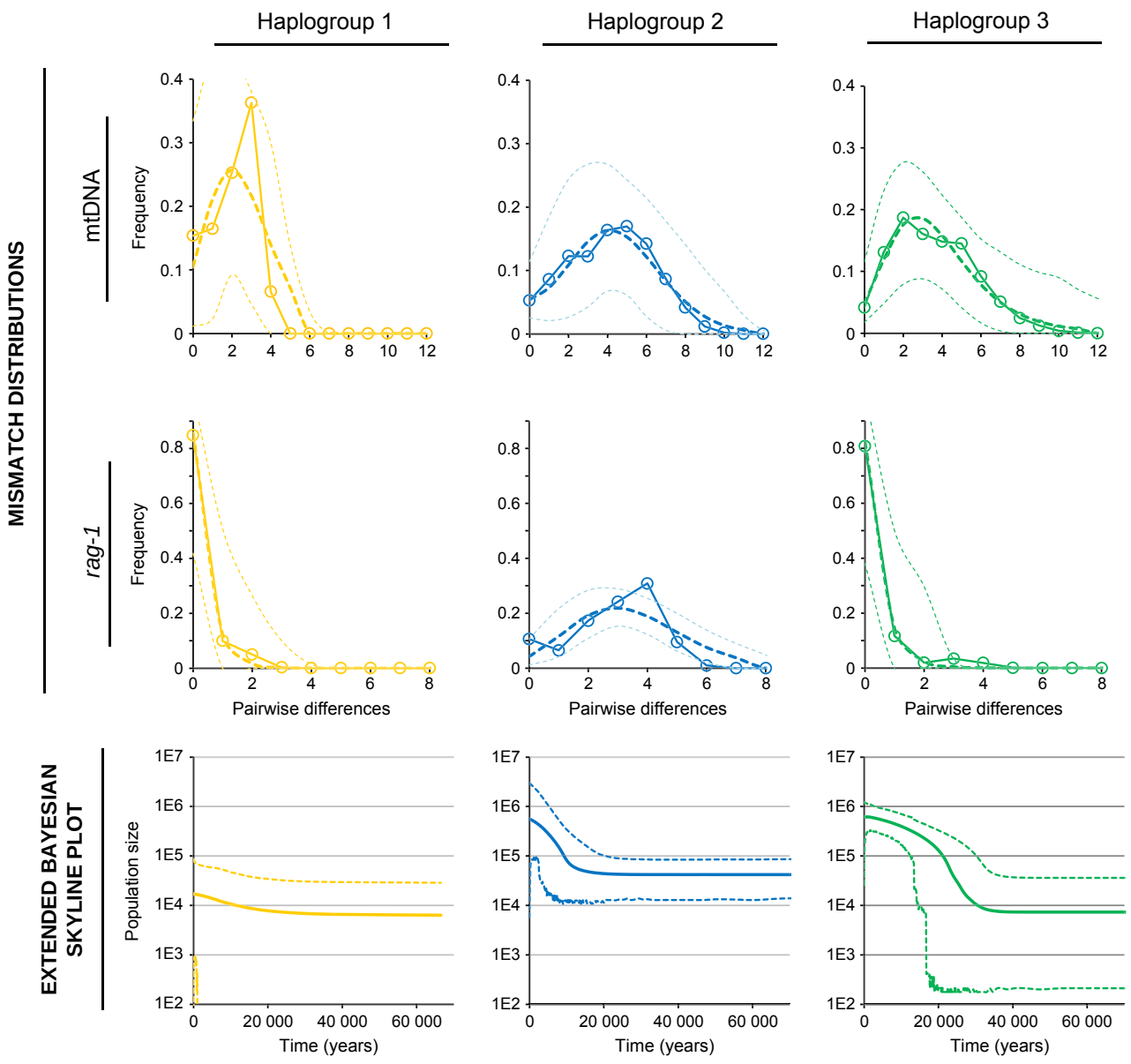

Fig. 5 Demographic analyses of the three main haplogroups defined from sequence data. Upper charts display the distribution of observed (circles) and expected (bold dash lines) pairwise nucleotide differences under models of sudden demographic expansion (Arlequin 3.5). 95% Confidence intervals of the expected distributions are indicated (thin dash lines). Mismatch distributions simulated under models of spatial expansion yielded similar expected curves (not shown). Tests for goodness-of-fit are available in Table 1. Lower charts display extended Bayesian skyline plots (EBSP) representing the recent demographic trends of each haplogroups, based on combined sequence data (solid lines: median estimates, dash lines: 95% confidence intervals). Y axes display estimated population size, as the product of effective population size per generation length.

haplotypes), pointing out recent contact between Crete and the Peloponnese (probably when sea levels dropped in the Ionian Sea; Perissoratis & Conispoliatis 2003), and population differentiation across recently colonized areas (Central, North-Western Europe) which might stem from isolation by distance (as reported), gene surfing (Excoffier *et al.* 2009) or post-glacial climate and range changes (e.g. the Younger Dryas, 11 kya; see Taberlet & Cheddadi 2002 and references therein). The latter could particularly explain why the most northern areas were colonized by a last, most recent wave of invasion, as depicted by the mtDNA phylogeographic inference.

Our findings are thus in line with the view that southern refugial areas host 'refugia within refugia' (Gómez & Lunt 2007), generating cryptic structure and maintaining

Table 1 summary statistics of the mismatch distribution analyses and neutrality tests. For both models (sudden demographic expansion, spatial expansion), tests of goodness-of-fit are provided (Sum of Square Deviation / Harpending's raggedness index) with their significance (^{NS} = nonsignificant; * = significant, $P < 0.05$). For mtDNA, the time since expansion (in years) was calculated from τ, using mutation rates of 0.036 and 0.064 substitutions/My for *cyt b* and the *D-loop* respectively (estimated in BEAST). Neutrality test statistics and their significance are shown (R^2 = Ramos-Onsins and Rozas's R^2). Marker informativeness is also provided for each haplogroup

	mtDNA (1547 bp)			*rag-1* (737 bp)			
	Haplogroup 1	Haplogroup 2	Haplogroup 3	Haplogroup 1	Haplogroup 2	Haplogroup 3	Haplogroup 4
Number of sequences	14	59	165	38	59	109	10
Number of haplotypes	7	34	71	3	12	10	2
Variable (informative) sites	6 (4)	37 (20)	78 (44)	3 (1)	8 (8)	11 (7)	3 (0)
Demographic							
Tau	2.6 (0.7–4.2)	4.9 (2.2–7.4)	2.1 (1.7–2.5)	3.0 (0.4–3.5)	3.5 (1.9–4.5)	3.0 (0.4–3.5)	—
Goodness-of-fit	$0.033^{NS}/0.112^{NS}$	$0.002^{NS}/0.011^{NS}$	$0.002^{NS}/0.018^{NS}$	$0.002^{NS}/0.564^{NS}$	0.028*/0.075*	$0.345^{NS}/0.488^{NS}$	—
Time since expansion	18 000 (5000–29 000)	34 000 (15 000–51 000)	15 000 (12 000–17 000)	—	—	—	—
Spatial							
Tau	2.6 (0.6–4.3)	3.9 (2.0–6.2)	2.1 (1.0–4.5)	0.8 (0–3.5)	3,5 (1.1–5.0)	2.8 (0.0–7.7)	—
Goodness-of-fit	$0.031^{NS}/0.112^{NS}$	$0.004^{NS}/0.011^{NS}$	$0.002^{NS}/0.019^{NS}$	$0.002^{NS}/0.564^{NS}$	$0.025^{NS}/0.075^{NS}$	$0.483^{NS}/0.488^{NS}$	—
Time since expansion	18 000 (4000–30 000)	27 000 (14 000–43 000)	15 000 (7000–31 000)	—	—	—	—
Fu's Fs	-2.04^{NS}	−1.60*	−2.36*	−1.57*	-1.82^{NS}	−2,15*	—
Tajima's D	0.257^{NS}	−27.2*	−91.2*	-1.43^{NS}	-1.43^{NS}	−10.8*	—
R^2	0.156^{NS}	0.050*	0.0206*	0.108^{NS}	0.182^{NS}	0.0211*	—

high genetic diversity. Increasing empirical evidence in support of this pattern is coming from taxa across a diversity of regions (Iberia: e.g. Martínez-Solano *et al.* 2006; Rowe *et al.* 2006; Gonçalves *et al.* 2009; Italy: e.g. Canestrelli *et al.* 2012; Nearctic: e.g. Nielson *et al.* 2001). So far, the Balkans have received less attention (Hewitt 2011), but signatures of multiple refugia have been found for a few species, both in Mediterranean (e.g. Ursenbacher *et al.* 2008; Bužan *et al.* 2010) and nonMediterranean parts of the Peninsula (Provan & Bennett 2008; e.g. Kryštufek *et al.* 2009; Fijarczyk *et al.* 2011). In particular, the northern Adriatic coast seems to have acted as a remote sanctuary for several Balkanic species (e.g. green toads, Stöck *et al.* 2008a; nose-horned viper, Ursenbacher *et al.* 2008), including tree frogs. There and in other parts (i.e. southern Balkans), population maintenance might have been tightly linked to the glacial distribution of deciduous forests (supposedly restricted to the southern and eastern Balkans, the Carpathian belt and south of the Alpine Glaciers, Adams & Faure 1997), and post-glacial expansions were probably associated with the recolonization of tree species (Taberlet & Cheddadi 2002; Stöck *et al.* 2012). Our results then support the view that the Balkan Peninsula is an important centre of European biodiversity, especially for the herpetofauna (Crnobrnja-Isailović 2007).

Multiple subrefugia are typical for low-vagility organisms such as newts (Sotiropoulos *et al.* 2007) or snakes (Ursenbacher *et al.* 2008) and are usually easier to detect given that populations have experienced long periods of allopatry. In contrast, tree frogs have a relatively high dispersal capacity for an amphibian (up to 4 km / year, Vos *et al.* 2000) so that ancestral populations may have regularly merged during interglacials, engulfing traces of multiple refugia (e.g. Stöck *et al.* 2012). The increasing appeal to multilocus genomic data will give exciting insights into the glacial and post-glacial phylogeography of genetically uniform taxa that might similarly hide cryptic population differentiations (Emerson *et al.* 2010).

Defining recently diverged management units

An important question raised by this cryptic diversity is whether conservation units can be defined from such recently diverged lineages, that is, do they match the criteria for ESUs or evolutionary populations' paradigms? Evolutionarily significant units can be defined provided that genetic distinctiveness of populations matches other features (e.g. geographic distribution, adaptive phenotypic variation) and is supported by mitochondrial monophyly and reciprocal significant nuclear divergence (Moritz 1994). Here, we did find some concordance between multiple molecular

polymorphisms and their geographic distribution. However, only the Adriatic mitochondrial clade was significantly supported, and populations carrying this mitochondrial lineage did not form a distinct cluster based on microsatellites. Our groups also exhibited considerable mixing where they come into contact, consistent with ongoing gene flow. Therefore, these clusters hardly fulfil the requirements of ESUs. On the other hand, despite a rough choice of population boundaries that should have led to overestimate the amount of gene flow (particularly since haplogroup 2 and 3 seem to have originated from proximate areas), patterns still reflect a departure from panmixia: even though most migration estimates are above the stringent cut-off value of $xN_em < 1$, they indicate isolation with migration ($xN_em < 25$) compatible with the evolutionary population concept proposed by Waples & Gaggiotti (2006).

Thus, although our clusters might be too young and insufficiently resolved to be considered ESUs, they feature enough genetic isolation to deserve special attention for wildlife management. The demographic approach thus seems more powerful to assess the evolutionary significance and conservation values of young subspecific radiations. Nevertheless, management design based only on the levels of gene flow may still miss significant divergences. In our case, the northern Adriatic coast can be viewed as a hotspot of genetic diversity, but experienced the highest levels of migration. New estimators combining both phylogeographic and demographic information (as well as adaptive variation, when applicable) might allow considering all valuable entities. In addition, the future development of specific criteria for discriminating different levels of population cohesion would be useful to set priorities for the definition of conservation units (Reilly *et al.* 2012).

Biogeographic loss of diversity and regional vulnerability

One main goal of our study was to assess whether the vulnerability of Western European populations was consistent with a biogeographic loss of genetic diversity. Higher amounts of microsatellite and sequence polymorphisms were indeed found in Southern Europe, which, from our analyses, seems largely explained by the maintenance of distinct ancestral demes and demographic stability of these populations. We could also document post-glacial expansions across most parts of the range (see also Stöck *et al.* 2012), leading to a severe loss of variability resulting from colonization-associated drift (Excoffier *et al.* 2009). Thus, the low levels of genetic diversity in Western and Northern Europe, also reported from previous regional studies (Edenhamn *et al.* 2000; Andersen *et al.* 2004; Arens *et al.* 2006; Dubey *et al.* 2009), might stem from historic/phylogeographic reasons more than from human disturbances.

From our results, this geographic trend in genetic diversity approximately corresponds to regional conservation status: south-eastern *H. arborea* populations are not considered threatened, which clearly contrasts with the precarious situation of the species in the north and west of Europe (Fig. S1, Supporting information). Although the level of threat must largely depend on the intensity of disturbances (especially land use and pollution), it is tempting to suggest that biogeographic history predisposes population vulnerability to current environmental pressures, even though this cannot be formally tested with the data in hand. Such natural genetic impoverishment might impact the sensitivity of populations to identified threats, like habitat destruction and pesticide exposure (an alarming cause of decline, Brühl *et al.* 2013), and accelerate their deterioration. Furthermore, Northern and Western European amphibian communities are especially challenged by the chytrid fungus, which does not yet affect Balkanic regions (RACE 2013). Whether the amount of genetic diversity conditions the susceptibility to infection is unclear (May *et al.* 2011), but it is known to improve fitness in our study species (Luquet *et al.* 2011). Experimental work aiming to understand the interaction between variability, fitness and known threats (e.g. pollutants) would be particularly relevant to test these hypotheses (e.g. Pearman & Garner 2005).

A few studies have empirically explored the link between genetic variation and population trends in plants (e.g. Brütting *et al.* 2012) and animals (e.g. Kvist *et al.* 2011). However, to our knowledge, only Schmitt & Hewitt (2004) had previously investigated this relationship in a biogeographic context. These authors showed that European butterflies performed better in refugial areas (eastern and southern Europe) than in recently deglaciated regions and discussed the lack of adaptability of the genetically poor northern and western populations. Many widespread species thrive in the central part of their distribution but are regionally endangered at the periphery (Hoffmann & Blows 1994). This range limit effect might have biogeographic origins in post-glacial areas, at least in species where differences in genetic variation could be detected across the range (Channell 2004).

Future studies should not only assess the role of landscape changes, but also account for the basal diversity historically present in a region. Especially, large-scale phylogeographic surveys of widespread taxa, where knowledge of regional population dynamics is available, would be most relevant to address this issue. In our case, we benefited from the relatively

well-covered conservation situation of *H. arborea* (although assessment data were too heteroclite for accurate analyses), most likely because it can be considered as an umbrella species, and populations are easy to detect and monitor by call census (Pellet & Schmidt 2005). Amphibians are especially good candidates to test this relationship, because they seem particularly susceptible to diversity loss (Allentoft & O'Brien 2010).

Phylogeographic analyses such as ours thus lay the ground work for conservation planning and identification of the potentially most sensitive regions. Even without fine-scale genetic data, general ideas of the main historical events (e.g. range expansion from a putative refugia) could be used to draw assumptions regarding their impact on diversity. In complement to the commonly used criteria (observed and forecast species abundance, population trends, potential threats, e.g. IUCN 2001), we propose that the geographic distribution (i.e. refugial vs post-glacial) should be considered when assessing regional risks and red lists.

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C.D. and N.P. designed research. C.D., J.W., K.G., M.St., P.L. and J.C.I. performed research. C.D., M.St. and A.B. contributed new markers. C.D. analysed data and drafted the manuscript, which was improved by N.P., A.B. and M.St.

Data accessibility

Sequences were deposited on GenBank, and Accession nos. listed in Table S3 (Supporting information). Microsatellite genotypes and sequences alignments were archived on Dryad (doi:10.5061/dryad.2vk30). Sequences of new microsatellite markers are available on GenBank (Accession nos. can be found in Table S4, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 Laboratory protocols for the amplification of the markers used in this study.

Data S2 Phylogeographic reconstruction in continuous space using relaxed-random walks (Lognormal), based on mitochondrial sequences.

Fig. S1 Conservation status of *Hyla arborea* on national and regional red lists, when available.

Fig. S2 Maximum-Likelihood phylogeny of 112 mitochondrial haplotypes (concatenated *D-loop* and *cyt b*).

Fig. S3 Maximum-Likelihood phylogeny of 27 haplotypes from the nuclear *rag-1*.

Fig. S4 Likelihood probability of the data L(*K*) and Δ*K* ad hoc statistics computed from Structure runs with Structure Harvester (ten replicates per *K*).

Fig. S5 Principal Component (PCA) of microsatellite allelic frequencies.

Fig. S6 Intergroups gene flow xN_em estimated by Migrate (first values: from sequence data / second values: from microsatellite data).

Table S1 National and regional red lists assessments of *Hyla arborea*, and population trends.

Table S2 Information on the localities included in this study, and their genetic diversity.

Table S3 GenBank Accession nos of *cytochrome b*, *D-loop* and *rag-1* sequences, and availability of microsatellite genotypes.

Table S4 Details on the genetic markers used in this study.

Table S5 Log marginal likelihood estimated from path (PS) and stepping stone (SS) sampling from mtDNA Bayesian inferences under different continuous spatial diffusion models.

Table S6 Demographic parameters θ and M, estimated with Migrate from sequences and microsatellites. 95% confidence intervals are given in brackets.

CHAPTER II

RANGE-WIDE SEX-CHROMOSOME SEQUENCE SIMILARITY SUPPORTS OCCASIONAL XY RECOMBINATION IN EUROPEAN TREE FROGS (*HYLA ARBOREA*)

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* equal contribution

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Range-Wide Sex-Chromosome Sequence Similarity Supports Occasional XY Recombination in European Tree Frogs (*Hyla arborea*)

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Abstract

In contrast with mammals and birds, most poikilothermic vertebrates feature structurally undifferentiated sex chromosomes, which may result either from frequent turnovers, or from occasional events of XY recombination. The latter mechanism was recently suggested to be responsible for sex-chromosome homomorphy in European tree frogs (*Hyla arborea*). However, no single case of male recombination has been identified in large-scale laboratory crosses, and populations from NW Europe consistently display sex-specific allelic frequencies with male-diagnostic alleles, suggesting the absence of recombination in their recent history. To address this apparent paradox, we extended the phylogeographic scope of investigations, by analyzing the sequences of three sex-linked markers throughout the whole species distribution. Refugial populations (southern Balkans and Adriatic coast) show a mix of X and Y alleles in haplotypic networks, and no more within-individual pairwise nucleotide differences in males than in females, testifying to recurrent XY recombination. In contrast, populations of NW Europe, which originated from a recent postglacial expansion, show a clear pattern of XY differentiation; the X and Y gametologs of the sex-linked gene *Med15* present different alleles, likely fixed by drift on the front wave of expansions, and kept differentiated since. Our results support the view that sex-chromosome homomorphy in *H. arborea* is maintained by occasional or historical events of recombination; whether the frequency of these events indeed differs between populations remains to be clarified.

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Introduction

Sex chromosomes have evolved along dramatically divergent pathways among vertebrates, depending on lineages. Most mammals and birds present strongly differentiated sex chromosomes, with highly degenerated Y and W chromosomes in males and females, respectively; in sharp contrast, many fishes, reptiles and amphibians present morphologically undistinguishable sex chromosomes. Two non-exclusive causes have been invoked to account for this homomorphy. On one hand, occasional turnovers may replace established sex chromosomes before they had time to decay (e.g. [1,2]). On the other hand, occasional XY recombination may rejuvenate senescing Y chromosomes by purging the load of deleterious mutations that accumulate in non-recombining genomic regions [3]; very rare events of X-Y recombination seem sufficient to prevent Y degeneration [4].

The XY-recombination model recently received support from studies of European tree frogs. Several species of the *Hyla arborea* radiation inherited the same pair of sex chromosomes from a common ancestor (>5 Mya); despite arrest of recombination in males, the X and Y allelic sequences of sex-linked genes cluster by species, not by gametologs [5,6], pointing to occasional events of recombination. Guerrero et al. [7] reached the same conclusion by analyzing with Approximate Bayesian Computations the patterns of XY divergence at sex-linked microsatellite loci. Surprisingly, however, no single event of male recombination could be detected by sibship analyses, despite thousands of offspring obtained in controlled crosses (e.g. [5,6,8]). Furthermore, West-European populations of the nominal species (*Hyla arborea*) consistently display sex-specific allelic frequencies at series of sex-linked microsatellite markers, often with male-diagnostic alleles (e.g. [5,8]), pointing to the absence of XY recombination in their recent history.

To address this apparent paradox, we decided to extend investigations on the patterns of XY differentiation to a broader phylogeographic framework. West-European populations of *H. arborea* are of recent origin [9,10]. The patterns of mitochondrial and nuclear diversity testify to a post-glacial expansion from southeastern Europe, where three main haplogroups (with ~200 ky divergence) survived in distinct refugia across the Balkan Peninsula. While two of these mitochondrial lineages remained limited to the Balkans (Adriatic coast and southern Balkans;

orange area in Fig. 1a), a third one expanded after the Last Glacial Maximum (~15 kya) to recolonize first the Pannonian Basin (violet area), then from there Western and Northern Europe (green area), losing much of its diversity during this process [10]. In the present study, we use intraspecific sequence polymorphism of three sex-linked markers to seek evidence for possible events of XY recombination during this species' late Pleistocene history.

Methods

DNA Sampling and Study Animals

DNA was sampled in 91 adult *H. arborea* (37 females, 54 males) using non-invasive buccal swabs in 87 live individuals or ethanol-preserved tissues in four collection specimens, and extracted manually (DNeasy kit, Qiagen) or with the Qiagen Biosprint robotic workstation. Samples were chosen to represent populations from the entire distribution range, from southern Greece to Western Europe (Fig. 1a, Table S1, Supporting Information). Live individuals were captured during the breeding period, and could be sexed unambiguously through secondary sexual traits; males being characterized by dark vocal sacs, nuptial excrescences, and mating calls. The four collection specimens were also sexed unambiguously by anatomical observation of either ovaries or testes. Our study was approved by the relevant Institutional Animal Care and Use Committee (IACUC), namely the Service de la Consommation et des Affaires Vétérinaires du Canton de Vaud (Epalinges, Switzerland); no live animal was sacrificed for the study (sampling only using non-invasive buccal swabs); other samples came from scientific collections specified in Table S1, Supporting Information.

PCR Amplification, Cloning and Sequencing

We amplified three sex-linked markers: parts of the transcription co-factor *Med15* (including two exons and two introns; n = 55), the non-coding *Ha-A103* (n = 33), and one intron from the gene *Smarcb1* (n = 16), as described [5,11]. Except for four *Smarcb1* samples, which we sequenced directly, all PCR products were cloned using the Promega pGEM-T Easy or Invitrogen TOPO-TA cloning kits. In most cases, at least eight clones per sample could be sequenced on an ABI3730 (Applied Biosystems), and consensuses were produced with Seaview [12]. When fewer than eight clones were obtained (24 cases) individuals were considered homozygous if at least six clones were identical. Two individuals only yielded three identical clones; we labeled the other allele as missing data and discarded these two individuals from the pairwise distance analyses. For directly sequenced *Smarcb1*, haplotypes

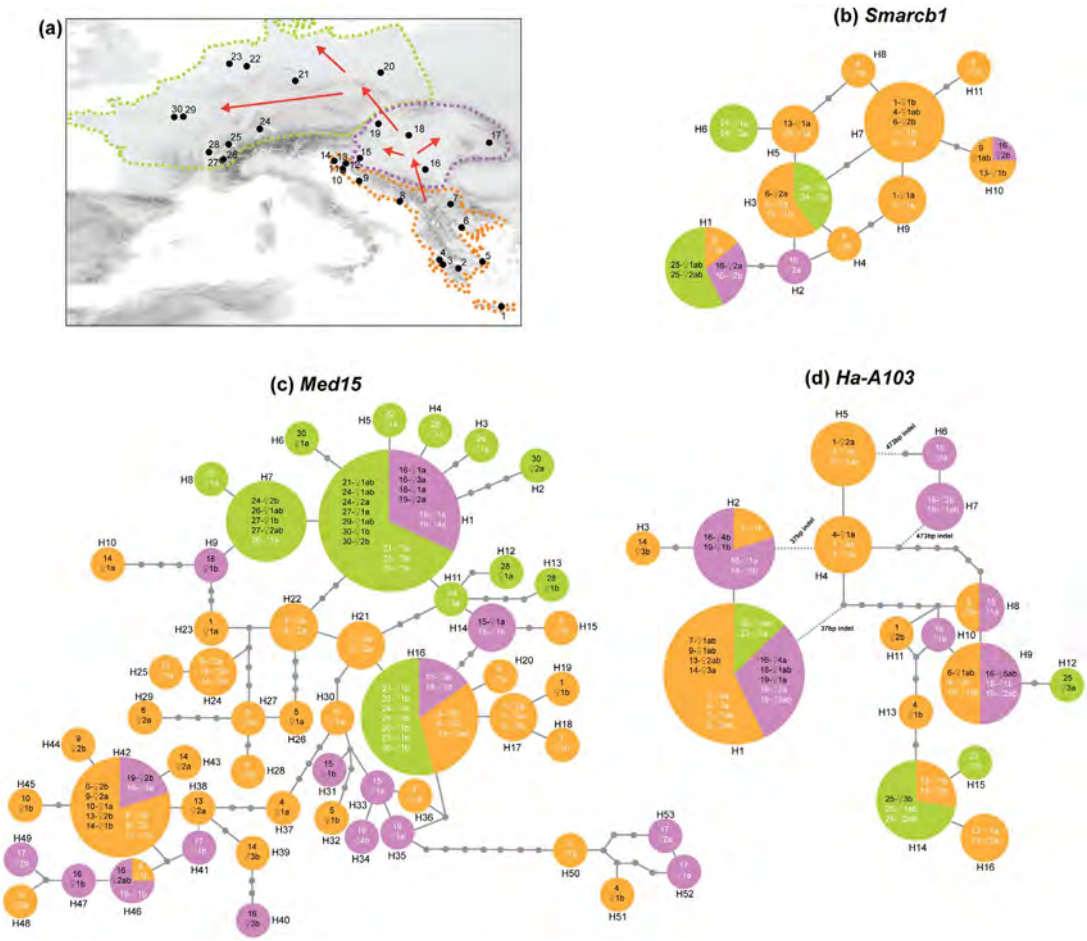

Figure 1. Sampling localities (a) and haplotype networks of *Smarcb1* (b), *Med15* (c) and *Ha-A103* (d). For each allele, labels indicate locality number, followed by the sex of the individual (black for females and white for males), a sample number, and the letter a or b (discriminating two alleles of heterozygotes; written "ab" for homozygotes). The colors of haplotypes correspond to the main phylogeographic regions across *H. arborea's* present distribution range (as described by Stöck et al. 2012, and Dufresnes et al. 2013), delimited by thin dashed lines on the map (orange: southeastern European refugia; violet: Pannonian basin; green: NW Europe). Arrows show post-glacial recolonization routes.
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could be reconstructed manually: two individuals were homozygous, and the other two shared identical genotypes with closely related alleles (differing by two polymorphic sites).

Data Analyses

Sequences were manually aligned in Seaview, and analyzed by statistical parsimony networks with TCS (v. 1.21 [13]; parsimony limit set to 95%), which allows taking indel polymorphisms into account (option "gap as fifth base"). In addition to SNPs, *Med15* and *Ha-A103* contain microsatellite-like repeats [5], which were re-coded so that one tandem repeat difference corresponds to one mutational step. To confirm proper amplification and observed length differences within *Med15* and *Ha-A103*, we compared this data with results from microsatellite genotyping at these loci (details in [5,8]). We followed the same re-coding strategy for two large indels (37 bp and 473 bp) within *Ha-A103* (Fig. 1d).

Although the two copies of a male necessarily comprise an X and a Y allele, it was *a priori* not possible to assign any specific copy to either the X or the Y chromosome (except for *Med15* in NW Europe populations; see Results and Discussion). Thus, X-Y and X-X divergence were compared by measuring, for each marker, the pairwise nucleotide differences p_d (in DnaSP v. 5 [14]) between the two copies of every male (X-Y difference) and female (X-X difference). Comparisons between sexes and regions were assessed by analyses of variance (ANOVA) of p_d, using a non-parametric permutation procedure to test the significance of differences (10′000 replicates; performed in *R* 2.13.1 [15]). The geographic regions were defined according to the phylogeographic structure of the species as inferred by multilocus nuclear and mtDNA data by Dufresnes et al. [10].

Results and Discussion

Sequences (Alignment S1, Supporting Information) of *Med15* (992 bp) were the most polymorphic (64 variable sites, 33 parsimony-informative), followed by *Ha-A103* (504 bp without a 473 bp indel found in 2 individuals, 19 variable sites, 15 parsimony-informative) and *Smarcb1* (411 bp, 8 variable sites, all parsimony-informative). Most of the diversity was found in southeastern Europe, and the Pannonian Basin (Fig. 1: orange and violet areas); for all three markers, postglacial populations from Northern and Western Europe (green area) harbor fewer haplotypes. These results corroborate evidence from autosomal and mitochondrial data of a large-scale post-glacial colonization of the Pannonian Basin from several glacial refugia in southeastern Europe, namely the southern Balkans and the Adriatic coast, followed by a later expansion to Western and Northern Europe, during which much variance was lost [10].

Haplotypic networks (Fig. 1b–d) show a generally large mix of male and female alleles. Averaged over all individuals and populations, alleles were not more diverged in males (X-Y) than in females (X-X) at all three loci (for *Ha-A103*: $F_{1,31} = 0.43$, p-value = 0.55; for *Smarcb1*: $F_{1,14} = 0.14$, p-value = 0.72; for *Med15* ($F_{1,51} = 0.44$, p-value = 0.50). Many males presented identical X and Y haplotypes, or shared both of their alleles with females (Fig. 1b–d). Interestingly, two large indels in *Ha-A103* were shared by X and Y haplotypes: the same 37 bp deletion occurred on the two copies of males from southeastern Europe (e.g. 3-♂1, 7-♂1 and 9-♂3), the Pannonian Basin (e.g. 18-♂2, 19-♂3) and NW Europe (20-♂1); similarly, the same 473 bp insertion was shared by the X and Y alleles of two males from the Pannonian Basin (15-♂2 and 18-♂1). Given that *Ha-A103* (as well as *Med15*) display perfect lineage sorting by species in *Hyla arborea, H. molleri, H. intermedia* [5], these patterns cannot be accounted for by the maintenance of ancestral polymorphisms predating speciation, and thus provide clear evidence for XY recombination events, postdating species divergence.

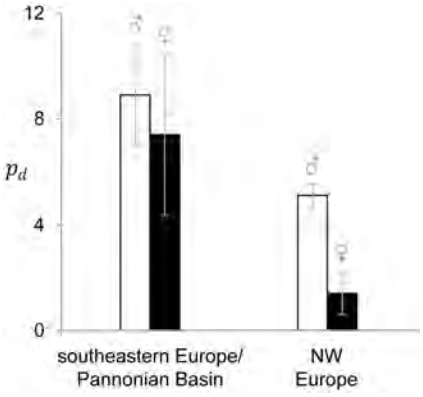

Figure 2. Pairwise nucleotide differences (p_d) between the two *Med15* alleles of every male (white bars) and female (black bars). Larger values are found in southeastern Europe and the Pannonian Basin (left) than in NW Europe (right), and, in the latter region, in males than in females.
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However, closer inspection reveals some differences between phylogeographic regions. In particular, the patterns of diversity and differentiation at *Med15* display a clear trend with geography (Fig. 1c). Whereas male alleles from southeastern refugial (orange) and Pannonian (violet) populations mix randomly with female alleles in the network, sex-differences occur for NW Europe (green, loc. 20–30). With the single exception of individual 29-♂1, all males harbor one and only one copy of allele H_{16}, otherwise identified from sibship analyses as the Y allele in several Swiss and French populations (referred to as allele *Ha5-22* 236 in [8,16]. This allele was only found in males throughout the *H. arborea* range covered by our study, including some from the Pannonian Basin (violet) and southeastern European glacial refugia (orange), where it might also occur on the Y. The second allele (i.e., the X copy) from all Western and Northern European *Hyla* males belongs to the H_1–H_{15} haplogroup, otherwise shared by all females from these populations, plus some from the Pannonian Basin. This was the only haplogroup found in females from Western and Northern Europe, but many more segregated in females from the Pannonian Basin and southeastern European refugial populations. Accordingly, a two-way ANOVA, with sex and geographic region (North-Western Europe *versus* Pannonian and southeastern populations) as factors, identified significant effects for both the region ($F_{1,49} = 12.5$, p-value = 0.009) and the interaction between sex and region ($F_{1,49} = 3.38$, p-value = 0.023). As shown in Fig. 2, this reflects respectively the higher diversity of southeastern European and Pannonian populations ($\overline{p_d} = 8.2$ as opposed to 3.3 in Northern and Western Europe) and the strong XY differentiation in North-Western Europe ($\overline{p_d} = 5.1$ in males *versus* 1.4 in females) compared to the rest of the range ($\overline{p_d} = 8.9$ in males *versus* 7.4 in females). The only exception (male 29-♂1, from the westernmost part of the distribution range) deserves special mention: given the strong differentiation between the haplotypes H_3 and H_4 harbored by this male on one hand, and the haplotype H_{16} fixed on the Y of other males on the other hand, the pattern observed is most likely to result from a recent (post-glacial) event of XY recombination.

No such interaction was identified for *Ha-A103* and *Smarcb1*, which might however result from a lower power due to smaller sample sizes, and to lower levels of polymorphism. Markers *Ha-*

A103 and *Smarcb1* were clearly less variable than *Med15*, and our range-wide samples appear to represent most of their diversity. The XY overlap found in NW Europe populations for these two markers are compatible with post-glacial XY recombination, but might also stem from ancestral polymorphisms shared by the X and Y chromosomes that contributed to the post-glacial expansion towards NW Europe.

Conclusion

Our study provides range-wide empirical evidence that X and Y chromosomes have exchanged genetic material until recently, and are possibly still recombining occasionally in the southern part of *H. arborea* geographic range. Although our results solve the apparent paradox mentioned in the Introduction, they also raise new questions, by suggesting that XY recombination rate might vary phylogeographically, being higher in refugial populations than in post-glacial-origin populations of NW-Europe. Patterns at *Med15* in particular suggest that different X and Y alleles have been fixed by drift in the wave of expanding populations, and were maintained differentiated over the last 10 ky. Whether the amount of recombination significantly between regions, however, remains to be clarified. More accurate reconstruction of the phylogeography of Y haplotypes, using a larger number of fast-evolving markers, should help to better characterize the spatial and temporal dynamics of recombination episodes in *H. arborea*.

Supporting Information

Table S1 Detailed sample information, including origin, nature, sex, number of clones sequenced and GenBank accession numbers.
(XLSX)

Alignment S1 Alignments of all three sequences markers (*Ha-A103*, *Med15* and *Smarcb1*) are provided along with this paper as Supporting Information.
(ZIP)

Acknowledgments

We are very grateful to the numerous people involved in the sampling and/or providing access to scientific collections (credited in Table S1, Supporting Information), and especially thank Z. Dumas and R. Sermier for their great help in the lab. **Data accessibility:** Sequences have been deposited in GenBank (*Smarcb1*: KJ789987–KJ790014; *Ha-A103*: KJ790015–KJ790056; *Med15*: KJ790057–KJ790148, and their accession numbers inserted in Table S1, Supporting Information.

Author Contributions

Conceived and designed the experiments: NP MS. Performed the experiments: MS CD AB. Analyzed the data: CD MS. Wrote the paper: CD MS. Contributed details and improvements: CD MS AB NP.

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CHAPTER III

SEX-CHROMOSOME DIFFERENTIATION PARALLELS POSTGLACIAL RANGE EXPANSION IN EUROPEAN TREE FROGS (*HYLA ARBOREA*)

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Sex-chromosome differentiation parallels postglacial range expansion in European tree frogs (*Hyla arborea*)

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Occasional XY recombination is a proposed explanation for the sex-chromosome homomorphy in European tree frogs. Numerous laboratory crosses, however, failed to detect any event of male recombination, and a detailed survey of NW-European *Hyla arborea* populations identified male-specific alleles at sex-linked loci, pointing to the absence of XY recombination in their recent history. Here, we address this paradox in a phylogeographic framework by genotyping sex-linked microsatellite markers in populations and sibships from the entire species range. Contrasting with postglacial populations of NW Europe, which display complete absence of XY recombination and strong sex-chromosome differentiation, refugial populations of the southern Balkans and Adriatic coast show limited XY recombination and large overlaps in allele frequencies. Geographically and historically intermediate populations of the Pannonian Basin show intermediate patterns of XY differentiation. Even in populations where X and Y occasionally recombine, the genetic diversity of Y haplotypes is reduced below the levels expected from the fourfold drop in copy numbers. This study is the first in which X and Y haplotypes could be phased over the distribution range in a species with homomorphic sex chromosomes; it shows that XY-recombination patterns may differ strikingly between conspecific populations, and that recombination arrest may evolve rapidly (<5000 generations).

KEY WORDS: **Amphibians, phylogeography, sex-chromosome evolution, XY recombination.**

The strongly differentiated sex chromosomes of many bird, mammal, and insect species have attracted much attention from evolutionary biologists very early on (Muller 1914, 1918; Fisher 1935; Ohno 1967). Converging empirical and theoretical studies concluded that sex chromosomes originate from autosomes (Ohno 1967). A first step in this evolution occurs when an autosomal gene, involved in the sex-determination cascade, mutates to such a form that individuals heterozygous for the mutation develop into one sex, whereas homozygotes develop into the other. As a second step, this new sex determinant will favor the spread of sexually antagonistic mutations in its vicinity. If autosomal, such mutations can only spread if the benefits conferred to one sex exceed the costs paid by the other; when tightly linked to the sex-determining mutation, however, they can spread even if strongly detrimental to the homogametic sex, being only rarely transmitted to this sex. Accruing sexually antagonistic genes will then, as a third step, favor a progressive arrest of XY recombination, so that male-beneficial alleles are always transmitted to sons, and female-beneficial alleles to daughters (Rice 1987, 1996;

Data archival: Adult and family microsatellite genotypes, including males phased sex haplotypes, have been archived http://doi.org/10.5061/dryad.45j84

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Charlesworth and Charlesworth 2000; Charlesworth et al. 2005). Note that, when evolving from a hermaphroditic state, genetic sex determination involves more than one mutation (one to suppress male fertility, the other to suppress female fertility), so that recombination arrest is also required to prevent the production of neuter individuals (Bachtrog et al. 2011).

As a side effect, however, recombination arrest necessarily induces a drastic drop in the effective population size of Y chromosomes due to a fourfold decrease in the number of copies per mating pair and Hill–Robertson interferences. Deleterious mutations will accumulate under the combined forces of Muller's ratchet, background selection, and selective sweeps, resulting in the progressive decay of the nonrecombining Y chromosome. Importantly, loss-of-function mutations create a gene imbalance that is bound to reduce fitness in the heterogametic sex. Some lineages accommodate this decay via dosage compensation, through either a global mechanism (such as found in mammals, flies, or nematodes), or a more subtle gene-by-gene regulation, such as found in birds or moths (Mank 2009). Alternatively, as theory suggests, this accumulating deleterious load may induce sex-chromosome turnovers (Blaser et al. 2013, 2014), or select for some nonzero level of XY recombination (Grossen et al. 2012). The evolutionarily stable rate of recombination that results from the conflicting forces of sexually antagonistic selection and deleterious mutations is expected to be quite low: very rare events of XY recombination seem sufficient to purge the load (reviewed in Grossen et al. 2012). Sex-chromosome turnovers and XY recombination are not mutually exclusive processes: both are thought to contribute to the rarity of differentiated sex chromosomes among poikilothermic vertebrates (e.g., Stöck et al. 2013a; van Doorn 2014).

In this context, European tree frogs present an interesting case. Several species from this radiation (namely *Hyla arborea*, *H. orientalis*, *H. intermedia*, and *H. molleri*; see Stöck et al. 2012 for phylogenetic relationships and species distributions) share the same pair of homomorphic sex chromosomes, inherited from a common ancestor more than five million years ago (Stöck et al. 2011, 2013b). The sequences of sex-linked genes along this chromosome cluster by species, not by gametologs, which implies some nonzero rate of XY recombination throughout all lineages (Stöck et al. 2011; Guerrero et al. 2012). Surprisingly, however, male recombination has never been documented in laboratory crosses, despite genotyping of thousands of offspring with known pedigree from several populations of all four species (e.g., Berset-Brändli et al. 2008a; Stöck et al. 2011, 2013b). In addition, males and females are genetically differentiated in all West- and North-European *H. arborea* populations investigated so far, with Y-specific alleles found at several sex-linked markers, attesting the absence of XY recombination in their recent evolutionary history.

To address this paradox, we undertook to seek insights into deeper events of *H. arborea*'s evolutionary history, by extending the phylogeographic scope of our investigations. North- and West-European *H. arborea* populations are very recent. As shown from autosomal markers and mitochondrial DNA (Dufresnes et al. 2013), refugial populations survived the last glaciations in southern Balkans and at the Adriatic coast, where three main haplogroups, with some 200 ky divergence, were maintained in distinct regions. Although two of these lineages remained limited to refugial areas (Adriatic coast and southern Balkans), a third one expanded after the Last Glacial Maximum (about 12–15 kya) to recolonize first the Pannonian Basin (a large lowland area of Central Europe surrounded by the Alpine, Carpathian, Dinaric, and Balkan mountain ranges), followed by Western and Northern Europe, losing much of its diversity in the process (Dufresnes et al. 2013). By analyzing the sequences of three sex-linked markers throughout the whole species distribution, Dufresnes et al. (2014) uncovered a significant effect of geography on XY differentiation at the *Med15* gene: contrasting with recent NW-European populations, which showed a clear pattern of XY differentiation, populations from ancient refugia (southern Balkans, Adriatic coast) and Pannonian Basin displayed a mix of X and Y alleles in haplotype networks.

The question then arises: Is this pattern specific to a single gene, or does it extend to other sex-linked markers? And if general, does the increased XY differentiation stem from the enhanced genetic drift that accompanies colonization events, or does it instead signal a decrease in XY recombination rate with range expansion? In the present study, we provide a phylogeographic analysis of sex-chromosome haplotypes in *H. arborea*. By sampling adults throughout their geographic range as well as offspring from family crosses, and genotyping them with a series of sex-linked microsatellite markers, we could phase X and Y haplotypes throughout the species distribution, and thereby reconstruct the recent evolutionary history of *H. arborea* sex chromosomes. Our specific aims were to further document the consequences of the species' recent range expansion on the genetic diversity of X and Y chromosomes, genetic differentiation between sexes, and dynamics of XY recombination.

Methods

SAMPLING, DNA EXTRACTION, AND GENOTYPING

Our phylogeographic survey included 670 field-captured individuals (416 adult males, 175 adult females, and 79 larvae) from 42 populations, all but one (loc. 66) analyzed by Dufresnes et al. (2013), and from which we kept the same locality numbers. File S1 summarizes information on sample types, localities, and sizes. DNA samples were collected using noninvasive buccal swabs

(adults, Broquet et al. 2007) or ethanol-fixed tissues (larvae). In addition, some males and females were mated in the field (see Dufresnes et al. 2011 for methods), and a subset of their progeny fixed in ethanol after hatching (on average ~20 offspring per family). As there is no sperm storage in frogs (fertilization is external), all offspring obtained were necessarily full sibs (as otherwise confirmed by genotypes). A total of 55 families (1176 offspring) from 17 populations were suitable for sibship analyses. For one family from population 1 (Crete), we additionally raised 16 offspring to several weeks after metamorphosis for anatomical/histological sexing (following Ogielska and Kotusz 2004; Haczkiewicz and Ogielska 2013).

DNA was extracted with the Qiagen Biosprint workstation. For all individuals (field samples and families), we amplified (using four multiplex PCR reactions) and genotyped 14 microsatellite markers from linkage group 1, shown to be sex-linked in all species from the *H. arborea* group studied so far (see Arens et al. 2000; Berset-Brändli et al. 2008a,b; Stöck et al. 2011; Brelsford et al. 2013; and File S2 for details and methods). To identify the genetic sex of the 79 larvae sampled in the field, we genotyped an SNP in the sex-linked gene *Dmrt1* (Brelsford et al. 2013), which perfectly associates with phenotypic sex across the whole *H. arborea* range (A. Brelsford et al. unpubl. ms.).

RECOMBINATION RATES, SEX LINKAGE, AND SEX HAPLOTYPES

Sex-specific recombination rates were measured from family data using CRIMAP (Green et al. 1990). The order of loci was determined using the function *all* based on the entire dataset because some markers were not polymorphic in all geographic regions. Sex-specific recombination distances were then calculated using the function *build*, and linkage maps constructed with MAPCHART (Voorrips 2002) independently for the main phylogeographic regions identified by Dufresnes et al. (2013). Morton's *M*-tests were computed to test whether recombination rates differ between geographic regions (Morton 1956).

In the absence of XY recombination, X and Y chromosomes are expected to diverge, allowing assessment of sex linkage through sex-specific differences in allele frequencies. Sex linkage was indeed obvious in many Central- and NW-European populations (as previously shown, e.g., by Berset-Brändli et al. 2006; Stöck et al. 2011). In one population with low XY differentiation (loc. 1, Crete), sex linkage was independently confirmed by phenotypic sexing of offspring from a full sibship.

Sex haplotypes were inferred from males in two ways. In Central- and NW-Europe populations, X and Y alleles were readily identified from adult genotypes based on sex differences in allele, genotype, and haplotype frequencies: males were consistently heterozygous for certain alleles, and these male-specific alleles formed identifiable Y haplotypes that recurred within populations or in neighboring ones (see File S3, loc. 56, for an illustration). In refugial populations (southern Balkans and Adriatic coast), X and Y chromosomes often shared the same alleles (see Results), but could still be phased based on sex differences in allele and haplotype frequencies (the same Y allele or haplotype was often fixed in all males from the same population) combined with pedigrees: thanks to the extremely low rate of recombination in males (see Results), genetic maps obtained from family data allowed us to infer the entire phase whenever the Y allele at any sex-linked locus was identifiable (see File S4, loc. 1, for an illustration). Independent validation of this approach was provided in the population of loc. 1 (Crete) by direct phenotypic sexing of offspring in one family.

XY DIFFERENTIATION, GENETIC STRUCTURE AND DIVERSITY

Genetic differentiation between males and females (F_{ST} per locus) was computed for all populations with at least seven individuals of each sex (FSTAT 2.9.3; Goudet 1995). Furthermore, in populations where sex haplotypes could be recovered from at least five males, we calculated the average overlap (over the n loci) between X and Y allele frequencies as $\frac{1}{n}\sum_i \sum_j \mathrm{Min}\left[p_{ijX},\ p_{ijY}\right]$, where p_{ijX} and p_{ijY} represent the frequency of allele j at locus i in X and Y haplotypes, respectively.

We conducted individual-based Bayesian clustering of X and Y haplotypes with Structure 2.3.3 (Pritchard et al. 2000), testing from one to 11 groups (K). Ten replicates were performed for each K value and the Evanno method (Evanno et al. 2005) was applied to identify the most appropriate clustering solution (STRUCTURE HARVESTER, Earl and VonHoldt 2012). For graphical visualization, we first combined replicate runs with CLUMPP (Jakobsson and Rosenberg 2007), and then used DISTRUCT (Rosenberg 2004) to build barplots. We also performed a principal component analysis (PCA) to visualize the main factors of haplotype differentiation (R package *adegenet*; Jombart 2008). Allelic richness k and expected heterozygosity H for X and Y haplotypes were calculated (FSTAT) in all populations with at least five males, and compared with published autosomal data (Dufresnes et al. 2013). Allelic richness measures the average number of alleles per locus in a population (standardized for sample size), and is expected to be more sensitive to founder effects than H. Furthermore, the genetic diversity index θ was calculated from H as $\theta = \frac{1}{2}\left(\frac{1}{(1-H)^2} - 1\right)$, assuming a stepwise mutation model (Kimura and Ohta 1975). At neutral equilibrium, the θ value for locus i is expected to reflect the effective population size N_e, mutation rate μ_i, and number of copies per breeding pair c_i: $\theta_i = c_i N_e \mu_i$. Thus, values for X- and Y-linked markers should represent ¾ and ¼ of autosomal values, respectively, assuming similar effective population sizes and

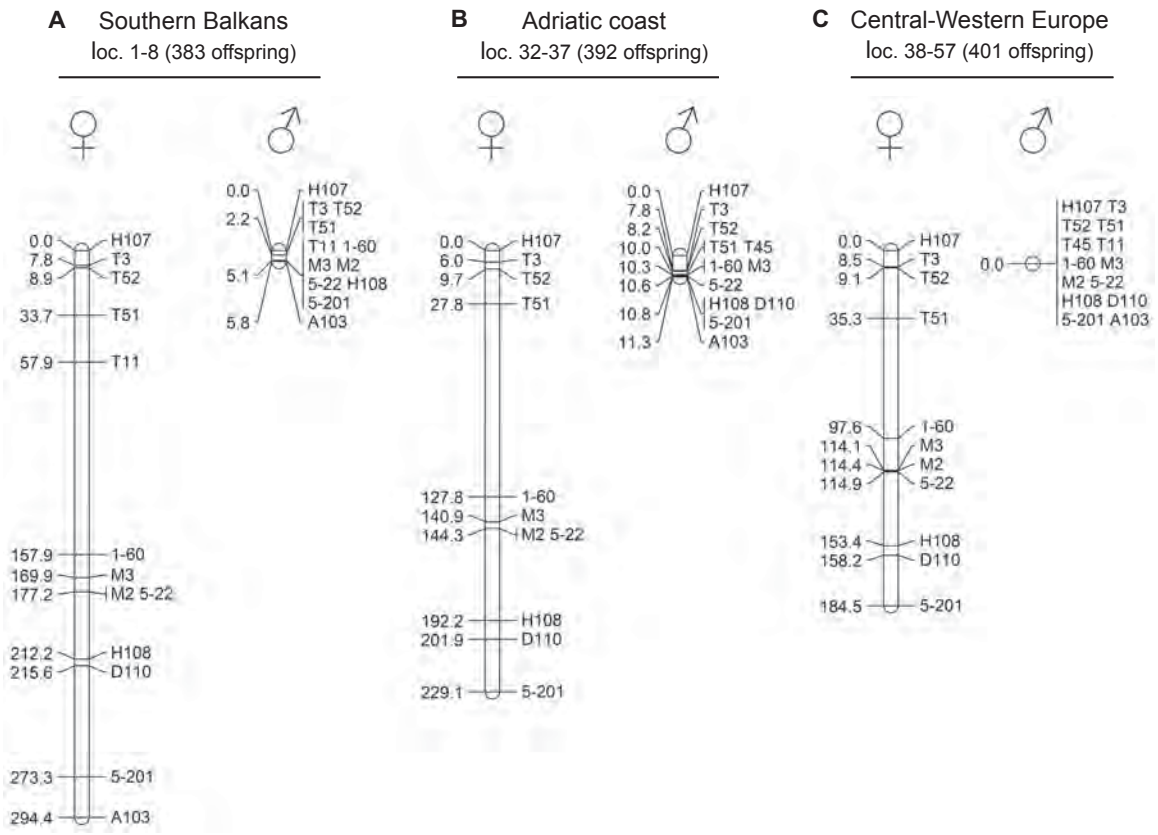

Figure 1. **Regional sex-specific linkage maps of *H. arborea*'s sex chromosomes, based on 14 microsatellites. Recombination distances between markers (centiMorgan) are indicated. For clarity, simplified marker labels are used.**

mutation rates, and absence of recombination. Geographic distance of each population from the refugial area (delimited as south of the Dinaric and Balkan mountain ranges) was computed to analyze the effect of postglacial recolonization on allelic richness and sex-chromosome differentiation. The geographic distances calculated were corridor-type, taking into account the possible routes for migration connecting populations (see main text and Data S2 in Dufresnes et al. 2013 for details).

Results

RECOMBINATION MAPS

From our sibship analyses, recombination rates differed significantly not only between sexes, but also between geographic regions (Fig. 1; Table 1). XY recombination occurred at low levels in males from refugial populations of the southern Balkans (Fig. 1A) and Adriatic coast (Fig. 1B), but was absent in postglacial populations of Central and NW Europe (loc. 38–57; Fig. 1C, Table 1). Female recombination was high over all regions, although slightly reduced in postglacial populations (Fig. 1C) relative to refugial ones (Fig. 1A, B), but these differences were not significant based on Morton tests (Table 1).

Table 1. **Morton tests of recombination rate heterogeneity between regions, combined over sexes, and for males and females separately.**

	χ^2	df	*P*
Sexes combined			
S-Balkans versus N-Adriatic	72.6	56	0.07
S-Balkans versus Central/NW Europe	133.8	71	**<0.001**
N-Adriatic versus Central/NW Europe	193.0	73	**<0.001**
Among all regions	297.4	176	**<0.001**
Females			
S-Balkans versus N-Adriatic	25.7	39	0.95
S-Balkans versus Central/NW Europe	29.4	45	0.97
N-Adriatic versus Central/NW Europe	26.6	48	0.99
Among all regions	57.6	106	1.00
Males			
S-Balkans versus N-Adriatic	52.4	43	0.15
S-Balkans versus Central/NW Europe	108.3	62	**<0.001**
N-Adriatic versus Central/NW Europe	160.6	66	**<0.001**
Among all regions	238.9	170	**<0.001**

Statistically significant values are indicated in bold.

GENETIC DIFFERENTIATION BETWEEN THE SEXES

Differentiation between the sexes varied significantly with geography, with a trend of increased F_{ST} with distance from refugia

(File S5; Fig. 2B). Differentiation was strong in postglacial populations (Pannonian Basin and NW Europe), where all loci but three carried male-diagnostic alleles (File S6). The exceptions were *Ha*-T52, *Ha*-T3, and *Ha*-H107, which mapped to the terminal position on the linkage map (Fig. 1) and featured low male–female F_{ST} in all populations (File S5). This strong sex differentiation allowed identification of one sex-reversed (XY) female, caught in amplexus with an XY male in population 40 (Pannonian Basin), which carried a male allele at each informative marker (File S7). This individual was removed from the subsequent population genetics analyses.

In contrast, sex differentiation was much lower in refugial populations (Adriatic coast and southern Balkans), with a sharing of male and female alleles at most loci (File S6). However, sex linkage was still unambiguous in all populations, with significant sex differences in allele frequencies at several loci (File S6). In the population from Crete investigated for offspring phenotypic sexes, sibship data unambiguously confirmed sex linkage, with a clear pattern of male heterogamety: one paternal haplotype (Y) was inherited by all sons, and the other (X) by all daughters ($\chi^2 = 16.0$, $P < 0.0001$, df $= 1$).

XY DIVERSITY AND DIFFERENTIATION

X and Y haplotypes could be phased for 11 markers in a total of 458 males from 41 populations. The θ values of diversity, calculated from expected heterozygosity *H*, were significantly lower for Y than for X haplotypes (0.15 ± 0.11 vs. 0.73 ± 0.35, respectively, averages and SD over loci and populations; ANOVA: $F_{1,44} = 55.8$, $P < 0.0001$, based on 10,000 permutations), and both were reduced compared to autosomal values (1.44 ± 0.77). In both cases, the ratios obtained (0.10:0.51:1) are lower than expected (¼:¾:1) assuming similar effective population sizes and mutation rates. Allelic richness was also significantly lower for Y than for X haplotypes, and both had reduced values compared to autosomal markers (Fig. 2C, data from Dufresnes et al. 2013). Richness was homogeneously low for Y markers over the entire geographic range, but decreased significantly with distance from refugial areas for X and autosomal markers (Fig. 2C). Diversity statistics of X and Y haplotypes are available for each locus and population in File S8. In line with the patterns of sex differentiation (Fig. 2B), XY overlap in allele frequencies decreased significantly with distance from refugial areas (Fig. 2A), being highest in southern Greece, and lowest in NW Europe (see also Fig. 3B).

The same trend in XY differentiation was uncovered by Bayesian assignments of individual haplotypes using STRUCTURE (Fig. 3): X and Y haplotypes cluster together in southernmost populations (Crete and Peloponnese), but not in the postglacial populations of Central and NW Europe, a result that holds across different *K* values (File S9). The best clustering

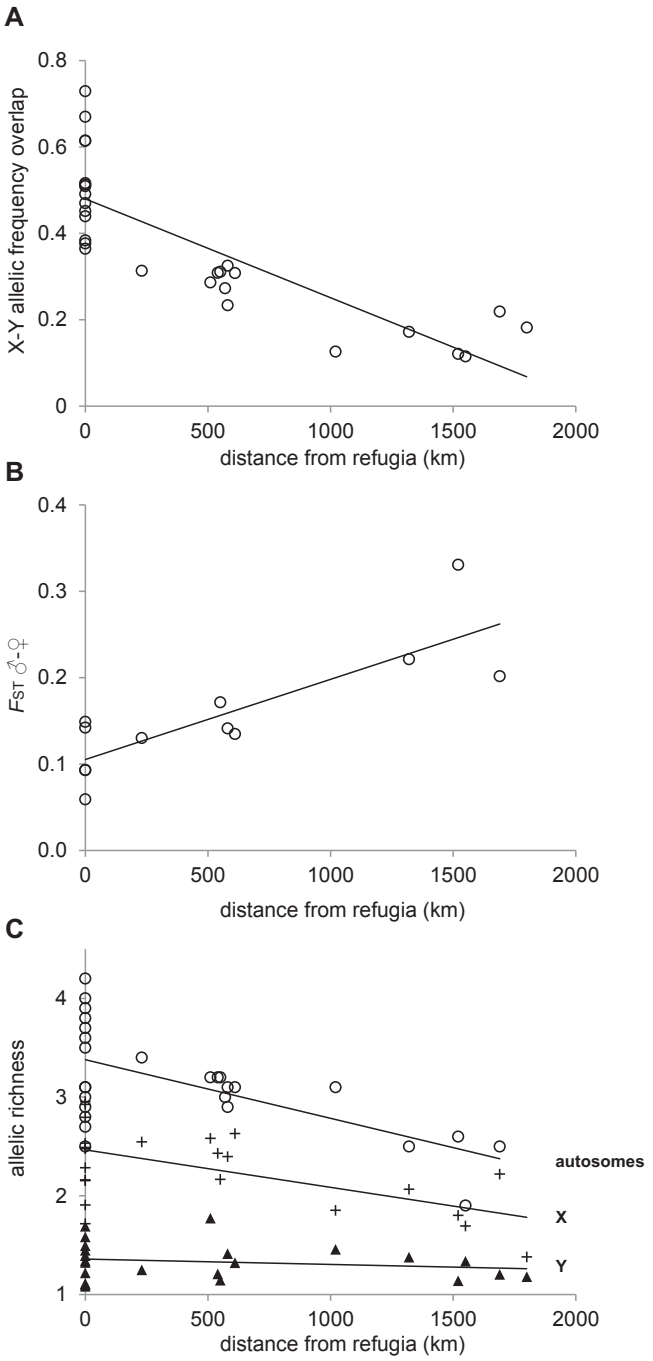

Figure 2. **Relationship between geographic distance from glacial refugia and X–Y overlap of allele frequencies (A), male–female F_{ST} (B) and allelic richness (C). Allelic richness was computed for autosomes (data from Dufresnes et al. 2013), X and Y haplotypes, all scaled to five individuals. Linear regressions are significant for autosomes ($R^2 = 0.39$, $F_{1,25} = 16.1$, $P < 0.001$) and X haplotypes ($R^2 = 0.35$, $F_{1,21} = 11.2$, $P = 0.003$), but not Y haplotypes ($R^2 = 0.03$, $F_{1,21} = 0.74$, $P = 0.40$). Allelic richness, and its relationship with the distance from refugial areas both significantly differ between marker sets (marker sets: ANCOVA's $F_{2,67} = 172.1$, $P < 0.001$; interaction between marker sets and distance: ANCOVA's $F_{2,67} = 5.6$, $P = 0.005$).**

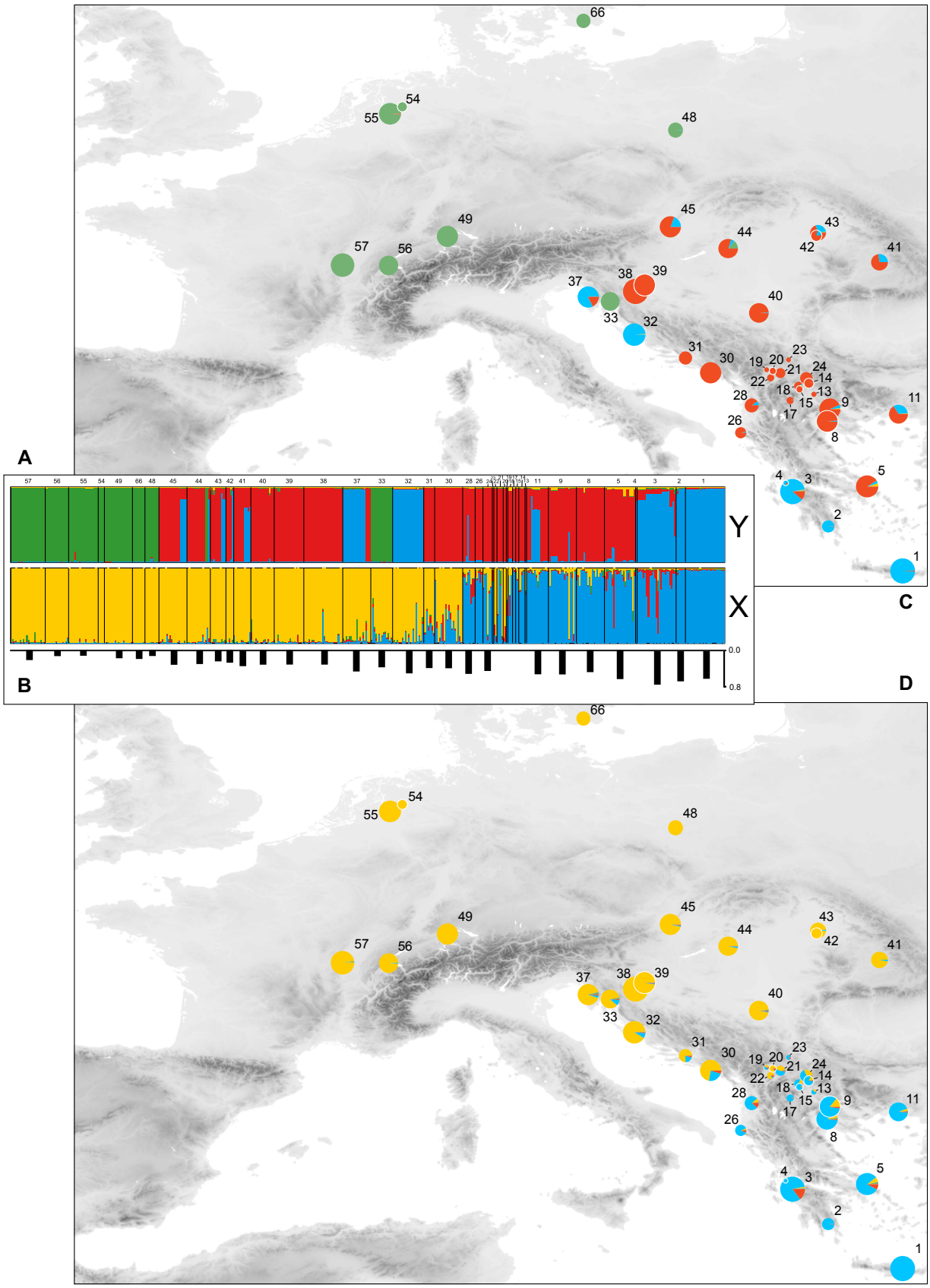

Figure 3. Best individual clustering assignment (STRUCTURE, K = 4) of male sex-linked haplotypes (A) and overlap of X and Y allelic frequencies (B). The maps show the average assignment (STRUCTURE) of each population for Y haplotypes (C) and X haplotypes (D).

solution comprises four groups ($K = 4$), associated with gametologs and geography (Fig. 3). X haplotypes are split in two clusters (Greece vs. N-Adriatic/Central/NW Europe) and Y haplotypes in three (southern Greece/N-Adriatic vs. Balkans/Central Europe vs. NW Europe), with the southernmost cluster (blue in Fig. 3) including both X and Y haplotypes. Interestingly, this cluster also comprises Y haplotypes north of the Adriatic coast (loc. 32 and 37), whereas other males from the region (including the close-by population of Krk island, loc. 33) harbor the Y haplotypes characteristic of the Pannonian Basin (red cluster) and NW-Europe populations (green cluster), the latter being also present at low frequency in the Pannonian loc. 44.

Similar patterns result from the PCA analyses (Fig. 4). The first axis (horizontal in the upper panel; 7.2% of variance explained) accounts for the progressive differentiation of X haplotypes from southern Greece (blue crosses on the right) to NW Europe (green crosses on the left). The second axis (vertical in the upper panel, horizontal in the lower panel; 6.1% of variance explained) opposes the Y haplotype group that expanded to NW Europe (dark green triangles; green cluster on STRUCTURE graphs) to all others. The third axis (vertical in the lower panel; 3.4% of variance explained) accounts for the high genetic diversity within the southern-Greece group of X and Y haplotypes (dark blue crosses and triangles; blue on the STRUCTURE graphs). This high diversity may seem to contrast with the low richness values documented for Y haplotypes throughout refugial areas (Fig. 2C), but this apparent discrepancy is easily solved by noting that Figure 2C plots within-population diversity (allelic richness per locality), whereas the spread of haplotypes along the third PCA factor (Fig. 4) expresses among-population diversity: Y chromosomes within populations tend to fix one or few alleles from the local distribution, which differ between populations. The three axes considered here only account for $\sim$17% of the total variance, meaning that a large part of the intra- and interpopulation variance is not accounted for in these plots. This is however to be expected because each of the 106 axes (corresponding to the total number of alleles) accounts for $<$1% of the variance on average (see plot of ordered eigenvalues in Fig. 4). Both panels clearly illustrate the strong XY differentiation in NW Europe (dark green symbols), contrasting with the high XY overlap in southern Balkans. The least differentiated X and Y haplotypes are found in southern Greece (dark blue), whereas haplotypes from northern Greece and the Pannonian Basin show intermediate levels of differentiation. Three discrete Y clusters of the Adriatic coast are visible (yellow triangles), with one haplogroup (loc. 32 and 37) clustering with Balkanic X haplotypes (yellow and purple crosses), the second shared with Y from the Pannonian Basin (light green triangles), and the third (loc. 33, Krk island) clustering with NW-European Y haplotypes (dark green triangles).

Discussion

PATTERNS OF XY GENETIC DIVERSITY AND GEOGRAPHIC STRUCTURE

Genetic diversity was much lower on sex chromosomes than on autosomes (Fig. 2C). The θ values for X and Y chromosomes only represent one-half and one-tenth of autosomal values, respectively, pointing to the action of Hill–Robertson interferences in addition to the mere decrease in the number of copies per mating pair. Notwithstanding this lower overall diversity, X chromosomes displayed very similar patterns to those found in autosomes (Dufresnes et al. 2013). The same signature of expansion could be identified, namely a significant decline in allelic richness with distance from refugia (Fig. 2C). Geographic structures also showed strong parallelism: STRUCTURE identified two main groups, one dominant in Greece (pops 1–28), the other spread through the Adriatic coast, Pannonian Basin, and NW Europe (compare our Fig. 3D with Fig. 4 in Dufresnes et al. 2013).

In contrast, Y chromosomes displayed different patterns from autosomes and X chromosomes, revealing their distinct dynamics. First, allelic richness was homogeneously reduced, independent of distance from refugia (Fig. 2C). Second, Bayesian clustering identified three main groups of haplotypes, with strong geographic structuring but complex distribution (Fig. 3C). One haplogroup with high similarity to local X haplotypes dominates in the refugial populations of southern Greece and Adriatic coast. A second group, mildly differentiated from local X haplotypes, is largely spread from the northern Greek refugium to the Pannonian Basin. A third main haplogroup, strongly differentiated from local X haplotypes, characterizes the populations that expanded into NW Europe, but also occurs in one population from the Pannonian Basin (loc. 44), and seems fixed on the island of Krk (Adriatic coast, loc. 33), which was also recently invaded by expanding Central-European frogs (Data S2 in Dufresnes et al. 2013). Such patterns are expected from the neutral processes of genetic drift and gene surfing on the front wave of expansions (Excoffier et al. 2009), but one cannot a priori exclude a role for selective sweeps of haplotypes favored by male-beneficial combinations of sexually antagonistic genes.

PATTERNS OF XY DIFFERENTIATION AND RECOMBINATION

Two lines of evidence point to a geographic trend in XY recombination. First, the genetic maps obtained from 55 families (17 populations) reveal a significant reduction in recombination with postglacial range expansion. Male recombination, which occurs at a nonzero level in refugial populations, is completely absent in postglacial populations from the Pannonian Basin and NW Europe. Second, indirect evidence comes from the increased XY differentiation with distance from refugia. Negligible in southern

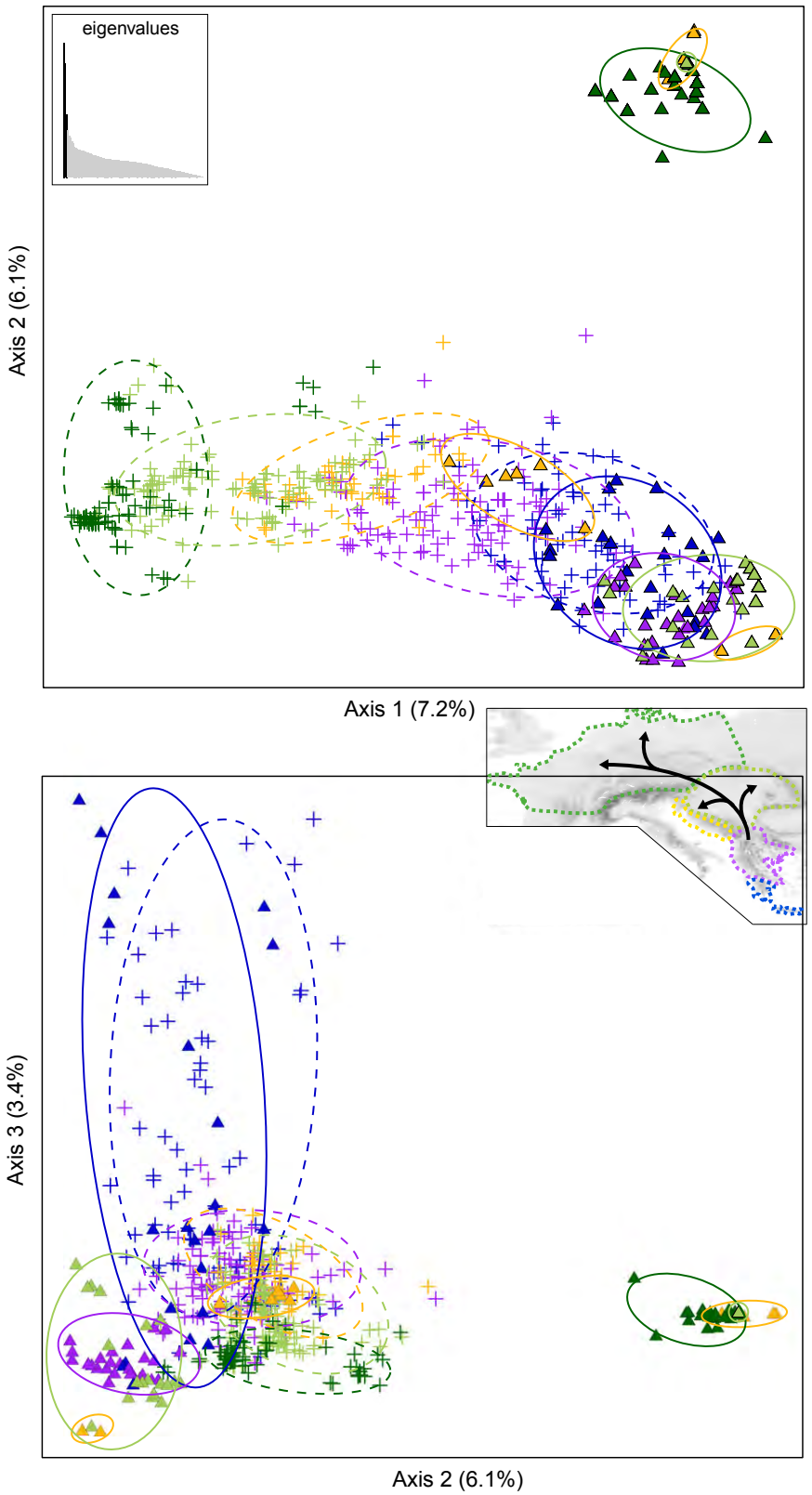

Figure 4. Principal component analysis (PCA) on allele frequencies for X (crosses) and Y (triangles) microsatellite haplotypes (based on 11 loci, 106 alleles in total). Colors discriminate between the main biogeographic regions: S-Balkans refugia (blue, purple), N-Adriatic coast refugium (yellow), Pannonian Basin (pale green), and NW Europe (dark green). Arrows indicate routes of postglacial recolonization. Also provided is the distribution of the 106 eigenvalues.

Greece (despite direct evidence for sex linkage gathered from sibship analyses), differentiation becomes marked in the Pannonian Basin, and is strongest in the most recently established populations of NW Europe, except for three loci in a distal position that might still display occasional recombination (although too rare to be observed in family studies). This corroborates sequence data from the sex-linked gene *Med15* (Dufresnes et al. 2014), for which NW-European populations show a male-specific allele, whereas both sexes share the same alleles in the Balkans. From our results, therefore, the stronger XY differentiation in NW Europe does not result solely from the genetic drift that affects expanding populations, but clearly associates with a more complete arrest of male recombination. It should be noted that despite similar absence of recombination in family studies, X and Y haplotypes are less differentiated in the Pannonian Basin than in NW Europe. This difference might stem from lower genetic drift (fewer bottlenecks during range expansion and larger local population sizes), residual recombination (although still too rare to be detected in our family studies) or inflow of Y haplotypes from southern (refugial) populations.

Using an approximate Bayesian computation (ABC) framework, Guerrero et al. (2012) estimated the amount of XY recombination to be about 10^{-5} lower than XX recombination in European tree frogs. This value was obtained assuming a homogeneous rate across times and lineages. As our present results show, this latter assumption may not entirely hold, and parameter estimates might require some reevaluation. Interestingly, however, XY recombination is clearly too rare, even in the southernmost populations, to counteract the strong genetic drift affecting Y chromosomes and refuel their allelic richness (Fig. 2C). A new analysis implemented using the values of within- and between-population diversity and differentiation of X and Y haplotypes in an ABC framework might help to further characterize the interplay between drift and recombination, and their relative influences on the evolutionary dynamics of young sex chromosomes.

This geographic trend in XY recombination raises a series of questions regarding ultimate causes and evolutionary implications. Several alternative interpretations are compatible with our data. On one hand, the trend might simply result from phenotypic plasticity. Postglacial populations are experiencing novel environments; differences from refugial areas, notably in terms of climate, are likely to affect many physiological processes, the more so in ectotherms. Meiotic recombination has been shown to vary with temperature in a diversity of organisms, including insects (e.g., Plough 1917; Hayman and Parsons 1960; Grell 1978), nematodes (Rose and Baillie 1979; Lim et al. 2008), and ascomycetes (McNelly-Ingle et al. 1966; Lamb 1969; Saleem et al. 2001). Other environmental factors have been shown to play a role, leading up to the idea that recombination rate increases with several forms of stress (Parsons 1988; Zhong and Priest 2011). In the present case, however, a reverse trend would be expected, namely an increased recombination rate in the new, stressful postglacial environments. Reciprocal transplant experiments should help to test this point.

On the other hand, regional differences might be genetic. Recombination is known to differ between *Drosophila* lines, and to respond quickly to selection (Chinnici 1971a,b; Kidwell 1972; Charlesworth and Charlesworth 1985; Brooks and Marks 1986; Brooks 1988). In mammals, multiple loci contribute to genome-wide recombination (Murdoch et al. 2010); allelic variation at the *Prdm9* gene was recently shown to account for a large component of intra- and interpopulation variance in recombination patterns (Baudat et al. 2010; Berg et al. 2010; Fledel-Alon et al. 2011). Crossing individuals from refugial and postglacial populations and analyzing recombination in the offspring should reveal the genetic architecture of XY recombination in tree frogs.

Assuming a genetic component, recombination arrest might merely arise from the fixation by genetic drift of recombination suppressor alleles in postglacial populations. This hypothesis seems plausible if recombination is controlled by one or two genes with few alleles, but much less so if multiple loci are involved (recombination arrest would require the fixation of the no-recombination allele at each locus). Alternatively, recombination arrest might arise from selection: evolutionarily stable rates of recombination result from a balance between sexually antagonistic selection (which favor a complete arrest of recombination) and the accumulation of deleterious mutations (which favor some recombination). Selection was invoked to account for the recent extension of the nonrecombining region of *Silene latifolia* sex chromosomes, based on the short evolutionary time involved (few million years; Bergero et al. 2013). In the case of tree frogs, the time involved is two orders of magnitude shorter, suggesting stronger selective pressures. A possible scenario might be that Y haplotypes with male-beneficial alleles, spread by selective sweeps during range expansion, indirectly selected for recombination arrest. It is not clear, however, why sexual selection would be stronger in postglacial than in refugial populations.

CONCLUSIONS AND PERSPECTIVES

This study provides several important new insights on the evolutionary dynamics of young sex chromosomes. Recombination arrest is generally assumed to constitute a crucial initial step in this context, and takes a core place in classical models of sex-chromosome evolution. Recent work on European tree frogs has challenged this view by providing indirect but compelling evidence that recombination arrest is not a necessary step in the evolution of sex chromosomes (Stöck et al. 2011; Guerrero et al. 2012). Here, we provide direct evidence not only for XY recombination, but also for differences between conspecific populations that diverged $<$15 ky ago (i.e., $<$5000 generations). This shows that populations from different geographic or environmental

contexts may differ with respect to this crucial feature, and that changes in XY recombination rates can proceed quickly. These results open unique opportunities to investigate the selective forces acting on sex-chromosome recombination and the evolutionary consequences of recombination arrest. Contrasting with the XY recombination in southern populations, which occurs at a level sufficient to prevent the differentiation and decay of Y chromosomes (although insufficient to refuel their genetic diversity), the complete arrest of recombination documented in NW-European populations should favor the accumulation of both sexually antagonistic and deleterious mutations. Reciprocally, the limited recombination rate in southern populations opens opportunities to identify the sex-determination gene(s), by searching genomic regions displaying fixed sex differences (such an approach would not be feasible in NW-European populations, given that sex haplotypes are transmitted as a whole). Moreover, our study is the first in which X and Y haplotypes could be phased over the distribution range of a species with homomorphic sex chromosomes. This result provides access to the contrasting dynamics of nascent X and Y haplotypes during range expansion, and similarly opens unique opportunities to investigate the dynamics of X and Y diversity and differentiation under the evolutionary forces of genetic drift and XY recombination.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

File S1. Detailed sampling information; locality number corresponds to Dufresnes et al. (2013); larvae were genetically sexed (see main text).
File S2. Information on the microsatellite markers used and protocol.
File S3. Identification of Y haplotypes from adult data (here loc. 56); note that this was not possible for a few peripheral markers (here *Ha*-H107, *Ha*-T3) with only little male–female differences in allelic frequencies; M, male; F, female; 0, null allele.
File S4. Identification of sex-linked haplotypes from sibship data (here a family from loc. 1); knowing that *Ha*-H108 allele 263 is fixed on the Y in this population (and that WHA5-201 allele 252 segregates almost exclusively in males), it is possible to reconstruct the phase of the male X (pink) and Y (blue) based on linkage disequilibrium; 0, null allele.
File S5. Male–Female F_{ST} (in populations when $n > 7$ individuals of each sex); relative position on the female linkage map (as estimated from all families; cM, centiMorgan) is given.
File S6. Sex-specific allelic frequencies of microsatellite markers in populations where $n > 7$ individuals of each sex.
File S7. Sex-linked genotypes of adult tree frogs from loc. 40; identified Y haplotypes are colored; female BF03 (bottom) possesses male genotypes and corresponds to a sex-reversed XY female (F*); 0, null alleles.
File S8. Diversity indices of X and Y haplotypes per locus and per population with at least five phased males.
File S9. Evanno's Delta *K* test (best for $K = 4$) and Bayesian clustering of males' X and Y microsatellite haplotypes in two to six groups.

PART 2

EVOLUTION OF SEX CHROMOSOMES IN PALEARCTIC TREE FROGS

CHAPTER IV

CRYPTIC DIVERSITY AMONG WESTERN PALEARCTIC TREE FROGS: POSTGLACIAL RANGE EXPANSION, RANGE LIMITS, AND SECONDARY CONTACTS OF THREE EUROPEAN TREE FROG LINEAGES (*HYLA ARBOREA* GROUP)

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Cryptic diversity among Western Palearctic tree frogs: Postglacial range expansion, range limits, and secondary contacts of three European tree frog lineages (*Hyla arborea* group)

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ABSTRACT

We characterize divergence times, intraspecific diversity and distributions for recently recognized lineages within the *Hyla arborea* species group, based on mitochondrial and nuclear sequences from 160 localities spanning its whole distribution. Lineages of *H. arborea, H. orientalis, H. molleri* have at least Pliocene age, supporting species level divergence. The genetically uniform Iberian *H. molleri*, although largely isolated by the Pyrenees, is parapatric to *H. arborea*, with evidence for successful hybridization in a small Aquitanian corridor (southwestern France), where the distribution also overlaps with *H. meridionalis*. The genetically uniform *H. arborea*, spread from Crete to Brittany, exhibits molecular signatures of a postglacial range expansion. It meets different mtDNA clades of *H. orientalis* in NE-Greece, along the Carpathians, and in Poland along the Vistula River (there including hybridization). The East-European *H. orientalis* is strongly structured genetically. Five geographic mitochondrial clades are recognized, with a molecular signature of postglacial range expansions for the clade that reached the most northern latitudes. Hybridization with *H. savignyi* is suggested in southwestern Turkey. Thus, cryptic diversity in these Pliocene *Hyla* lineages covers three extremes: a genetically poor, quasi-Iberian endemic (*H. molleri*), a more uniform species distributed from the Balkans to Western Europe (*H. arborea*), and a well-structured Asia Minor-Eastern European species (*H. orientalis*).

1. Introduction

For many European vertebrate species including amphibians, phylogeographic hypotheses have been established in the last decade (for review: Hewitt, 2011). While morphological or behavioral traits mark the boundaries of some species (e.g. Fijarczyk et al., 2011), the situation is less clear for sibling species and cryptic lineages, which are revealed only by the recent application of molecular markers (e.g. Stöck et al., 2006; Teacher et al., 2009; Hauswaldt et al., 2011; Recuero et al., 2012; Bisconti et al., 2011; Garcia-Porta et al., 2012). Western Palearctic tree frogs of the *Hyla arborea* group provide a good example (Faivovich et al., 2005; Smith et al., 2005; Wiens et al., 2005, 2010). Until recently, most European populations were considered to belong to a single species, *H. arborea* (e.g. Schneider and Grosse, 2009; http://www.iucnredlist.org/apps/redlist/details/10351/0), except for the Apennine Peninsula (plus Sardinia and Corsica), where *H. intermedia* (resp. *H. sarda*) had been assigned species status, confirmed by the lack of introgression at a contact zone with *H. arborea* (Verardi et al., 2009). A phylogenetic analysis based on 3200 bp of mitochondrial and 860 bp of coding nuclear DNA (Stöck et al., 2008a) revealed this former, wide-ranging *H. arborea* to comprise three highly diverged lineages: *H. arborea*, occurring from Greece to northwestern France including Central Europe with the restricted type locality (Zurich; Dubois, 1996); *H. molleri* (previously considered a subspecies of *H. arborea*), known from the Iberian Peninsula; and *H. orientalis*, ranging from Asia Minor to northeastern Europe, and not previously distinguished from *H. arborea*. Phylogenies based on mtDNA show that *H. molleri* and *H. orientalis* are as much diverged from *H. arborea* as is the recognized species *H. intermedia*, hence supporting a similar taxonomic status.

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Based on phylogeographic patterns of European anurans (Stöck et al., 2006; Hofman et al., 2007), and given the constraints imposed by high altitudes and latitudes on the relatively thermophilic European tree frogs (e.g. Schneider and Grosse, 2009), we hypothesized that the Carpathians, Alps and Pyrenees play major roles in maintaining allopatry (leaving potential for secondary contacts and hybridization in surrounding lowlands). The age of hybridizing lineages experiencing secondary contact after Quaternary separation may vary from Late Pleistocene to the Late Miocene (cf. Hewitt, 2011). In the context of our previous work on tree frogs (Stöck et al., 2008a), we further expect to find varying amounts of geographic genetic structuring within these lineages, with distinctly lower genetic diversity in the northern regions and higher endemism in the southern ones, which have had a relatively more stable climate since the Last Glacial Maximum (Sandel et al., 2011).

Given that the whole *cytochrome b* has been shown to be the most informative of several tested mitochondrial markers (Stöck et al., 2008a; Gvozdik et al., 2010), we used this mitochondrial and one nuclear intronic marker to delineate the ranges of these three species lineages, evaluate intraspecific diversity, estimate divergence times, examine signatures of postglacial range expansions, and localize secondary contacts.

2. Methods

2.1. Amplification, cloning, and alignment of sequences

Samples of 462 frogs covering the whole European *Hyla* distribution (Fig. 1) were collected from living adults (buccal swabs), tadpoles (tail tips), or tissues from adult voucher individuals stored in scientific collections (Appendix S1). Buccal swabs were stored at −20 °C, tissue samples in 100% ethanol. DNA was extracted with Qiagen DNeasy Tissue Kit or the BioSprint robotic workstation (Qiagen), eluted in a 200 µl Qiagen Buffer AE and stored at −18 °C. The mitochondrial cytochrome *b* (ca. 1 kb) was amplified with primers L0 and H1046 as described (Stöck et al., 2008a). To amplify ca. 545 bp of intron 1 of *Fibrinogen A*, *alpha-polypeptide*, we used two primers (MVZ47: 59_AGTGAAAGATACAGTCACAG TGCTAGG_39; MVZ48: 59_GGAGGATATC-AGCACAGTCT-AAAAAG_ 39) and the following protocol: PCRs were performed in 12.5 µl reactions containing 7.55 µl H_2O, 1.25 µl of PCR buffer including 1.5 mM $MgCl_2$, 0.1 µl of dNTPs, 0.1 µl Taq QIAGEN, 0.75 µl of each primer having a concentration of 10 mM, and 2 µl of genomic DNA with a concentration of 20 ng/µl. For subsequent cloning, two such reactions from each individual were pooled to increase volume. The PCR protocol followed a "touch-up" approach with 10 cycles of annealing temperatures (55–60 °C) increasing by 0.5° each cycle (with 30 s at 95 °C, 30 s at annealing temperature, and 45 s at 72 °C), followed by 25 cycles with 30 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C, and a final extension of 7 min at 72 °C. All PCR-products (each clone of *Fibrinogen*; direct sequencing of PCR products of *cytochrome b*) were sequenced in both directions, visualized on an ABI 3730 sequencer, and aligned with Sequencher 4.9, followed by the algorithms as implemented in Seaview (Gouy et al., 2010).

2.2. Phylogenetic analyses

In a first step, we reduced the total number of mtDNA sequences to the number of haplotypes found at each locality. Maximum likelihood (ML) phylogenies were generated with PhyML 3.0 (Guindon et al., 2010) using the GTR model for *cytochrome b* and HKY model for the *Fibrinogen alpha* nuclear marker. For each case, we chose a BioNJ tree as a starting tree and used the combined subtree pruning and regrafting (SPR) plus nearest neighbor interchange (NNI) options for tree improvement. All other parameters were set as default (http://atgc.lirmm.fr/phyml/). Bootstrap values were based on 1000 (mtDNA) or 100 (nuDNA) resampled datasets. Bayesian phylogenetic analysis using the reported marker-specific substitution models was performed in MrBayes v3.1.0 (Ronquist and Huelsenbeck, 2003), with the default heating values for three out of four chains, running 20×10^6 generations separately for the mtDNA and nDNA datasets, with tree sampling every 1000 generations. The "burnin"-value was selected by visualizing the log likelihoods associated with the posterior distribution of trees in the program Tracer (http://tree.bio.ed.ac.uk/software/tracer/).

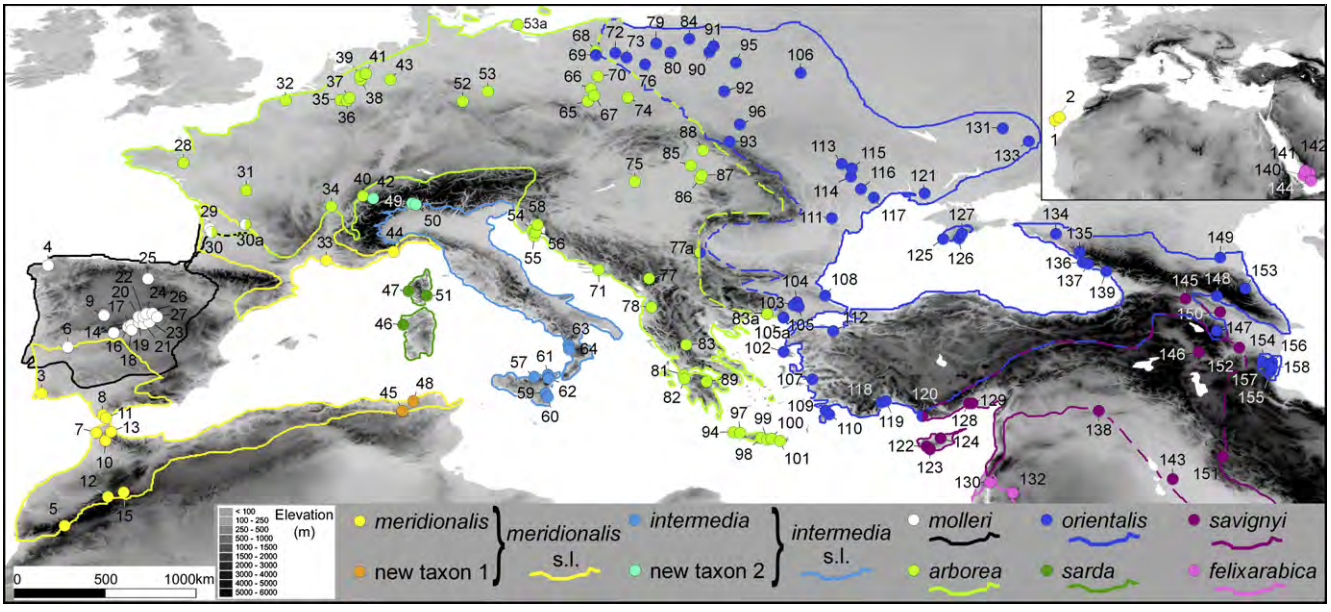

Fig. 1. Map with approximate range limits of Western Palearctic tree frogs (range limits according to maps available through the Global Amphibian Assessment http://www.iucnredlist.org/initiatives/amphibians) with sampling localities (see Map IDs in Appendix S1). *Hyla meridionalis* and "new taxon 1" (acc. Stöck et al., 2008a) are united as "*Hyla meridionalis* s.l."; *H. intermedia* and "new taxon 2" (Stöck et al., 2008a) as "*Hyla intermedia* s.l.". Approximated range limits in interleaved colors indicate parapatric ranges or deficiency of knowledge.

All trees generated before the log likelihood curve flattened were discarded.

2.3. Demographic analyses and estimates of divergence time

We used DnaSP v.5 (Librado and Rozas, 2009) to calculate and visualize the distributions of observed and expected pairwise nucleotide site differences ('mismatch distributions'), between all individuals within the mtDNA clades of *Hyla arborea, H. molleri*, and subclades within *H. orientalis*, as well as the respective expected values for growing populations (Librado and Rozas, 2009). We included only *cytochrome b* markers for which >904 bp 100% readable sequences were available (*H. arborea*: 86%, *H. orientalis*: 94% *H. molleri*: 100%, total: 92%).

Divergence times to the most recent common ancestors were estimated from the *cytochrome b* and *Fibrinogen alpha* markers independently, assuming an uncorrelated exponential relaxed molecular clock (BEAST v. 1.6; Drummond et al., 2006; http://beast.bio.ed.ac.uk/Main_Page). In the absence of appropriate fossils, we based our prior on results from previous work (Smith et al., 2005; Stöck et al., 2008a), assuming a normal distribution for the divergence time between *H. meridionalis* and other tree frogs, with a mean of 10 millions of years ago (Mya) and standard deviation of 1 My (thus effectively spanning a large range from 7.5 to 12.5 Mya).

We applied the marker specific models of sequence evolution as described for PhyML, and a Yule tree prior (constant speciation rate per lineage) as most appropriate for species-level divergences (Drummond et al., 2007). DNA *cytochrome b* data were analyzed both with and without codon partition, with different partitions for codons 1 + 2 and 3.

3. Results

Maximum likelihood and Bayesian phylogenetic analyses yielded mtDNA trees with congruent topologies (Figs. 2 and S1). The same clades were recovered for the nuDNA tree, but with markedly lower support (Fig. 3). Results turned out to be very robust regarding partitioning. In the following, we focus on each of the three species lineages of the *Hyla arborea* group.

3.1. European tree frog (Hyla arborea)

This species ranges from the Western Balkan Peninsula across Central into mainland Western Europe (Fig. 1), showing almost no genetic structure on the mitochondrial or the nuclear level (Figs. 2 and 3). Specifically, it occurs on Crete (locs. 94, 97–101), the Peloponnesus and mainland Greece (locs. 81–83, 89), along the eastern Adriatic coast (locs. 54–56, 58, 71, 77, 78), and throughout the Eastern Pannonian Basin (Hungary, NE-Romania, W-Ukraine: locs. 75, 85–87), where it is separated from the eastern tree frog (*H. orientalis*) by the Carpathian Arc. *Hyla arborea* is the only tree-frog taxon occurring from central Poland (west of the Vistula River: locs. 65–67, 70) throughout central (locs. 52 and 53) to northwestern (locs. 34–41, 43) and western Europe (locs. 28, 31, 32). The mtDNA mismatch distribution (Fig. 4a) shows significantly high matching of simulated and observed curves (Table 1), pointing to a recent and rapid expansion.

3.2. Eastern tree frog (Hyla orientalis)

Our data show that this lineage, whose old name was resurrected when molecular evidence showed its mitochondrial and nuclear divergence from *H. arborea* (Stöck et al., 2008a; Gvozdik et al., 2010), in fact represents a genetically very diverse and

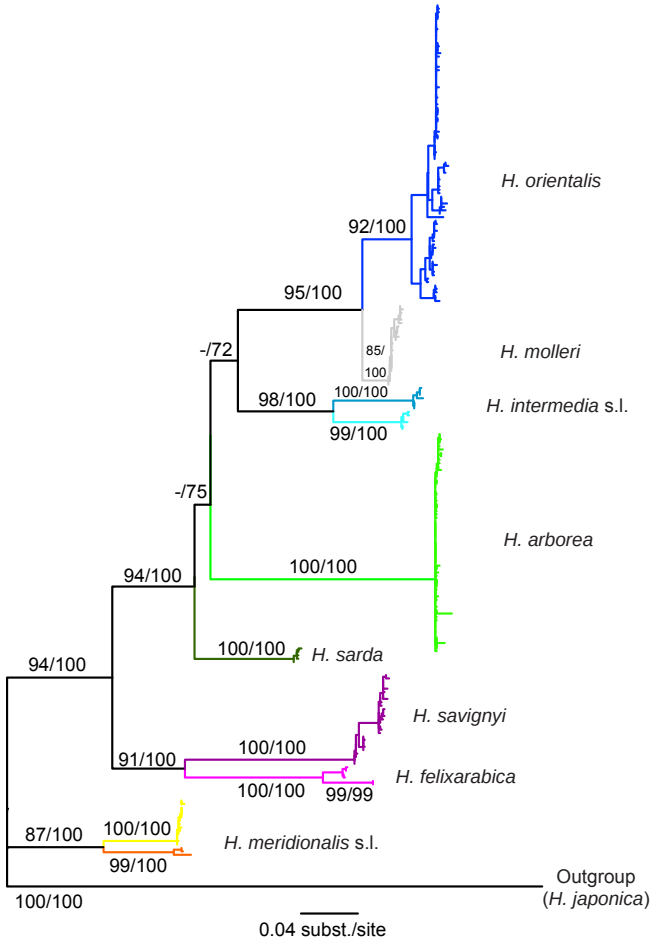

Fig. 2. Schematic maximum likelihood tree obtained with the program PhyML based on ca. 1 kb of mtDNA *cytochrome b* (a detailed version with all individual labels is shown in Fig. S1) with bootstrap support values obtained from 1000 resampled data sets (major nodes below 50% remained unlabeled; before "/"), followed by Bayesian posterior support values (%) for major respective nodes (after the "/") from analysis using Mr. Bayes v3.1.0. Color codes of clades correspond to those of localities in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

well-differentiated group of lineages based on both mtDNA (Figs. 2 and 5a) and nuclear DNA (Fig. 3). Using mtDNA, we found five well-supported subclades (Fig. 5): one in the Talysh Range (locs. 154–158), and a second well-structured one in the Caucasus and adjacent areas (locs. 134–139, 147–149, 153); for both, mismatch distribution analyses (Figs. 5b and c) failed to reach significance (Table 1). Another well-structured mtDNA-group with two subclades inhabits western Asia Minor (locs. 107, 118–120) and the western coast of the Black Sea (locs. 108, 111, 112, 114, 121), without signs of recent demographic changes (Table 1). Finally, a well-supported, widespread haplotype clade with almost no substructure inhabits the Crimea, the northwestern coast of the Black Sea and the entire northeastern European region including Ukraine, Belarus, Russia, and Poland, with the Vistula River as its approximate western border. For this latter group, demographic analyses revealed an almost perfect match of simulated to empirical data (Fig. 5e and Table 1), also pointing to a recent expansion.

The two groups of subclades based on mtDNA (i: Caucasus and Talysh vs. ii: Asia Minor and Black Sea, Eastern Europe and Crimea; Fig. 5a) are not entirely recovered based on nuDNA, where two weakly supported subclusters (Fig. 3) unite Eastern European and Talysh with western Asia Minor frogs.

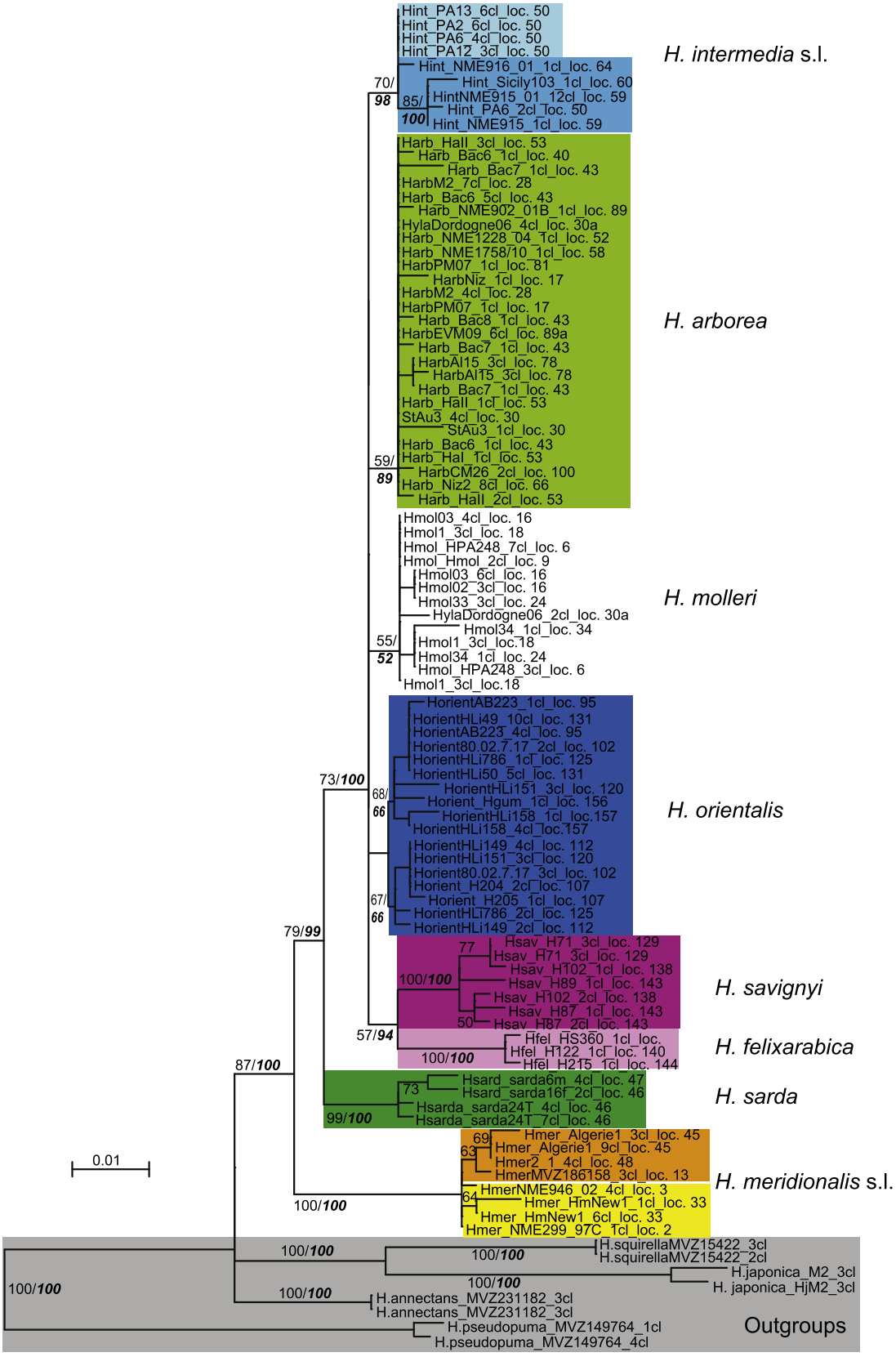

Fig. 3. Maximum likelihood tree obtained with the program PhyML based on 545 bp of nuDNA *fibrinogen alpha* (*intron 1*). The number of identical clones obtained for each sequence is given after the sample ID (as in Appendix S1), and before the locality ID (as in Fig. 1 and Appendix S1). Bootstrap support values from 100 resampled data sets (normal font) for this tree are followed by Bayesian posterior support values (%) for major respective nodes in ***bold italics*** (after the "/") from analysis using Mr. Bayes v3.1.0.

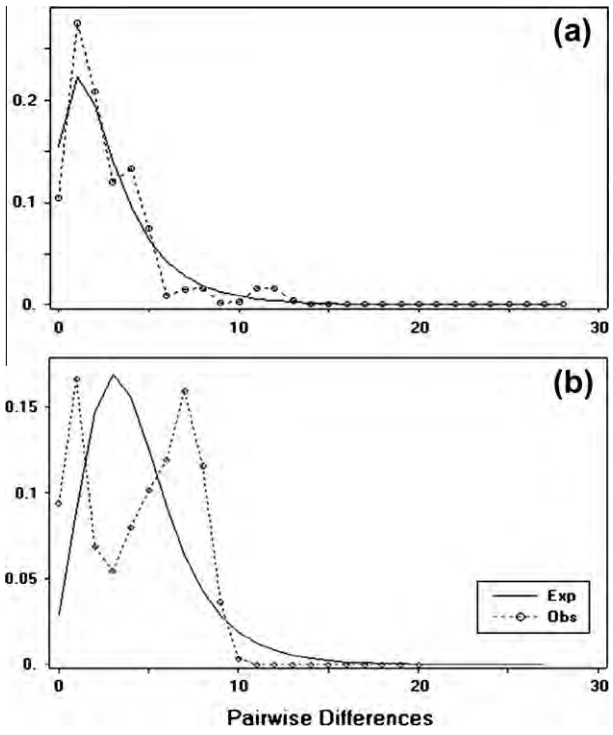

Fig. 4. Mismatch distributions from 905 bp of mitochondrial *cytochrome b*. The dotted lines show the frequency distribution of the observed pairwise differences; the solid lines show the frequency distribution of the expected pairwise differences under the sudden expansion model, performed in DnaSP v.5.

Table 1
Demographic analyses of mitochondrial haplotypes in three species (six clades) of European tree frogs as obtained using DnsSP v.5 (Librado and Rozas, 2009); for details see text.

Species	*N*	Theta	R^2	Tajima's *D*
Hyla molleri	24	4.41	0.088 NS	−0.786 NS
Hyla arborea	56	2.75	0.036**	−2.48***
Hyla orientalis				
Talysh	6	2.47	0.156 NS	−0.351 NS
Caucasus	19	5.65	0.098 NS	−0.823 NS
Asia Minor and W-coast of Black Sea	16	8.38	0.119 NS	−0.168 NS
Eastern Europe and Crimea	46	1.67	0.035**	−2.290***

** $p < 0.01$.
*** $p < 0.001$.

3.3. Iberian or Moller's tree frog (*Hyla molleri*)

Samples from Spain (locs. 4, 9, 14, 16–27), Portugal (loc. 6), and southwestern France (locs. 29–30a) harbor *H. molleri* (including hybrids, as concluded from occurrence in both nuDNA clades, e.g. sample Fig. 3: HylaDordogne06, loc. 30a, or mismatch between mtDNA and nuDNA clade membership, Figs. S1 and S3), with a relatively shallow genetic structure across frogs from all localities. Demographic analyses using DnaSP did not yield significant results (Fig. 4b and Table 1).

3.4. Secondary contact zones in Western Palearctic tree frogs

We newly localized five major contact zones: First, in northeastern Greece, we narrowed the potential contact between *H. arborea* and *H. orientalis* to less than 70 km (locs. 83a and 105a), without evidence of genetic interactions. Second, the Western Carpathians of Serbia (loc. 77a) show co-occurrence of *H. arborea* and *H. orientalis* mtDNAs. Thirdly, to the north of the Carpathian Arc, in the lowlands of central Poland, we have evidence for parapatric ranges of *H. arborea* and *H. orientalis* (locs. 68 and 69), with the Vistula River representing a reasonable approximation for range borders of both lineages. Fourth, near the Atlantic coast of SW-France (locs. 30–30a), we found range overlap and hybridization of *H. arborea* and *H. molleri*, as nuclear intron alleles from *H. arborea* were detected in two individuals with *H. molleri* mtDNA; one frog also possessed nuclear alleles from both *H. arborea* and *H. molleri* (Fig. 5). Fifth, we found a contact zone between *H. orientalis* and *H. savignyi* in SW-Anatolia (loc. 120), where we indentified an apparently re-combined nuclear allele, seemingly stemming from successful hybridization of both species that occur in geographic proximity. *Hyla orientalis* and *H. savignyi* have close geographic proximity in the south of the Great Caucasus (locs. 145–148), but no documented hybridization.

3.5. Divergence-time estimates

The posterior predictions for the divergence time between *Hyla meridionalis* and other Western Palearctic *Hyla* lineages were very close to the mode assumed for the prior, and very consistent between mtDNA and nuDNA (namely, 9.7 and 9.8 Mya for the *cytochrome b* and *Alpha-Fibrinogen*, respectively; Table 2). For the inner groups, the mtDNA and nuDNA markers also yielded similar and widely overlapping ranges of the divergence-time estimates (Table 2), with most lineages formed between late Miocene and lower Pliocene time periods (*H. sarda*, *H. savignyi*, *H. felixarabica*, *H. arborea*), while the remaining lineages (*H. molleri*, *H. orientalis*) are suggested to be of Pliocene age. The mean substitution rates predicted for *cytochrome b* and *Alpha-Fibrinogen* were 0.0161 and 0.00262 per lineage per million years respectively, similar to those found in other anurans (e.g. Mulcahy and Mendelson, 2000; Hoegg et al., 2004).

4. Discussion

4.1. Cryptic diversity

Throughout the European range of *H. arborea*, we show great mtDNA homogeneity (Figs. 2 and S1), and also nuDNA-uniformity. A fast postglacial range expansion of the *H. arborea* mtDNA haplotype group from a Balkanian refugium into its entire current range is very well documented by the mtDNA mismatch distribution (Fig. 4a and Table 1) and the corresponding haplotype network (Fig. S2), which shows the most frequently represented haplotype (Fig. S2: rectangle) ranging from western France to Western Ukraine (Fig. 1: locs. 28 and 88) with its closest relatives at the Adriatic coast (Albania: loc. 78; Croatia: e.g. locs. 71) and in Greece (e.g. loc. 83). The Balkanic region harbors a greater diversity of haplotypes than does the rest of Europe (Fig. S2). Our previous study based on the coding nuclear *Rag-1* for a small subset of samples (Stöck et al., 2008a) also detected a larger amount of genetic diversity in the south of the range, interpreted as diversity in the proposed Pleistocene refugium, the Balkan Peninsula and Adriatic coast.

Despite limited sampling from some regions for *H. molleri*, we covered most geographic extremes of the range including its northern limits in southwestern France. As recently concluded by Barth et al. (2011), who had larger sample sizes for the western range, we find that the Iberian endemic *H. molleri* exhibits little mtDNA diversity throughout its range. As for many Iberian species, it could circumvent the Pyrenees only to the West but has spread to northern latitudes much less than has *H. arborea*.

In sharp contrast to *H. arborea* and *H. molleri*, we found substantial mtDNA but also nuDNA-based genetic structure within

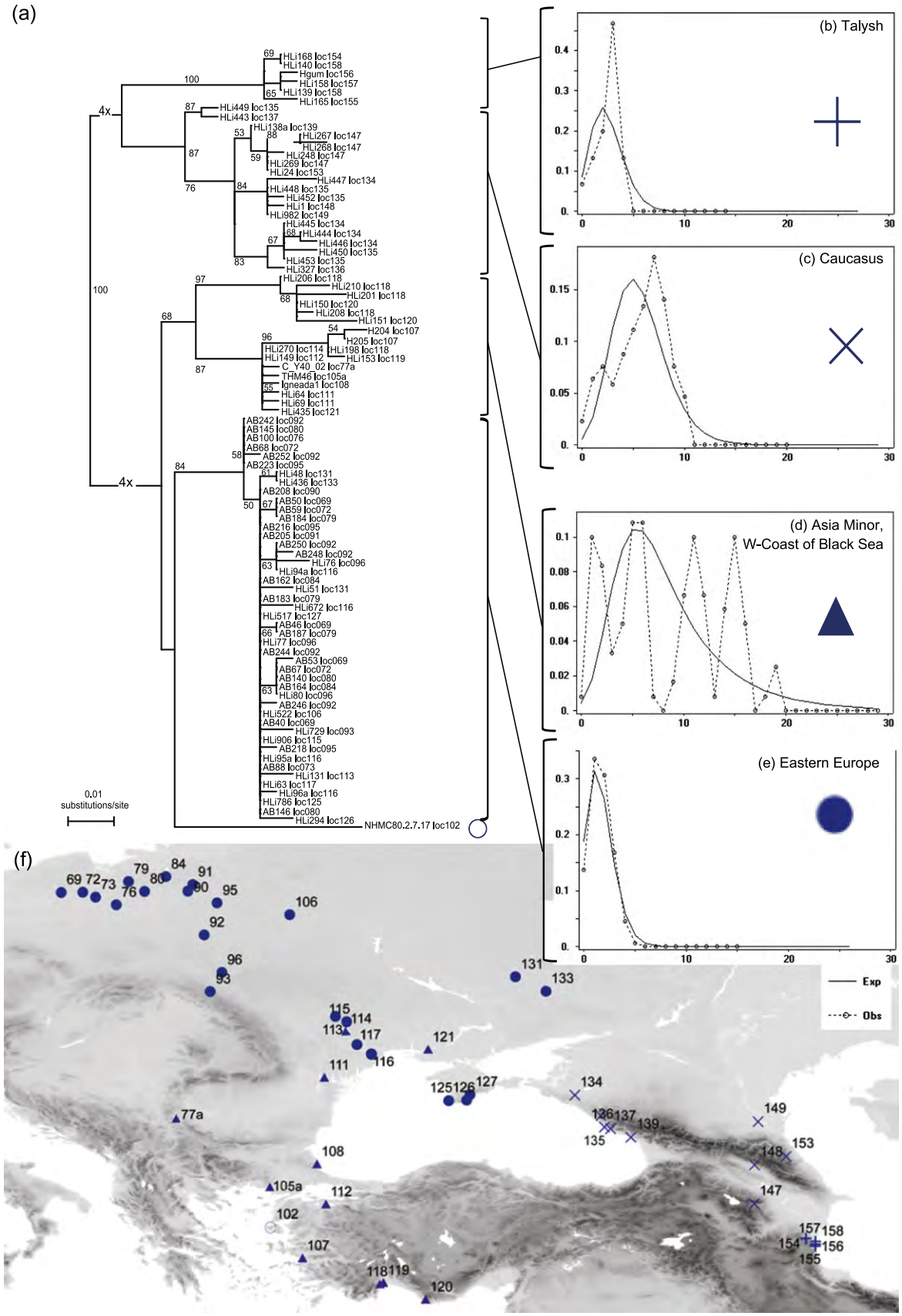

Fig. 5. Mitochondrial DNA-diversity within *Hyla orientalis*. (a) Unrooted maximum-likelihood tree for 905 bp of *cytochrome b* (for details: legend of Fig. 2). (b–e) Mismatch distributions from 905 bp of mitochondrial *cytochrome b* for the corresponding haplotype clades as shown in (a). The dotted line shows the frequency distribution of the observed pairwise differences; the solid line shows the frequency distribution of the expected pairwise differences under the sudden expansion model, performed in DnaSP v.5. (f) Geographical representation of clades shown in (a–e).

Table 2
Divergence time (millions of years, My) to the most recent common ancestor (including stem), estimates based on the program BEAST using mitochondrial (*cytochrome b*; mean rate: 0.0161 per lineage per million years) and nuclear (*Fibrinogen alpha*; mean rate 0.00262 per lineage per million years) DNA sequences.

Species	*H. meridionalis*		*H. sarda*		*H. savignyi*		*H. felixarabica*		*H. arborea*		*H. intermedia*		*H. molleri*		*H. orientalis*	
Marker	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA
My	9.7 (7.7–11.6)	9.8 (7.6–12.0)	6.1 (4.3–8.3)	7.7 (4.5–11.1)	6.2 (4.3–8.4)	5.2 (2.9–7.9)	6.2 (4.3–8.4)	3.9 (2.0–6.1)	6.1 (4.3–8.1)	4.5 (2.3–7.0)	6.1 (4.5–8.2)	3.6 (1.7–5.8)	3.7 (2.6–5.0)	3.5 (1.7–5.4)	3.7 (2.6–5-5)	4.6 (2.4–6.9)

the recently recognized Eastern tree frog *H. orientalis* (Figs. 3 and 5). Much of *H. orientalis*' diversity occurs in Asia Minor and suggests circum-Black Sea Pleistocene refugia. The clade that post-glacially colonized the northern latitudes shows high mtDNA-uniformity and significant signs of recent range expansion (Fig. 5e), similar to the signature across all of *H. arborea*'s mtDNA (Fig. 4a). The reason that Gvozdik et al. (2010) found all their "*H. orientalis* samples (to) form a compact cluster with substantial genetic variation, although without any deep divergences" appears to result from sampling only some Asia Minor and Caucasian regions.

4.2. Divergence times

The posterior predictions for the divergence time with *H. meridionalis* are extremely close to the mode assumed for the prior and very consistent between *cytochrome b* and *Alpha-Fibrinogen*. Furthermore, the associated mean substitution rates are similar to those found in other anurans (e.g. Mulcahy and Mendelson, 2000; Hoegg et al., 2004), providing support for our calibration of the phylogenies with *Hyla meridionalis*

Despite previous estimates of divergence time for some Western Palearctic tree frog species (Canestrelli et al., 2007: *H. intermedia*; Recuero et al., 2007: *H. meridionalis*), Gvozdik et al., 2010: *H. orientalis, H. savignyi, H. felixarabica, H. meridionalis*), our study is the first that includes all extant species, and that uses mitochondrial and nuclear sequence markers. As far as comparable (divergence of *H. orientalis* vs. *H. savignyi* + *H. felixarabica*) our estimates are compatible, given the highest posterior density interval spanning "the period from the Early Pliocene through the Miocene, between 4.9 and 23.0 My" (Gvozdik et al., 2010).

Some of the discrepancies between our mtDNA and nuDNA-based estimates (Table 2) may be explicable by fewer data on the nuclear than on the mtNDA level for several clades. We confirm considerable divergences between two subclades of both "*H. meridionalis* s.l." and "*H. intermedia* s.l." (Fig. 2), as previously shown by other authors and markers (Recuero et al., 2007; Canestrelli et al., 2007), and temporarily called "new taxa1 and 2" (Stöck et al., 2008a). More work is needed to understand potential taxonomic implications for these lineages but is beyond the scope of this paper.

4.3. Contact and hybrid zones

The Eastern Mediterranean contains several major Pleistocene refugia, with the territory of Greece representing a meeting zone of faunal elements of Asia Minor and of Balkan Peninsular (plus African) origin (Lymberakis and Poulakakis, 2010). Western Greece, Crete and some western Aegean islands are colonized by the mitochondrial lineage that also occurs on the western Balkan Peninsula and stretches into Central and even Western Europe (*H. arborea*), while the eastern Greek provinces of Macedonia, Thrace and the eastern Aegean islands are phylogenetically close to the clade of Asia Minor origin (*H. orientalis*). Although we narrowed the potential contact to ca. 70 km (locs. 83a and 105a), our data are not sufficient yet to reveal potential contacts of tree-frog lineages in northeastern Greece and the Aegean islands. To the north of Greece, the Carpathians represent a major barrier for tree frogs. West of this mountain range occurs the *H. arborea* haplotype group, and to the east of the Carpathian Arc that of *H. orientalis*, which also inhabits the entire rest of the Eastern European *Hyla* range. Large Carpathian river valleys provide rare opportunities for secondary contacts, with so far one locality of co-occurrence of both mtDNAs (loc. 77a). To the north of the Carpathian Arc, secondary contact and hybridization between *H. arborea* and *H. orientalis* are documented by mtDNA and microsatellite data from the lowlands of Poland (Borzée, 2010, in prep.). Interestingly, the mtDNA subclade of *H. orientalis* that meets the uniform *H. arborea* in Poland (Fig. 1 and 5) differs from the subclade (Figs. 1 and 4d: triangles) that is in potential contact in Serbia and northeastern Greece. This offers interesting comparative research opportunities on secondary contacts of differently, but quite closely related populations.

Since the splitting of *H. molleri* from *H. arborea* by Stöck et al. (2008a), occurrence and range limits at the Atlantic coast of SW-France, in the Aquitaine region, have been ambiguous with respect to species (see also Barth et al., 2011). Our new data (locs. 29–30a) not only revealed the only Western Palearctic region with three co-occurring tree frog taxa but also (at least) F_1-hybridization between *H. molleri* and *H. arborea*. As in the overlapping distributions of *H. meridionalis* and *H. molleri*, in the Spanish Sistema Central Mountains, few hybridization events have been reported (Oliveira et al., 1991; Barbadillo and Lapena, 2003); even genetic interactions between three species appear possible, but more research is required.

In addition to the three newly localized contact zones of *H. arborea* with *H. orientalis* (NE-Greece, Poland), and with *H. molleri* (SW-France), a well-known contact zone with *H. intermedia* exists in NE-Italy (Verardi et al., 2009), where neither hybrids nor backcrosses were identified, indicating a lack of current gene exchange between the two species. However, introgressed alleles appeared in both species, indicating past introgressive hybridization. Using bioacoustic inference, pending genetic confirmation, Schneider (2001) narrowed the contact between *H. orientalis* (as "*H. arborea*") and *H. savigny* to less than 10 km in the Anamur plain of southwest Anatolia. Parapatry with one documented locality of hybridization (Karkom, Israel) has been shown between *H. savignyi* and *H. felixarabica* (Gvozdik et al., 2010).

4.4. Comparisons with phylogeographic patterns of other terrestrial groups

Our data contribute to knowledge of the evolutionary history of Western Palearctic tree frogs as well as the comparative phylogeography of Europe, and should improve conservation measures. As recently noted by Rissler and Smith (2010) for North America: "Identifying congruence in the geographical position of lineage breaks and species range limits across multiple taxa is a focus (...) of comparative phylogeography. These regions are biogeographical hotspots for investigations into the processes driving divergence at multiple phylogenetic levels". Indeed, the postglacial colonization routes and resulting

secondary hybrid zones of tree frogs in the Western Palearctics coincide with several of those known from other terrestrial species. Namely, the postglacial colonization route of *H. arborea* resembles that of the grasshopper *Chorthippus parallelus*, and the advance of beech (*Fagus sylvatica*) and black alder (*Alnus glutinosa*) from their Balkanian refugia (King and Ferris, 1998; Hewitt, 1999, 2004; Magri, 2008), with the broad-leaf forest providing direct summer habitats for tree frogs, suggesting partial co-colonization. As do *H. arborea* and *H. molleri*, these three species meet Iberian counterparts in the Pyrenees (Hewitt, 1999) and form hybrid zones in their vicinity. Postglacial colonization of northeastern Europe to the east of the Carpathians by *H. orientalis* resembles that by the green toad *Bufo variabilis* (Stöck et al., 2006, 2008b).

4.5. Implications for conservation of European tree frogs

Amphibians are undergoing a massive and extensive crisis (Wake and Vredenburg, 2008; Hoffmann et al., 2010), with complex causes that include land-use changes (Hof et al., 2011). The remaining amphibian biodiversity should thus be especially assessed and protected in regions with industrial agriculture and intense land use and fragmentation (such as Western Europe) or currently facing major land-use changes due to political and economic transformations (such as Eastern Europe). While most *Hyla* species are still common in parts of their Western Palearctic range, habitats are fragmented, and these frogs are in significant decline over much of their Western European distribution (http://www.iucnredlist.org/apps/redlist/details/10351/0), mainly by "loss of breeding habitats, habitat isolation, fragmentation, and pollution". Tree frogs are considered less threatened in Eastern Europe (www.amhibiaweb.org, incl. refs.). However, land-use changes caused by ongoing political and economic transformation pose upcoming threats also for the latter regions. Our data therefore support conservation efforts by fine-tuning measured locations of refugia harboring great genetic diversity (e.g. Moritz, 2002), which are "essential refuges for Earth's many small-ranged species" (Sandel et al., 2011). The localized areas of secondary contact should be considered "natural arenas to investigate processes driving speciation" (Rissler and Smith, 2010), which require special conservation efforts.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.05.014.

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CHAPTER V

TRANS-SPECIES VARIATION IN *DRMT1* IS ASSOCIATED WITH SEX DETERMINATION IN FOUR EUROPEAN TREE-FROG SPECIES

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Brelsford A, Dufresnes C, Perrin N. Trans-species variation in *Dmrt1* is associated with sex determination in four European tree-frog species.

Trans-species variation in *Dmrt1* is associated with sex determination in four European tree-frog species

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ABSTRACT

There is accumulating evidence that a small number of genes repeatedly become the master sex determination switch in many systems with otherwise different sex chromosomes. The gene *Dmrt1* and its paralogs are known to control sex determination in birds and at least one fish and one frog species, and *Dmrt1* is located on the sex chromosomes of several other amphibian species. Here, we demonstrate that *Dmrt1* is also the master sex determining gene in four European tree-frog species. We identified trans-species variation specific to Y chromosomes in *Dmrt1* exon 1, but not in exon 3 or other neighboring genes. A genome scan of over 29,000 SNPs identified no additional trans-species Y-specific markers. This discovery lends strong support to the hypothesis that certain genes in the conserved sex determination pathway recurrently become the master sex determining gene. Moreover, the sex-specific markers we identify will enable research on environmental sex reversal in a wider range of frog species.

INTRODUCTION

The field of sex chromosome evolution has recently expanded beyond the typically old and stable sex determination systems of mammals, flies, and birds, to include organisms with genetic sex determination controlled by undifferentiated chromosome pairs (Bachtrog et al. 2014). Most amphibians and fish fall into the latter category, and there is an emerging consensus that sex-chromosome turnovers contribute to this pattern (Kikuchi and Hamaguchi 2013, Malcom et al. 2014). Against this background of rapid change, we are interested in whether any consistent patterns emerge in the genes or genomic regions responsible for sex determination.

Graves and Peichel (2010) noted that some ancestral vertebrate chromosomes have repeatedly become sex-linked in different organisms, hypothesizing that there are "limited options" for sex determination, and that a few important genes are particularly likely to take over the function of sex determination. Of the small but growing set of identified sex-determining genes, many have been shown to be involved in the sex differentiation cascade across diverse vertebrate groups. In therian mammals, *Sry* is known to be the master sex determination gene (Sekido & Lovell-Badge 2008), and recent evidence suggests that the gene from which it is derived, *Sox3*, also plays a major role in sex determination in the fish *Oryzias dancena* (Takehana et al. 2014) and the frog *Rana rugosa* (Oshima et al. 2009). Several fish have converged on another pathway in the same cascade. *Amh*, which is essential for testis development throughout vertebrates, is a strong candidate for the sex-determining gene in monotremes (Cortez et al. 2014); a paralog controls sex differentiation in the Patagonian pejerrey *Odontesthes hatcheri* (Hattori et al. 2012), and its receptor *Amhr2* provides a similar function in the fugu *Takifugu rubripes* (Kamiya et al. 2012). *Dmrt1* is another gene that is likely to play a role in sex determination across a diverse range of species (Matson & Zarkower 2012). This gene contributes to sex differentiation in organisms as diverse as nematodes, insects, and mammals; it is the master sex determination gene in birds (Smith et al. 2009), and likely a dosage-dependent sex-determining gene in the tongue sole *Cynoglossus semilaevis* (Chen et al. 2014). Partial duplicates of *Dmrt1* are also the master sex determiners in the African clawed frog *Xenopus laevis* (Yoshimoto et al. 2008) and the medaka fish *Oryzias latipes* (Nanda et al. 2002; Matsuda et al. 2002). The chromosome harboring *Dmrt1* has been independently co-opted for sex in three deeply divergent lineages of frogs, the green toad (*Bufo viridis*), common frog (*Rana temporaria*), and European tree frog (*Hyla arborea*) species groups (Brelsford et al. 2013).

Within the *H. arborea* radiation, *Dmrt1* is sex-linked in four species that share a common ancestor 5-7 million years ago, namely *H. arborea*, *H. intermedia*, *H. molleri* and *H. orientalis* (Stöck et al. 2011, 2013; Brelsford et al. 2013). Stöck et al. (2011) pointed out that, despite the absence of recombination between X and Y chromosomes in experimental crosses (Berset-Brändli et al. 2008, Stöck et al. 2011, 2013), the Y chromosomes of each species are more similar to the X chromosomes of the same species than to Y chromosomes of related species, strongly suggesting a history of X-Y

recombination after the species diverged. However, as pointed out by Blaser et al. (2014), such a clustering of alleles by species (rather than by gametologs) might in principle also stem from recurrent homologous transitions (*sensu* van Doorn & Kirkpatrick 2007, 2010); namely, recurrent masculinizing mutations of the female-determining *X* allele. Fully excluding this possibility requires a phylogenetic analysis of the sex-determining gene itself: the homologous-transition hypothesis predicts shallow phylogenies for all sex-linked genes, including the sex-determining ones (Figure 1A), while the XY-recombination hypothesis predicts shallow phylogenies for all genes except those involved in sex determination. If the four tree-frog species retain an ancestral sex-determining gene, the Y-linked alleles of this gene should cluster together across all four species, with the X-linked alleles forming a separate cluster (Figure 1B). Finding a genomic region where coalescence time for the X and Y gametologs predates species divergence would not only help identify the sex-determination locus itself, but also provide further support for the XY-recombination model, by falsifying the homologous-transition alternative.

Here, we search the *Hyla* genome for trans-species Y-specific polymorphism using two approaches, one focused on the candidate gene *Dmrt1* and another genome-wide scan using genotyping-by-sequencing. Our aims are to identify the sex-determining locus of European tree frogs, to test whether sex-chromosome homomorphy in this group stems from XY recombination or from homologous transitions, and to demonstrate the utility of trans-species association studies for fine-scale genetic mapping, particularly in regions of low recombination.

METHODS

DMRT1 *CANDIDATE GENE APPROACH*

We used multiple rounds of marker development and genotyping to isolate the small genomic region that shows no evidence of recombination between X and Y chromosomes in four related tree frog species. Initially, we genotyped a single-nucleotide polymorphism in *Dmrt1*, which showed sex-diagnostic variation across all sampled populations of *H. arborea*. We then scanned other genes flanking *Dmrt1*. For each subsequent scan, we identified fixed differences between X and Y gametologs, and then developed new markers to search a reduced region of the sex chromosome around the marker(s) displaying this pattern (see Table S1 for a complete list of markers; see results section for additional details of the order of marker development).

New genomic resources developed for *H. arborea* facilitated our scans of the sex chromosome. We used both a transcriptome (Brelsford et al. 2013) and a newly developed low-coverage draft genome sequence. Genome sequencing and assembly followed methods described in Purcell et al. (in press). Briefly, we assembled quality-trimmed reads from a single Illumina paired-end library, sequenced to 5x depth, using Abyss (Simpson et al. 2009). We then scaffolded the resulting assembly with SSPACE (Boetzer et al. 2011). To identify genetic differences between X and Y gametologs, we combined Sanger sequencing of markers developed from the draft genome sequence, and SNPs from the transcriptome.

GENOTYPING-BY-SEQUENCING GENOME SCAN APPROACH

We also conducted a genome scan using a genotyping-by-sequencing approach, seeking markers that showed consistent differences between four males and four females across the four related frog species (32 frogs in total). We used the genotyping-by-sequencing procedure described by Parchman et al. (2012) with slight modifications. Briefly, genomic DNA was digested with restriction enzymes EcoRI-HF and MseI (New England Biolabs), ligated to Illumina sequencing adapters, and PCR amplified. The PCR reaction used one selective primer designed to amplify a subset of restriction fragments; we modified the primer design of Parchman et al. (2012) to include phosphorothioate bonds at both ends of the primer to prevent degradation by high-fidelity polymerase, which can otherwise reduce the specificity of PCR amplification (Brelsford et al. 2011). PCR reactions were pooled, and amplicons of 400-500 bp were isolated by agarose gel extraction. The library was sequenced on the Illumina Hiseq 2000 platform at the Lausanne Genomic Technologies Facility.

Raw reads were demultiplexed and quality filtered using the process_radtags module of Stacks (Catchen et al. 2013), and mapped to the *H. arborea* draft genome using Bowtie2 (Langmead and Salzberg 2012). Variants were identified using Samtools mpileup (Li et al. 2009). These variants were then quality-filtered using Vcftools (Danacek et al. 2011). Genotypes with quality scores <20 were converted to missing data, and loci with >20% missing data were removed. Finally, loci with a minor allele frequency <3% were removed. We then

used Vcftools and custom shell scripts to identify markers polymorphic within one species, polymorphic in multiple species, or fixed between species, and to search for markers heterozygous in all males and homozygous in all females.

RESULTS

DMRT1 *CANDIDATE GENE APPROACH*

First, we genotyped a single-nucleotide polymorphism (SNP) previously identified in *Dmrt1* (Brelsford et al. 2013) in 217 samples distributed throughout the *H. arborea* species range (Table S1). This non-synonymous SNP was heterozygous for the C and T alleles in all adult males and homozygous for the C allele in all adult females, strongly suggesting that the C allele (encoding threonine) is fixed on the X chromosome and the T allele (encoding alanine) on the Y chromosome.

We then tested whether the range-wide sex-diagnostic region of the chromosome encompassed two genes located on each side of *Dmrt1* on the *Xenopus* genome sequence, *Dock8* and *Map1b*, by genotyping four SNPs in these genes (genotyping details in Table S2) in populations from Greece, which exhibit occasional recombination and low differentiation between X and Y chromosomes (Dufresnes et al. in press). None of the four SNPs were sex-diagnostic (Table S1). Assuming that gene order is conserved between *X. tropicalis* and *H. arborea*, this narrowed the potentially sex-diagnostic region to the interval between *Dock8* and *Map1b*, which spans 5.4 Mbp and contains 52 genes in *X. tropicalis* genome.

Next, we used the *H. arborea* transcriptome sequence (Brelsford et al. 2013) to identify SNPs in six genes between *Dock8* and *Map1b*. These SNPs were then genotyped in the same adults from Greece (SNP and genotyping information in tables S1 and S2). All SNPs presented genotype patterns similar to *Dock8* and *Map1b*, with no sex-diagnostic variants found. This narrowed the potentially sex-diagnostic region to the 0.5 Mbp interval between *Kank1* and *Smarca2*, which contains three genes: *Dmrt1*, *Dmrt3*, and *Dmrt2*.

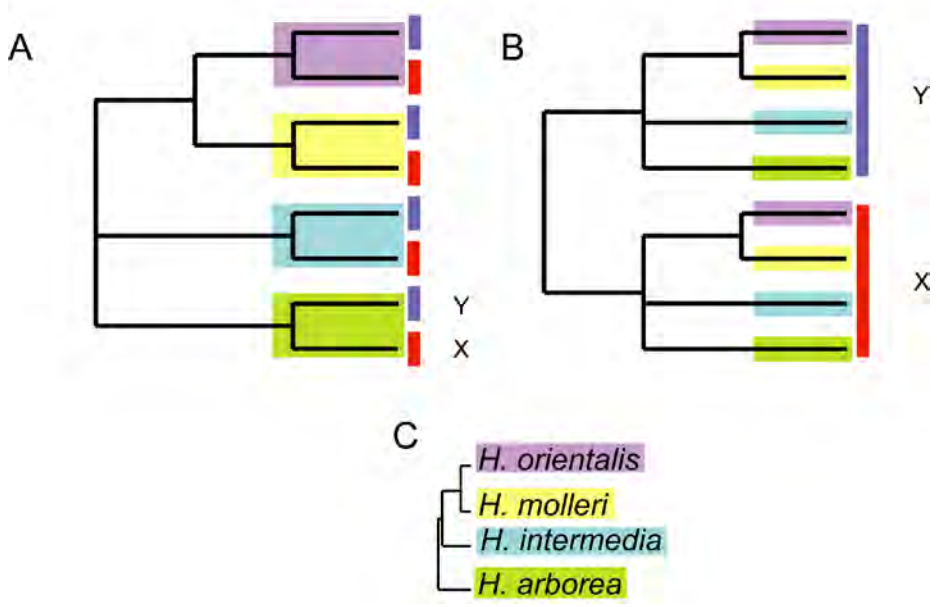

FIGURE 1: Hypothetical gene trees illustrate alternative scenarios of *Hyla* sex chromosome evolution. Clustering by species (A) is observed across most of the sex chromosome (Stöck et al. 2011), and is expected even at the sex-determining locus under the "homologous transitions" model. In contrast, the "fountain of youth" model predicts clustering by gametolog (B) at the sex-determining locus, and clustering by species (A) elsewhere on the sex chromosome. A tree based on mitochondrial DNA (C) illustrates the colors used to represent each species.

We then designed primers from a low-coverage draft assembly of the *H. arborea* genome (Supp. Mat.) to amplify exons 1 and 3 of *Dmrt1* and intron 10 of *Kank1*, located in the region of *Kank1* closest to *Dmrt1*. We sequenced these three loci in males and females of four *Hyla* species that share the same sex chromosome (*H. arborea*, *intermedia*, *molleri*, *orientalis*; Stöck et al. 2011, 2013), as well as in *H. meridionalis* as an outgroup. Amplicons were sequenced on both strands by Microsynth (Balgach, Switzerland) on an ABI 3730XL sequencer. Haplotypes were inferred with PHASE (Stephens and Donnelly 2003). For each male, the haplotype most similar to a female haplotype was designated a putative X-linked sequence, and the other allele was designated a putative Y-linked sequence. We then inferred a gene tree at each locus using PhyML (Guindon et al. 2009). For *Dmrt1* exon 1, all putative Y haplotypes of the *H. arborea*, *intermedia*, *molleri*, and *orientalis* formed one clade, which excluded all known X-linked sequences from females and putative X-linked sequences from males. In contrast, *Dmrt1* exon 3 and *Kank1* intron 10 showed a more complex pattern with some clades containing both X-linked and Y-linked sequences (Figure 2).

To confirm the male-specificity of the putative Y-linked sequences, we identified a SNP that was heterozygous in all sequenced males and homozygous in all sequenced females, 248 bp upstream of *Dmrt1* exon 1. Based on comparison with the *H. meridionalis* outgroup, the SNP is a G-to-C substitution on the Y chromosome. We genotyped this SNP in 20 males and 20 females of three of the four focal *Hyla* species; in the fourth, *H. orientalis*, we genotyped 20 males and eight females (see Supp. Mat. for genotyping details). All genotyped males were G/C heterozygous and all females G/G homozygous, with the exception of two heterozygous *H. molleri* females, confirming that this marker is sex-diagnostic across three species of tree frogs and strongly associated with sex in a fourth.

Genome sequencing

We obtained 28.8 Gbp of raw sequence data from a Swiss male *H. arborea*, 21.3 Gbp of which was retained after filtering and quality trimming. Assembly with Abyss (k=44) and scaffolding with SSPACE produced a highly fragmented low-coverage draft genome sequence, with length 1.2 Gbp and N50 1.2 kbp excluding gaps. This sequence is considerably smaller than the 5 Gbp expected genome size of *H. arborea* (Borkin et al. 2005), but contains four of the five exons of *Dmrt1*.

Genotyping-by-sequencing genome scan

We identified 29186 SNPs in 10536 scaffolds of the low-coverage genome assembly, including 21588 polymorphic within one species, 3031 fixed differences between species, and 4567 polymorphisms shared between multiple species. None of the shared polymorphisms matched the pattern of *Dmrt1* exon 1, with one allele being found in all males and absent in females. The *Hyla* genome is approximately 5 Gbp giving an expected interval of 475 kbp between scaffolds for which we obtained GBS data. This can be taken as an approximate upper limit of the size of the sex-diagnostic region of the genome, an order of magnitude larger than the 42 kbp separating *Dmrt1* exon 3 from *Kank1* intron 10 in *X. tropicalis*.

Discussion

Our results strongly suggest that *Dmrt1* is the master sex-determining gene in at least four European tree-frog species. The first exon of *Dmrt1* is the only one of 29186 examined loci where sequences cluster perfectly by gametolog rather than by species, as predicted for an ancestral sex-determining locus. This pattern does not hold at two tightly linked loci, *Dmrt1* exon 3 (22 kb downstream in *X. tropicalis*) and *Kank1* intron 10 (20 kb upstream in *X. tropicalis*): some Y haplotypes are closer to X haplotypes of the same species than to Y haplotypes of other species, indicating that recombination occurred between gametologs subsequent to species divergence. These contrasted topologies between neighboring genes also further support the XY-recombination model of sex chromosome homomorphy, by dispelling the alternative hypothesis of homologous transitions (recurrent masculinizing mutations of X alleles). As developed in the introduction, this latter model would predict shallow phylogenies for all sex-linked genes, including those involved in sex determination.

Our method, like that of Kamiya et al. (2012) shows that when the genetic basis of a trait is shared across multiple species, it is possible to fine-map the responsible locus to below the gene level using trans-species variation. Notably, this is true even when recombination rates are extremely low, as is the case in *Hyla* sex chromosomes (Guerrero et al.

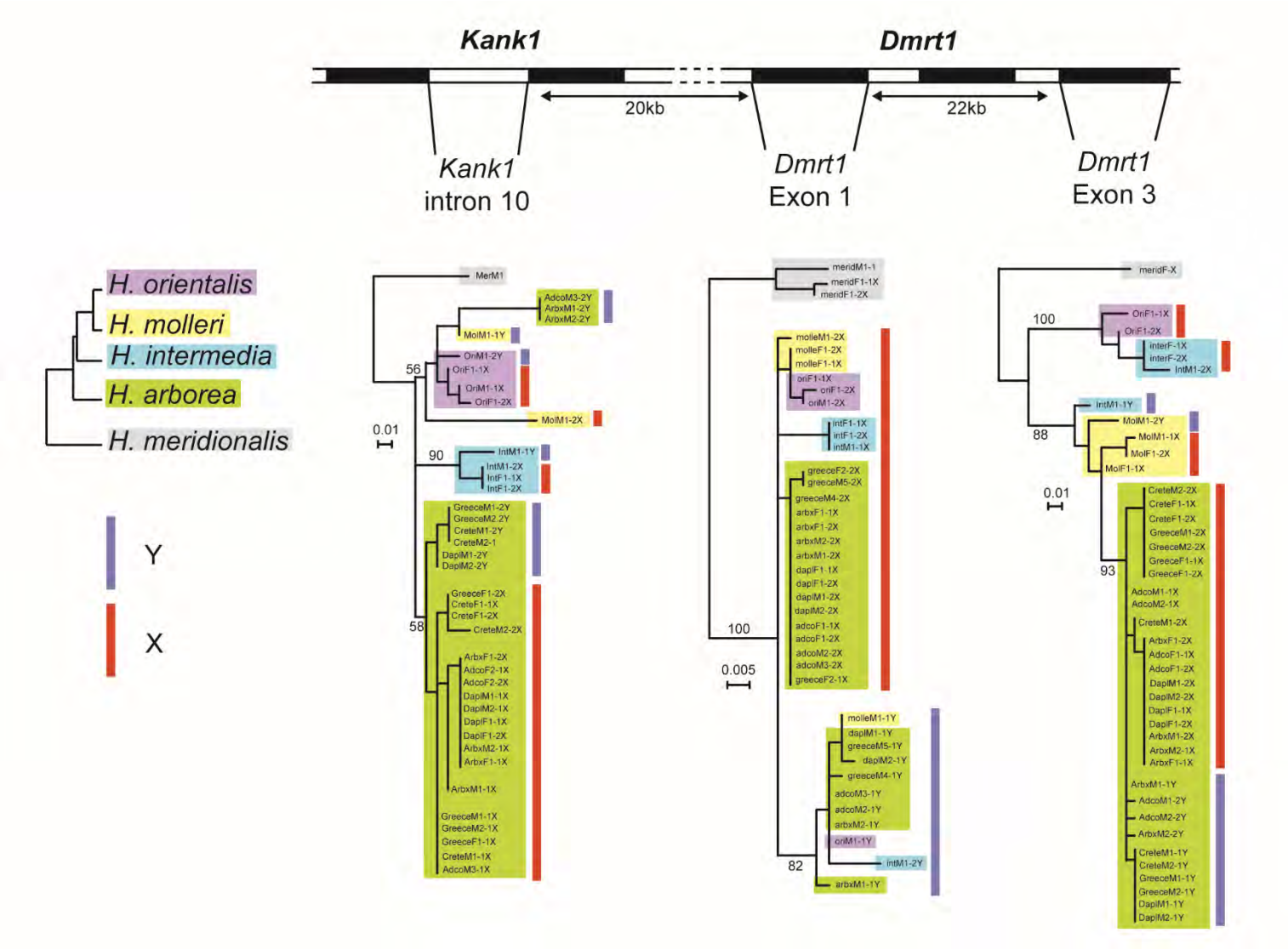

FIGURE 2: Sequences of four *Hyla* species at *Dmrt1* exon 1 cluster by gametolog and not by species. In contrast, at *Dmrt1* exon 3 and intron 10 of the adjacent gene *Kank1*, some Y-chromosomal sequences cluster with the X-linked alleles of the same species rather than with the Y-linked alleles of other species. Distances between loci are from the *X. tropicalis* genome; the *Hyla* genome is approximately three times larger so the true distances in *Hyla* may be greater.

2012). We also demonstrate the continued utility of candidate gene approaches, despite the increasing ease of generating large population-genomic datasets, since our genome survey with GBS markers failed to detect the small trans-species sex-diagnostic region.

This study contributes to a growing understanding that certain genes are especially likely to take over the role of sex determination. *Dmrt1* occupies a vital place in the highly conserved sex-determination pathway (Matson & Zarkower 2012), and this gene or its paralogs have been coopted as master sex determining genes in at least 4 independent cases (see Introduction). Future work should investigate the potential mechanism by which *Dmrt1* determines sex in tree frogs, by analyzing temporal expression patterns of the X and Y copies of *Dmrt1*.

Finally, we have documented an assay for molecular sexing of three European tree-frog species. Sex reversal due to extreme temperature events or synthetic endocrine disruptors is a conservation concern for amphibians (e.g. Hayes et al. 2002, 2006), but studies of this phenomenon are limited by the ability to reliably determine the genetic sex of individuals. Indeed, the two *H. molleri* females with anomalous male-like *Dmrt1* genotypes may indicate environmental sex reversal in this population, although we cannot rule out rare XY recombination as an alternative explanation. Our reliable and inexpensive SNP assay will facilitate future studies of environmental sex reversal in *H. arborea*, *H. intermedia*, *H. molleri*, and *H. orientalis*.

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Table S1 : List of markers used

gene	gene start position in *X. tropicalis* scaffold 1	primer f	primer r	restriction enzyme(s)	reference
phase 1 (*H. arborea*)					
Dmrt1	96303907	CACACAGCATTCCTTATGTTGA	CGATGAATAGCCATGGTTCC	*HinfI*	Brelsford et al 2013
Dock8	96078164	TTGAGCCACTGTTTGCAACT	GCCGTAGATATTGTTTGAATGC	*AluI, AvaII, MseI*	Brelsford et al 2013
Map1B	101456644	CCGCTTGTAAAATCAAAAACTAAAA	CGGCACACAGAAGCAAAATA	*AvaII*	Brelsford et al 2013
phase 2 (*H. arborea, H. orientalis*)					
Gldc	98254152	CATCTATGGCGACCAACATTT	CCACAGGGTTATGGAGAGGAT	*MluCI*	this study
Smarca2	96769565	ACCACAGATACCTCCAGCACA	TGCAGGTAGGGTTCTCTTTCC	*SacI*	this study
Smn2	101248089	GCACACAAAGTAAGGGTCTGG	TGAAGTTGAGGGCATTTCCTA	*BsiEI,Hpy166II*	this study
Kank1	96235063	TGGTGCTGATGAAAACATGAA	GCAGCTTCCAACAGAACAGTC	*TaqI,XmnI*	this study
Cdc37l1	97709307	GCAGAGGAACAAGGGTATTTTG	TGGCTAAAACATTGTACTTTCATATT	*Hpy166II*	this study
Kiaa0020	97016505	GGATTCTGCAGTACGACAACG	TTGCCACCAGGTAGAAAATCT	*RsaI*	this study
phase 3 (5 species)					
Dmrt1Ex1	96303907	ACCCTGCAGGTTTACCTATGA	CTTTCTGCACTGGCAGTCTCT	*Sanger sequence*	this study
Dmrt1Ex3	96303907	CATTTCAGGCCAGTTTCACTC	TTCATCCTACCAGATGGCTGT	*Sanger sequence*	this study
Kank1	96235063	ATGAGTGGTTCCGTGTGTCA	TCTGGTGGTTTACGTTGCAC	*Sanger sequence*	Brelsford et al 2013
phase 4 (4 species)					
Dmrt1	96303907	AAATATTTAACTTTTCATACCCTGCAGGTTTACCTATGA	TTTCMATATCCCTCCCACAATC	*BccI*	this study

Table S2 : SNP genotyping information

				number of individuals per genotype					
				male			female		
gene	enzyme	species	region	AA	AB	BB	AA	AB	BB
dmrt1	*hinfI*	*H. arborea*	Greece		63				30
dmrt1	*hinfI*	*H. arborea*	Adriatic		55				16
dmrt1	*hinfI*	*H. arborea*	Danube		14				9
dmrt1	*hinfI*	*H. arborea*	NW Europe		18				12
dock8	*aluI*	*H. arborea*	Greece	49	15			11	17
dock8	*avaII*	*H. arborea*	Greece		11	53		1	27
dock8	*mseI*	*H. arborea*	Greece		28	33			28
map1b	*avaII*	*H. arborea*	Greece		4	63			28
smarca2	*saci*	*H. arborea*	Switzerland		3				4
gldc	*mluci*	*H. arborea*	Switzerland		3	1	3	1	
smn2	*bsiei*	*H. arborea*	Switzerland	2	2			2	2
smn2	*hpy166II*	*H. arborea*	Switzerland		2	2	2	2	
cdc37l1	*hpy166II*	*H. arborea*	Switzerland	1	3			1	3
kiaa0020	*rsaI*	*H. arborea*	Switzerland		4				4
kiaa0020	*scrfI*	*H. arborea*	Switzerland		4		4		
kank1	*xmnI*	*H. arborea*	Switzerland		4		4		
kank1	*taqi*	*H. arborea*	Switzerland		4		4		
gldc	*mluci*	*H. arborea*	Greece		11	49			28
smarca2	*saci*	*H. orientalis*	Serbia	3		1	2	1	1
smarca2	*saci*	*H. arborea*	Greece	17	39		3	12	13
smn2	*bsiei*	*H. orientalis*	Serbia	4			4		
smn2	*bsiei*	*H. arborea*	Greece	7	30	23	4	9	13
smn2	*hpy166II*	*H. orientalis*	Serbia	2	1		2	1	
smn2	*hpy166II*	*H. arborea*	Greece	8	28	25	1	4	22
kank1	*taqi*	*H. orientalis*	Serbia			2			3
kank1	*taqi*	*H. arborea*	Greece			58			28
kank1	*xmni*	*H. orientalis*	Serbia	4			4		
kank1	*xmni*	*H. arborea*	Greece	60			28		
cdc37li	*hpy166II*	*H. orientalis*	Serbia	3	1		3	1	
cdc37li	*hpy166II*	*H. arborea*	Greece	33	24		22	5	
dmrt1	*bcci*	*H. arborea*	Greece		4				4
dmrt1	*bcci*	*H. orientalis*	Serbia		10				8
dmrt1	*bcci*	*H. molleri*	Spain		20			2	18
dmrt1	*bcci*	*H. intermedia*	Switzerland		20				20
dmrt1	*bcci*	*H. orientalis*	Greece		10				
dmrt1	*bcci*	*H. arborea*	Switzerland		5				5
dmrt1	*bcci*	*H. arborea*	Danube		5				4
dmrt1	*bcci*	*H. arborea*	Adriatic		5				5

CHAPTER VI

TURNOVERS OF EVER-YOUNG SEX CHROMOSOMES IN PALEARCTIC TREE FROGS

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Turnovers of ever young sex-chromosomes in Palearctic tree frogs

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ABSTRACT

Homomorphic sex chromosomes are hypothetically maintained through frequent sex chromosomes transitions or recurrent X-Y recombination. The latter have kept the sex chromosomes of closely-related European *Hyla* tree frogs ever-young for at least the past 5 My. Here we extend investigations of this chromosome pair (referred to as linkage group 1, LG1) to test whether the same pattern holds across ten Eurasian *Hyla* lineages, diverged by 3 to 40 Mya. Phylogenetic patterns of sex-linkage suggests several sex chromosome transitions (including one heterogametic switch) over the last 10 My, and sex-determination by LG1 in at least six out of nine resolved cases. As revealed by sibship analyses, males face a phylogenetic inertia of nearly zero recombination in all European species at LG1, regardless of sex-linkage, which may relate to its predisposition for sex. In parallel, phylogenies of sex-linked markers are consistent with chromosome-wide recurrent recombination within all species investigated. We conclude that both the frequent transitions and occasional recombination models operate in Palearctic tree frogs, and that the homomorphic *Hyla* linkage group LG1, which carries key genes for sexual development, is specialized in sex determination.

INTRODUCTION

Sex-chromosomes have evolved along drastically different trajectories between lineages. Mammals and birds feature remarkably stable genetic systems of sex determination, involving dimorphic sex chromosomes with respectively male (XY) and female (ZW) heterogamety. This dimorphism is the final stage of a long process initiated by recombination arrest between gametologs: more than 120 million years without recombination has driven the mammalian Y and avian W into progressive degeneration, by the accumulation of deleterious mutations through processes such as Muller's ratchet, hitchhiking with favorable mutations at other genes, background selection and insertion of repetitive elements, ultimately causing gene losses and drastic structural changes (reviewed by Charlesworth & Charlesworth 2000).

In contrast, in other groups genetic sex determination is way more labile. Many species of fish, amphibians and reptiles possess undifferentiated, seemingly nascent sex-chromosomes, testifying to distinct dynamics (Bachtrog et al. 2014). There are two main explanations for this pattern. On the one hand, it is widely accepted that senescing sex chromosomes may be frequently replaced through sex-chromosomes transitions (Schartl 2004, Volff et al. 2007). Comparative mapping showed that sex-determining systems can switch rapidly in amphibians and fishes (Malcom et al. 2014, Kikuchi & Hamaguchi 2013), leading to different sex chromosome pairs between closely-related species (Kitano & Peichel 2011, Mank & Avise 2009), or even between conspecific populations (Miura 2007). Theory predicts that several forces may drive these transitions, such as sex-antagonistic selection (van Doorn & Kirkpatrick 2007, 2010), sex-ratio selection (Grossen et al. 2011), and rescue of the Y genetic load (Blaser et al. 2013). Furthermore, the homologies noted between the sex-chromosomes of deeply diverged lineages have led up to the view that turnovers could be biased towards a limited set of chromosomes that carries important upstream genes from the sex-determining cascade (Graves & Peichel 2010, Brelsford et al. 2013), and so their dynamics may be more conserved than previously imagined.

On the other hand, it was recently proposed that old sex-determining systems can persist provided senescing Y chromosomes

rejuvenate via occasional events of recombination with the X, thus remaining "ever-young" (Perrin 2009, Stöck et al. 2011). Studies of X-Y differentiation at sex-linked markers provided convincing evidence for this model in a few amphibian groups (Stöck et al. 2011, 2013a). Evolutionary stable rates of XY recombination, which should be mediated by two main opposing forces (sex-antagonistic selection *versus* genetic load) may occur via two mechanisms. First, Perrin (2009) emphasized sex-reversal as a "fountain of youth" for Y chromosomes in ectothermic species (e.g. fish, reptiles and amphibians). As environmental factors (e.g. temperature) interfere with sex-determination, sex-reversed XY embryos developing into females will recombine sex chromosomes at meiosis, assuming recombination to depend on phenotypic rather than genetic sex (Perrin 2009). Second, Y copies may also recombine directly in some males (Stöck et al. 2013a, Dufresnes et al. 2014d). In theory, very little recombination is sufficient to renew and maintain old Y chromosomes in stable sex determining systems (Grossen et al. 2012).

The occasional recombination model received much support from the European tree frogs species complex (*Hyla arborea* group). In this group, X-Y recombination has kept sex chromosomes undifferentiated since the last 5 My: the same XY pair is shared among four recently-diverged lineages (namely *H. arborea*, *H. intermedia*, *H. molleri* and *H. orientalis*, Berset-Brändli et al. 2006, Stöck et al. 2011, 2013b; mapped as linkage group LG1, Berset-Brändli et al. 2008, Dufresnes et al. 2014c) and X and Y haplotypes are genetically homogeneous within species, indicative of recurrent gene exchange since they speciated (Stöck et al. 2011, Guerrero et al. 2012). Only a <50Kb portion surrounding the probable sex-determining gene *DMRT1* has remained X- and Y-specific through their diversification (Brelsford et al. in prep). This system being stable for at least 5 My, it becomes interesting to know how longer X-Y recombination have been at work to prevent genetic decay of this old sex chromosome pair. Does it still apply to deeper diverged *Hyla* species, or did some experienced sex chromosomes turnovers?

Despite substantial theoretical consideration, the evolutionary dynamics of turnovers and XY recombination are still largely unexplored in natural systems; estimating their relative contributions within a well-resolved radiation thus makes an important empirical question. Furthermore, in *Hyla*, one interesting aspect is that LG1 may be predisposed for sex: this pair carries genes that have been repeatedly co-opted to govern the vertebrate sex-determining cascade (e.g. *DMRT1*, *AMH*, Graves & Peichel 2010, Matson & Zarkower 2012, Breslford et al. 2013), and it is homologous to the sex-chromosomes of distant anuran species, including bufonids and ranids (Breslford et al. 2013). In this context, we were particularly interested to characterize how conserved sex-determination with LG1 was in *Hyla*.

To address these issues, we extended the survey to the rest of European radiation of tree frogs, namely the basal lineages *H. sarda*, *H. savignyi*, *H. felixarabica* and *H. meridionalis* (which arose 5-10 Mya, Stöck et al. 2012), as well as their Asian relatives *H. japonica* and *H. suweonensis* (diverged 30-40 Mya from European taxa, Smith et al. 2005). Phylogenetic relationships are shown in Figure 1. Through comparative linkage mapping, population genetic and phylogenetic analyses, (1) we test whether the same linkage group (LG1) is also sex-linked in these species; (2) we contrast its sex-specific recombination rates; and (3) we examine phylogenetic relationships of sex-linked sequence data to underline signs of recurrent X-Y recombination.

METHODS

SAMPLING AND DNA EXTRACTION

Adult samples comprised males and females from the same populations of *H. sarda*, *H. savignyi*, *H. meridionalis*, *H. japonica*, *H. suweonensis* and males from *H. felixarabica*. To obtain family samples (parents + offspring), mating pairs were caught during the breeding season and we raised their offspring to hatching (methods: Dufresnes et al. 2011), or for a subset, several months after metamorphosis, when phenotypic sex can be unambiguously determined by morphological identification of gonads (Haczkiewicz & Ogielska 2013). The final pedigree resource totalized 1896 offspring from 60 informative families of *H. meridionalis*, *H. sarda*, *H. savignyi* and *H. japonica*, including phenotypically sexed juveniles for the later three species. Details on sample sizes and origins are provided in File S1.

DNA was sampled using non-invasive buccal swabs (adults; Broquet et al. 2007), or ethanol fixed tissues (larvae, dissected froglets), and extracted with the Qiagen Biosprint Robotic workstation.

MARKER GENOTYPING AND SEQUENCING

In each species, we tested 15 microsatellite markers shown to be sex-linked in *H. arborea* and mapped as linkage group 1 (LG1, following Berset-Brändli et al. 2008 and Dufresnes et al. 2014c). These include the 14 loci used by Dufresnes et al.

(2014d; see their File S2 for the list of markers and methods), plus one additional microsatellite located within intron 7 of the gene *Smarcb1* (methods: Brelsford et al. 2013). Primers were re-designed for amplifying microsatellite *Ha-A103* in several species (forward: 5'-GGGACCTATGGATTAAAG-3'; reverse: 5'-CAATTCACACCCAAATCAGAT-3'). PCR products were run on an ABI-3100 genetic analyzer (Applied Biosystem, Inc.), and peaks were scored with Genemapper 4.0 (Applied Biosystems, Inc.). For each species, usable loci were genotyped in all adults and families.

We generated sequence data from four markers widely distributed across LG1 in adults from both sexes of each species under focus when possible, in complement to published sequences (Stöck et al. 2011, Dufresnes et al. 2014b). The four markers consisted of parts of the gene *Med15* (~1000bp, expect in *H. sarda* where it included a ~600bp insertion; encompassing introns 7, 8 and exons 7, 8), intronic sequences from the genes *SmarcB1* (~500bp encompassing intron 7) and *a-Fibrinogen* (~500bp encompassing intron 1), and flanking regions of the non-coding microsatellite *Ha*-A103 (~500bp, expect in *H. meridionalis* where it included a 700bp insertion). PCRs were carried out as described (*Med15, a-Fibrinogen* and *Ha*-A103: Stöck et al. 2011; *SmarcB1*: Brelsford et al. 2013), using re-designed primers for *Med15* (forward: 5'-TAGCATTAGCTATTAAGCATACTCG-3', reverse: 5'-TTACAGCAACAGCAAATGG-3'), *a-Fibrinogen* (forward: 5'-AGATACAGTCACAGTGCTAGGTTC-3', reverse: 5'-GGAGGATATCAGCACAGTCTAAA-3') and *Ha*-A103 (forward: 5'-ATGAATGGGCAAACTTTCCAT-3',

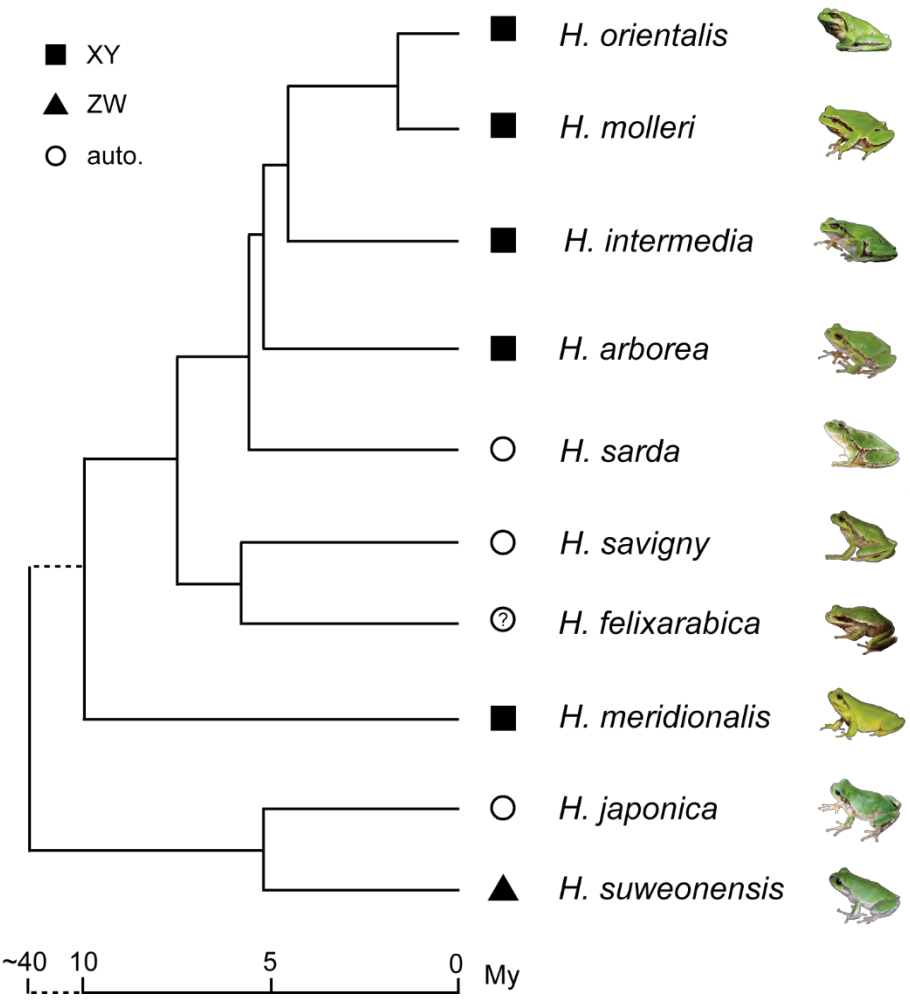

FIGURE 1: **LG1 tree of sex in Palearctic tree frog lineages.** Tree topology and approximate divergence times (My: Million years) were adapted from mitochondrial phylogenies and molecular dating by Smith et al. (2005) and Stöck et al. (2012). *H. suweonensis* unambiguously branched as a sister taxon of *H. japonica*. Symbols show patterns of sex-linkage for *H. arborea*'s linkage group LG1 (auto.: autosomal). Uncertainty remains for *H. felixarabica* since only adult males were analyzed.

TABLE 1: Sex-linkage of *Hyla* linkage group LG1 in the Eurasian tree frogs investigated

taxon	LG1 sex-linked?	evidence	references
H. arborea	yes, XY	sex-specific alleles in populations sex-specific inheritance of alleles	Berset-Brändli et al. 2006 Dufresnes et al. 2014d
H. intermedia	yes, XY	sex-specific alleles in populations	Stöck et al. 2011
H. molleri	yes, XY	sex-specific alleles in populations	Stöck et al. 2011
H. orientalis	yes, XY	sex-specific inheritance of alleles	Stöck et al. 2013
H. sarda	no	absence of sex-specific alleles in populations random inheritance of alleles	this study this study
H. savignyi	no	absence of sex-specific alleles in populations random inheritance of alleles	this study this study
H. felixarabica	prob not as XY	autosomal-like male genotypes in populations	this study
H. meridionalis	yes, XY	sex-specific alleles in populations	this study
*H. japonica**	no	absence of sex-specific alleles in populations random inheritance of alleles	this study this study
H. suweonensis	yes, ZW	sex-specific alleles in populations	this study

* an XY system was identified in this species (Kawamura & Nishioka 1977), although not on LG1.

reverse: 5'-GCCTAGAAATGTGCAGTGATC-3', for *H. meridionalis*, alternative forward: 5'-CCAAGACCTCTTGTCCAACATTAGT-3') optimized for cross-amplification. PCR products were cloned with the TOPO TA cloning kit (Life Technologies) or pGEM-easy vector system (Promega), from which at least 8 clones per sample were sequenced. A few samples were sequenced directly (*SmarcB1*, n=13). In these cases, heterozygous positions were visualized from electropherograms in MEGA 5.0 (Tamura et al. 2011). All sequences were edited and aligned in Seaview (Gouy et al. 2010). Only *Ha*-A103 sequences could not be generated in the two Asian species. Samples origins and GenBank accessions are provided in File S2.

Data analysis

We refer to Stöck et al. (2012) and Smith et al. (2005) for phylogenetic relationships and fossil-calibrated divergence times of the considered *Hyla* species (Figure 1). In complement, the position of *H. suweonensis* was inferred from published mitochondrial *cytochrome-b* sequences (GenBank KF564855-KF564864), branched on the *cyt-b* tree of Stöck et al. (2012) using a similar maximum-likelihood reconstruction.

Sex-linkage of LG1 was ascertained on two grounds. First, we compared adult male and female microsatellite genotypes directly, and by estimating allele frequencies and male-female pairwise differentiation (F_{ST}) in Fstat (Goudet 1995). Sex-linked loci are expected to carry sex-specific alleles and genotypes, i.e. heterozygotes in the heterogametic sex, and resulting in male-female differentiation. Potential patterns of sex-linkage were tested specifically with combinatory statistics. Second, in families with phenotypically sexed offspring, we tested whether the inheritance of maternal or paternal alleles was sex-specific by Fisher's exact tests (Rodrigues et al. 2013).

Species- and sex-specific microsatellite linkage maps of LG1 were computed using Crimap (Green et al. 1990). For each species, we first deduced the most likely order of loci with LOD scores (functions *all* and *flips*) and then calculated sex-specific recombination distances (function *build*). Final maps were produced with MapChart (Voorrips 2002).

We performed separate maximum-likelihood phylogenetic reconstructions of *Med15*, *SmarcB1*, *a-Fibrinogen* and *Ha*-A103 sequences with PhyML (Guindon et al. 2009), using respectively GTR+G, GTR+G, HKY+G and GTR models of sequence evolution (MrAIC, Nylander 2004) and 1000 bootstrap replicates. To avoid artefacts due to misalignments, microsatellite-like indels were discarded from the analyses. Following the rational of Stöck et al. (2011; see their Figure 1), males and female alleles should cluster together by species if LG1 is sex-linked and gametologs occasionally recombine. Obviously, such a pattern is also expected if LG1 is autosomal.

Results

Sex-linkage of LG1

LG1 mapped to the sex chromosomes of *H. meridionalis* and *H. suweonensis* with respectively male (XY) and female (ZW) heterogamety, based on sex-specific alleles and genotypes (File S3 and S4). In contrast, LG1 was autosomal in *H. sarda*, *H. savignyi* and *H. japonica*: similar alleles segregated in males and females and were not inherited in a sex-specific way (File S4). Male LG1 genotypes of *H. felixarabica* did not support an XY system (File S4). Overall, LG1 was thus confirmed as the sex chromosome pair in six cases (5 XY, 1 ZW) and as an autosomal pair in three (Figure 1, Table 1).

Sex-specific recombination rates

We documented extremely low male compared to female recombination rates at LG1 for the European *H. sarda* (36 fold difference), *H. savignyi* (26 fold difference), and *H. meridionalis* (23 fold difference). In these lineages, males featured a large non-recombining segment encompassing most of the linkage group, comparable to other European species (Figure 2). In contrast, on average the Asian *H. japonica* did not feature sex-specific recombination rates at LG1, neither a non-recombining segment, but limited crossovers in both sexes (Figure 2). Closer inspection of the family data suggests strong within-individual variation in recombination: some males and some females show almost complete absence of crossovers, whereas others freely recombine all markers.

Phylogeny of the LG1 markers

LG1 sequences distinguished between all the lineages considered, excepted for incomplete lineage sorting of the youngest *H. molleri* and *H. orientalis* in *Med15* and *Ha*-A103 trees (Figure 3). All male and female sex-linked alleles clustered by species, consistent with occasional XY and ZW recombination (Figure 3).

Discussion

We show that both XY recombination and frequent transitions model coexist within the *Hyla*

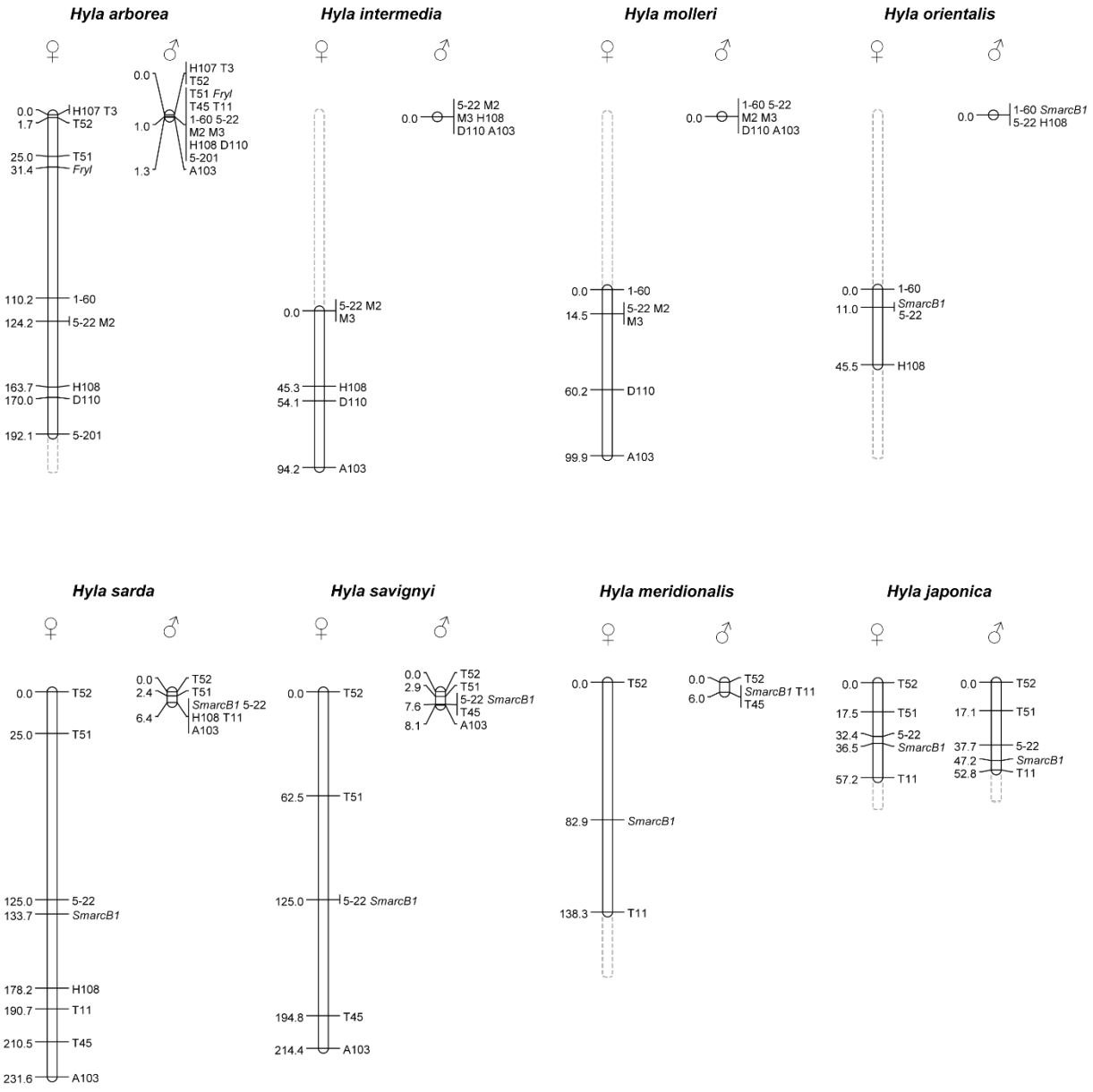

FIGURE 2: Sex-specific linkage maps of linkage group LG1. Loci orders were calculated separately for each species, and are based on the highest likelihood (CRI-MAP). The *H. arborea* map was adapted from Dufresnes et al. 2014c), including most of the loci cross-amplifying in congenerics. Maps of *H. molleri*, *H. intermedia* and *H. orientalis* were adapted from Stöck et al. 2011 and 2013b. Given trans-species similitudes in recombination rates, we extrapolated recombination distances to non-available peripheral markers for visual comparison (dash lines). Recombination distances are displayed in centiMorgan (cM).

tree frog radiation. As previously shown for *H. arborea*, *H. molleri* and *H. intermedia* (Stöck et al. 2011), all sex-linked sequences from both sexes grouped by species, reflecting strong X-Y and Z-W similarities, and supporting recurrent sex chromosomes recombination also within *H. orientalis*, *H. meridionalis* and *H. suweonensis* (Figure 3). This result yet contrasts with our failure to detect male recombination over most parts of LG1 in crosses of *H. meridionalis* (expect for terminal markers) and other European *Hyla* (Figure 2), suggesting cryptic mechanisms like sex-reversal events (Perrin 2009) or recent arrest of male recombination, as shown in some populations of *H. arborea* (Dufresnes et al. 2014d).

However, occasional XY recombination was apparently not sufficient to maintain the sex-determining system of tree frogs stable for more than 5 My. In parallel, patterns of sex-linkage at LG1 indicate several sex-chromosome transitions within the last 10 My, in both the European and Asian radiations (Figure 1). In Europe, assuming the mitochondrial phylogeny to reflect the true topology (Stöck et al. 2012; supported by genome-wide genotyping-by-sequencing data, A. Breslford, unpublished data), at least two turnovers are necessary to explain the LG1 tree of sex. (Figure 4). The different sex-determining systems between the two Asian species further involve a switch in heterogamety: we confirmed a ZW system in *H. suweonensis*, previously suggested from cytogenetics (Yu & Lee 1990), whereas the sister species *H. japonica* was shown to follow an XY system (Kawamura & Nishioka 1977), though not involving LG1.

Both alternative models of sex chromosome evolution can thus account for the maintenance of homomorphic sex chromosomes in *Hyla* i.e. frequent turnovers against a background of sex-chromosome recombination, highlighting their non-exclusiveness (Perrin 2009). Their combined actions raise interesting insights on the evolutionary dynamics of turnovers. We can rule out the accumulation of deleterious mutations (Blaser et al. 2013) as a transition's trigger in *Hyla* since the genetic load of the Y is recurrently purged by XY recombination. Nonetheless, the homomorphy maintained by occasional recombination may have promoted turnovers by other mechanisms, like sex-antagonistic selection (van Doorn & Kirkpatrick 2007), which is expected to induce shifts in heterogamety (van Doorn & Kirkpatrick 2010).

Our results bring significant support to the view that some chromosomes are better than other for sex determination (Graves & Peichel 2010, Bachtrog et al. 2011, O'Meally et al. 2012, Brelsford et al. 2013). Out of twelve chromosome pairs (Anderson 1991), *Hyla* LG1 was confirmed sex-linked in six out of nine resolved cases, despite a context of rapid changes. In the European radiation, it is unclear whether LG1 was recently co-opted for sex in most species (Figure 4a, c), or if it was conserved as the sex chromosome throughout the radiation, being replaced only recently in *H. sarda* and *H. savignyi* (Figure 4b). Phylogenetic analyses of *DMRT1* rather argue against the latter: trans-species X-Y differentiation shared by *H. arborea*/*intermedia*/*orientalis*/*molleri* at exon 1 is not shared by *H. meridionalis* (Figure 2 in Breslford et al. in prep), suggesting these lineages acquired sex-determination on LG1 independently (i. e. Figure 4a, c). It will be interesting to map the sex-chromosomes of *H. sarda*, *H. savignyi*, *H. felixarabica* and *H. japonica* to further reconstruct the history of turnovers. Testing cross-amplifying markers from other *H. arborea* linkage groups (Dufresnes et al. 2014a, 2014c) so far remained unfruitful (C. Dufresnes, unpublished data).

The frequent participation of LG1 in sex determination within hylids and other amphibians like bufonids and ranids (Brelsford et al. 2013) likely relates to its gene content. *DMRT1* and its paralogs have key roles in sex-differentiation in birds, fish and amphibians (Matson & Zarkower 2012). Assuming homology with *Xenopus* chromosome 1 (Brelsford et al. 2013), *Hyla* LG1 also carries the anti-Müllerian hormone gene *AMH*, required for testis development in mammals and probably determining sex in monotremes (Cortez et al. 2014). In addition, LG1 may have adapted to its recurrent role in sex. Chromosomes that are repeatedly sex-linked are expected to accumulate genes with sex-antagonistic effects, in turn making them more likely to recapture sex-determination functions in future turnovers (van Doorn & Kirkpatrick 2007, Blaser et al. 2014). Another presumed adaptation is the evolution of recombination rates, which can further predispose transitions towards former sex chromosomes. In the European radiation, LG1 faces a phylogenetic inertia of extremely reduced recombination in males from all species, even where it is autosomal (*H. sarda* and *H. savignyi*, Figure 2). Male recombination is globally repressed over the whole genome in *H. arborea* (Berset-Brändli et al. 2008, Dufresnes et al. 2014c), but ratios of female over male recombination are at least twice higher for LG1 (about 36 in *H. sarda*, 26 in *H. savigny*) compared to autosomes (in *H. arborea*: 14.3, Berset-Brändli et al. 2008; 13.7, Dufresnes et al. 2014c). It is then tempting to suggest that such inertia results from the specialization of LG1 as an XY pair of sex chromosomes in these lineages, and remained conserved across the entire radiation, suggesting

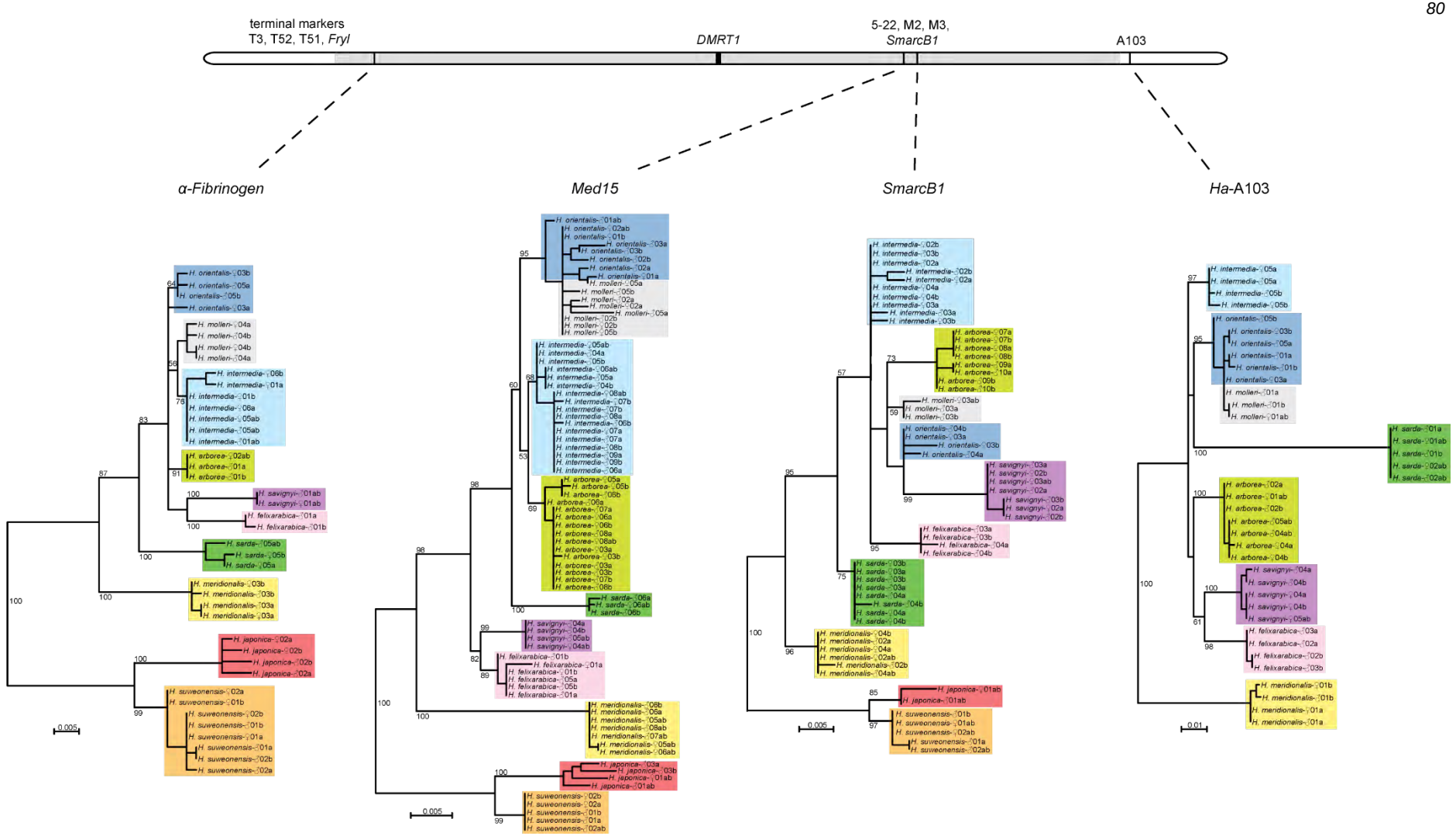

FIGURE 3: Maximum-likelihood phylogenies of four LG1-linked markers. The top schematic illustrates rough relative position of these markers across LG1, based on linkage analyses (Figure 2) and homologies with *Xenopus tropicalis* (Brelsford et al. 2013); the shaded area corresponds to the non-recombining region of European males. Bootstrap values are shown for the main branches, when above 50%. Colors code for species (following Stöck et al. 2012). Sequence labels feature species, sex, ID number and allele (*a*, *b* or *ab* for homozygotes). Male alleles correspond to X and Y copies in *H. arborea*, *H. molleri*, *H. orientalis*, *H. intermedia* and *H. meridionalis*, and female alleles correspond to Z and W copies in *H. suweonensis*; these always group together by species for all four markers, consistent with chromosome-wide occasional XY and ZW recombination. Only the youngest *H. orientalis* (dark blue) and *H. molleri* (grey) show incomplete lineage sorting for *Med15* and *Ha*-A103.

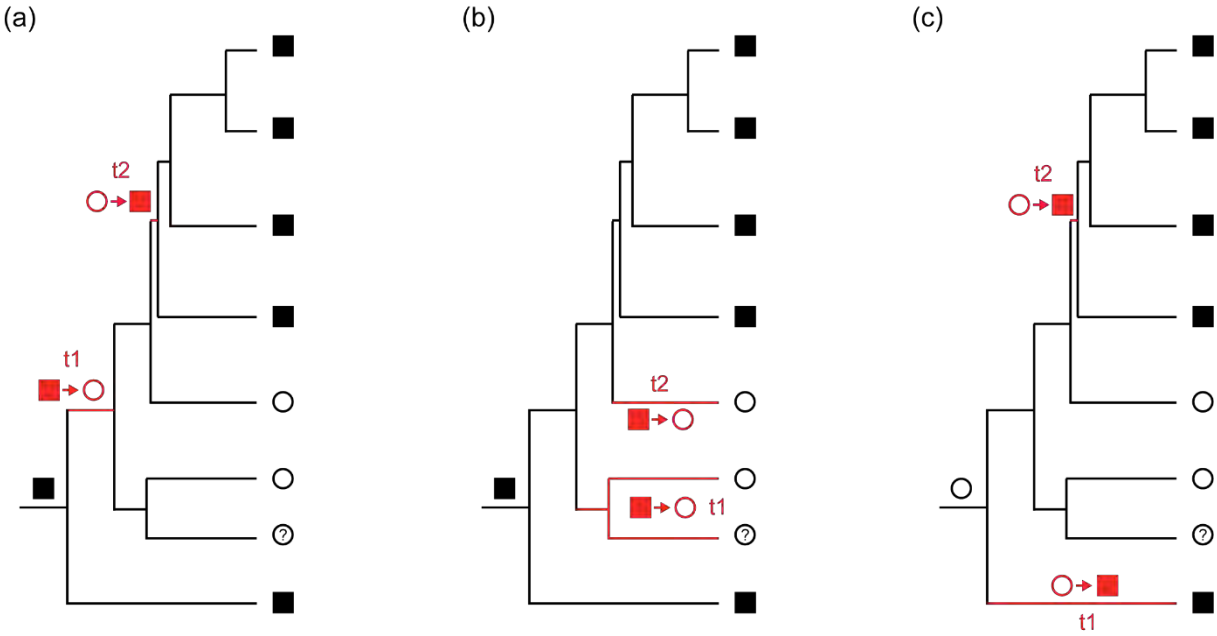

FIGURE 4: Parsimonious turnover scenarios accounting for patterns of sex-linkage at LG1 in the European *Hyla* radiation. Filled squares: sex-linked (XY), empty circles: autosomal. Turnover events are indicated in red. (a) Assuming that sex-linkage of LG1 is the ancestral state, a first turnover occurred after the split of *H. meridionalis* in the ancestor of *H. savignyi/felixarabica/sarda* (t1), and a second turnover re-established sex-linkage back to LG1 in the ancestor of *H. arborea/intermedia/orientalis/molleri* (t2). (b) LG1 is the sex chromosome in all ancestral species and two recent turnovers independently occurred within *H. savignyi/felixarabica* (t1) and *H. sarda* (t2) lineages. (c) Assuming autosomal location of LG1 as the ancestral state, two independent turnovers recently co-opted LG1 for sex determination within *H. meridionalis* (t1) and in the ancestor of *H. arborea/intermedia/orientalis/molleri* (t2).

some chromosome-specific regulation of recombination in males. In mammals, recombination hot spots were shown to evolve rapidly, being mostly specified by the gene *Prdm9* (Baudat et al. 2013), and sequence variation at *RNF212* directly associates with sex-specific recombination (Kong et al. 2008).

In contrast, the complex situation in *H. japonica*, involving strongly-reduced recombination at LG1 in several individuals from both sexes, is difficult to interpret. If LG1 recombination rates are indeed fine-tuned during episodes of sex-linkage, this polymorphism may relate to the co-occurrence of different heterogametic systems in the closely-related Asian species. Comparison with genome-wide recombination would be a first step to understand this pattern. Further investigations are obviously required, and should notably aim at measuring LG1 recombination rates in the ZW *H. suweonensis*, as well as estimating the contribution of LG1 for sex-determination in other Asian lineages.

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File S1: Origin and number of samples used in population and sibship analyses for each *Hyla* species investigated.

Species	Origin	Geographic coordinates (Lat/long)		loci	Nm	Nf	Np	No
Hyla sarda	France, Corsica	43.000°	9.404°	8	24	24	21	713 (80)
Hyla savignyi	Cyprus, Drouseia	34.971°	32.406°	6	18	18	17	504 (13)
	Cyprus, Kannaviou	34.933°	32.573°	6	-	-	4	116
Hyla felixarabica	Jordan, Canjon S Dana	30.661°	35.622°	5	10	0	-	-
Hyla meridionalis	France, La-Tour-du-Valat	43.500°	4.700°	4	20	20	13	361
Hyla japonica	South-Korea, Seocheon	36.076°	126.852°	5	11	9	-	-
	South-Korea, Andong	36.472°	128.167°	5	14	4	-	-
	South-Korea, Gwanak-gu	37.460°	126.952°	5	-	-	5	202 (107)
Hyla suweonensis	South-Korea, Siheung	37.410°	126.805°	1	5	7	-	-
	South-Korea, Geumcheon	37.750°	126.758°	1	18	0	-	-

Nm: number of males, Nf: number of females (used in population genetics analyses); Np: number of pairs (families); No: number of offspring (incl. sexed offspring in brackets)

File S2: Samples used in phylogenetic analyses of LG1

ID	Species	Sex	Origin	Latitude	Longitude	*α-Fibrinogen*	*SmarcB1*	*Ha-A103*	*Med15*
H. arborea-♀01	*H. arborea*	F	Croatia, Istria	45.34	13.81	-	-	×	-
H. arborea-♀02	*H. arborea*	F	Croatia, Karlovac	45.54	15.57	×	-	-	-
H. arborea-♀03	*H. arborea*	F	Greece, Crete	35.19	25.46	-	-	-	×
H. arborea-♀04	*H. arborea*	F	Netherlands, Lochem	52.12	6.48	-	-	×	-
H. arborea-♀05	*H. arborea*	F	Serbia, SE-Serbia	42.33	21.90	-	-	-	×
H. arborea-♀06	*H. arborea*	F	Serbia, Vojvodina	44.74	20.14	-	-	-	×
H. arborea-♀07	*H. arborea*	F	Switzerland, Vaud	46.50	6.42	-	×	-	-
H. arborea-♀08	*H. arborea*	F	Switzerland, Vaud	46.50	6.42	-	×	-	×
H. arborea-♂01	*H. arborea*	M	Croatia, Karlovac	45.54	15.57	×	-	-	-
H. arborea-♂02	*H. arborea*	M	Croatia, Krk	45.17	14.62	-	-	×	-
H. arborea-♂03	*H. arborea*	M	Greece, Crete	35.19	25.46	-	-	-	×
H. arborea-♂04	*H. arborea*	M	Netherlands, Lochem	52.12	6.48	-	-	×	-
H. arborea-♂05	*H. arborea*	M	Netherlands, Lochem	52.12	6.48	-	-	×	-
H. arborea-♂06	*H. arborea*	M	Serbia, SE-Serbia	42.33	21.90	-	-	-	×
H. arborea-♂07	*H. arborea*	M	Serbia, Vojvodina	44.74	20.14	-	-	-	×
H. arborea-♂08	*H. arborea*	M	Switzerland, Vaud	46.50	6.42	-	-	-	×
H. arborea-♂09	*H. arborea*	M	Switzerland, Zürich	47.58	8.60	-	×	-	-
H. arborea-♂10	*H. arborea*	M	Switzerland, Zürich	47.58	8.60	-	×	-	-
H. felixarabica-♀01	*H. felixarabica*	F	Syria, Jebbel Druz	32.66	36.73	-	-	-	×
H. felixarabica-♂01	*H. felixarabica*	M	Jordan, Canjon S Dana	30.66	35.62	×	-	-	×
H. felixarabica-♂02	*H. felixarabica*	M	Jordan, Canjon S Dana	30.66	35.62	-	-	×	-
H. felixarabica-♂03	*H. felixarabica*	M	Jordan, Canjon S Dana	30.66	35.62	-	×	×	-
H. felixarabica-♂04	*H. felixarabica*	M	Jordan, Canjon S Dana	30.66	35.62	-	×	-	-
H. felixarabica-♂05	*H. felixarabica*	M	Syria, Jebbel Druz	32.66	36.73	-	-	-	×
H. felixarabica-♂06	*H. felixarabica*	M	Yemen, S Yarim	14.22	44.39	-	-	-	×
H. intermedia-♀01	*H. intermedia*	F	Switzerland, Piazzogna	46.14	8.82	×	-	-	-
H. intermedia-♀02	*H. intermedia*	F	Switzerland, Piazzogna	46.14	8.82	-	×	-	-
H. intermedia-♀03	*H. intermedia*	F	Switzerland, Piazzogna	46.14	8.82	-	×	-	-
H. intermedia-♀04	*H. intermedia*	F	Switzerland, Piazzogna	46.14	8.82	-	×	-	-
H. intermedia-♀05	*H. intermedia*	F	Switzerland, Piazzogna	46.14	8.82	×	-	×	×
H. intermedia-♀06	*H. intermedia*	F	Switzerland, Piazzogna	46.14	8.82	×	-	-	×
H. intermedia-♀07	*H. intermedia*	F	Italy, Calabria	39.35	16.03	-	-	-	×
H. intermedia-♀08	*H. intermedia*	F	Italy, Calabria	-	-	-	-	-	-
H. intermedia-♂01	*H. intermedia*	M	Switzerland, Piazzogna	46.14	8.82	×	-	-	-
H. intermedia-♂02	*H. intermedia*	M	Switzerland, Piazzogna	46.14	8.82	-	×	-	-
H. intermedia-♂03	*H. intermedia*	M	Switzerland, Piazzogna	46.14	8.82	-	×	-	-
H. intermedia-♂04	*H. intermedia*	M	Switzerland, Piazzogna	46.14	8.82	-	-	-	×
H. intermedia-♂05	*H. intermedia*	M	Switzerland, Piazzogna	46.14	8.82	×	-	×	×
H. intermedia-♂06	*H. intermedia*	M	Italy, Sicily	37.29	15.00	-	-	-	×
H. intermedia-♂07	*H. intermedia*	M	Italy, Sicily	38.10	15.14	-	-	-	×
H. intermedia-♂08	*H. intermedia*	M	Italy, Sicily	38.14	15.05	-	-	-	×

H. intermedia-♂09	*H. intermedia*	M	Italy, Sicily	37.33	15.08	-	-	-	×
H. japonica-♀01	*H. japonica*	F	Japan, Hiroshima	34.43	132.74	-	×	-	×
H. japonica-♀02	*H. japonica*	F	Japan, Hiroshima	34.43	132.74	×	-	-	-
H. japonica-♂01	*H. japonica*	M	Japan, Hiroshima	34.43	132.74	-	×	-	×
H. japonica-♂02	*H. japonica*	M	Japan, Hiroshima	34.43	132.74	×	-	-	-
H. japonica-♂03	*H. japonica*	M	Japan, Hiroshima	34.43	132.74	-	-	-	×
H. meridionalis-♀01	*H. meridionalis*	F	France, Camargue	43.52	4.70	-	-	×	-
H. meridionalis-♀02	*H. meridionalis*	F	France, Camargue	43.52	4.70	-	×	-	-
H. meridionalis-♀03	*H. meridionalis*	F	France, Camargue	43.52	4.70	×	-	-	-
H. meridionalis-♀04	*H. meridionalis*	F	France, Camargue	43.52	4.70	-	×	-	-
H. meridionalis-♀05	*H. meridionalis*	F	France, Camargue	43.52	4.70	-	-	-	×
H. meridionalis-♀06	*H. meridionalis*	F	France, Camargue	43.52	4.70	-	-	-	×
H. meridionalis-♂01	*H. meridionalis*	M	France, Camargue	43.52	4.70	-	-	×	-
H. meridionalis-♂02	*H. meridionalis*	M	France, Camargue	43.52	4.70	-	×	-	-
H. meridionalis-♂03	*H. meridionalis*	M	France, Camargue	43.52	4.70	×	-	-	-
H. meridionalis-♂04	*H. meridionalis*	M	France, Camargue	43.52	4.70	-	×	-	-
H. meridionalis-♂05	*H. meridionalis*	M	Portugal, Algarve	37.31	-8.60	-	-	-	×
H. meridionalis-♂06	*H. meridionalis*	M	Portugal, Algarve	37.31	-8.60	-	-	-	×
H. meridionalis-♂07	*H. meridionalis*	M	Spain, Tenerifa	28.40	-16.53	-	-	-	×
H. meridionalis-♂08	*H. meridionalis*	M	Spain, Tenerifa	28.40	-16.53	-	-	-	×
H. molleri-♀01	*H. molleri*	F	Spain, Valdemanco	40.85	-3.65	-	-	×	-
H. molleri-♀02	*H. molleri*	F	Spain, Valdemanco	40.85	-3.65	-	-	-	×
H. molleri-♀03	*H. molleri*	F	Spain, Cantera	40.21	-4.65	-	×	-	-
H. molleri-♀04	*H. molleri*	F	Spain, Cantera	40.21	-4.65	×	-	-	-
H. molleri-♀05	*H. molleri*	F	Spain, Cantera	40.21	-4.65	-	-	-	×
H. molleri-♂01	*H. molleri*	M	Spain, Valdemanco	40.85	-3.65	-	-	×	-
H. molleri-♂02	*H. molleri*	M	Spain, Valdemanco	40.85	-3.65	-	-	-	×
H. molleri-♂03	*H. molleri*	M	Spain, Cantera	40.21	-4.65	-	×	-	-
H. molleri-♂04	*H. molleri*	M	Spain, Cantera	40.21	-4.65	×	-	-	-
H. molleri-♂05	*H. molleri*	M	Spain, Cantera	40.21	-4.65	-	-	-	×
H. orientalis-♀01	*H. orientalis*	F	Greece, Rhodos	36.36	28.12	-	-	-	×
H. orientalis-♀02	*H. orientalis*	F	Greece, Thrace	41.41	26.63	-	-	-	×
H. orientalis-♀03	*H. orientalis*	F	Serbia, SE-Serbia	43.06	22.68	×	×	×	-
H. orientalis-♂01	*H. orientalis*	M	Azerbaijan, Lenkoran	38.65	48.82	-	-	×	×
H. orientalis-♂02	*H. orientalis*	M	Greece, Lesvos	39.23	26.03	-	-	-	×
H. orientalis-♂03	*H. orientalis*	M	Greece, Rhodos	36.36	28.12	-	-	-	×
H. orientalis-♂04	*H. orientalis*	M	Serbia, SE-Serbia	43.06	22.68	-	×	-	-
H. orientalis-♂05	*H. orientalis*	M	Serbia, SE-Serbia	43.06	22.68	×	-	×	-
H. sarda-♀01	*H. sarda*	F	France, Corsica	43.00	9.40	-	-	×	-
H. sarda-♀02	*H. sarda*	F	France, Corsica	43.00	9.40	-	-	×	-
H. sarda-♀03	*H. sarda*	F	France, Corsica	43.00	9.40	-	×	-	-
H. sarda-♀04	*H. sarda*	F	France, Corsica	43.00	9.40	-	×	-	-
H. sarda-♀05	*H. sarda*	F	France, Corsica	43.00	9.40	×	-	-	-
H. sarda-♀06	*H. sarda*	F	Italy, Sardinia	40.82	8.49	-	-	-	×
H. sarda-♂01	*H. sarda*	M	France, Corsica	43.00	9.40	-	-	×	-
H. sarda-♂02	*H. sarda*	M	France, Corsica	43.00	9.40	-	-	×	-
H. sarda-♂03	*H. sarda*	M	France, Corsica	43.00	9.40	-	×	-	-
H. sarda-♂04	*H. sarda*	M	France, Corsica	43.00	9.40	-	×	-	-

H. sarda-♂05	*H. sarda*	M	France, Corsica	43.00	9.40	×	-	-	-
H. sarda-♂06	*H. sarda*	M	Italy, Sardinia	40.82	8.49	-	-	-	×
H. savignyi-♀01	*H. savignyi*	F	Cyprus, Drouseia	34.97	32.41	×	-	-	-
H. savignyi-♀02	*H. savignyi*	F	Cyprus, Drouseia	34.97	32.41	-	×	-	-
H. savignyi-♀03	*H. savignyi*	F	Cyprus, Kannaviou	34.93	32.57	-	×	-	-
H. savignyi-♀04	*H. savignyi*	F	Cyprus, Sotira	34.71	32.85	-	-	×	×
H. savignyi-♀05	*H. savignyi*	F	Cyprus, Sotira	34.71	32.85	-	-	×	-
H. savignyi-♂01	*H. savignyi*	M	Cyprus, Drouseia	34.97	32.41	×	-	-	-
H. savignyi-♂02	*H. savignyi*	M	Cyprus, Drouseia	34.97	32.41	-	×	-	-
H. savignyi-♂03	*H. savignyi*	M	Cyprus, Kannaviou	34.93	32.57	-	×	-	-
H. savignyi-♂04	*H. savignyi*	M	Cyprus, Sotira	34.71	32.85	-	-	×	×
H. savignyi-♂05	*H. savignyi*	M	Cyprus, Sotira	34.71	32.85	-	-	-	×
H. suweonensis-♀01	*H. suweonensis*	F	South-Korea, Siheung	37.41	126.80	×	×	-	-
H. suweonensis-♀02	*H. suweonensis*	F	South-Korea, Siheung	37.41	126.80	×	×	-	×
H. suweonensis-♂01	*H. suweonensis*	M	South-Korea, Siheung	37.41	126.80	×	×	-	×
H. suweonensis-♂02	*H. suweonensis*	M	South-Korea, Siheung	37.41	126.80	×	×	-	×

File S3: sex-linked microsatellite genotypes in adults of *H. meridionalis* (a) and *H. suweonensis* (b).

File S3a: genotypes of four LG1 markers in 20 females and 20 males of H. meridionalis

Sample	**Sex**	***Ha*-T52**		***SmarcB1***		***Ha*-T45**		***Ha*-T11**	
HmerF11	F	365	365	NA	NA	209	209	333	330
HmerF12	F	365	365	NA	NA	209	209	330	330
HmerF13	F	365	365	239	240	209	209	333	330
HmerF14	F	356	365	239	240	209	209	333	330
HmerF15	F	356	365	239	240	209	209	333	333
HmerF16	F	356	365	239	239	209	209	333	330
HmerF17	F	365	365	NA	NA	209	209	333	330
HmerF18	F	365	365	NA	NA	209	209	333	330
HmerF06	F	365	365	239	239	209	209	330	330
HmerF07	F	356	365	239	240	209	209	333	330
HmerF08	F	365	365	239	239	209	209	333	333
HmerF09	F	356	365	239	239	209	209	330	330
HmerF10	F	365	365	239	239	209	209	333	330
HmerF19	F	365	365	239	239	209	209	333	333
HmerF20	F	356	365	239	239	209	209	333	330
HmerF21	F	365	365	239	240	209	209	336	333
HmerF22	F	365	365	239	240	209	209	333	330
HmerF23	F	365	365	239	240	209	209	333	330
HmerF24	F	356	365	239	240	209	209	333	330
HmerF25	F	365	365	239	239	209	209	330	330
HmerM11	M	365	365	NA	NA	209	212	333	330
HmerM12	M	365	365	NA	NA	209	212	330	330
HmerM13	M	356	365	240	240	209	212	330	330
HmerM14	M	365	365	239	239	209	212	330	330
HmerM15	M	365	365	239	239	209	212	333	330
HmerM16	M	365	365	239	240	209	212	333	330
HmerM17	M	365	365	NA	NA	209	212	333	330
HmerM18	M	356	365	NA	NA	209	212	330	330
HmerM06	M	356	365	239	239	209	212	330	330
HmerM07	M	365	365	239	240	209	212	330	330
HmerM08	M	356	365	239	240	209	212	333	330
HmerM09	M	365	365	239	240	209	212	333	330
HmerM10	M	365	365	NA	NA	209	212	330	330
HmerM19	M	365	365	239	239	209	212	336	330
HmerM20	M	356	365	239	239	209	212	333	330
HmerM21	M	356	365	239	239	209	212	330	330
HmerM22	M	365	365	239	240	209	212	330	330
HmerM23	M	356	365	239	239	209	212	330	330
HmerM24	M	365	365	239	240	209	212	333	330
HmerM25	M	356	365	239	239	209	212	330	330

At locus *Ha*-T45, all females are homozygous *209*/*209* and all males are heterozygous *209*/*212*, suggesting an XY pattern. Males also possess at least one copy of *Ha*-T11 allele *330*, which co-segregates with *Ha*-T45 allele *212* on the Y (blue color). F: female, M: male, NA: missing data

File S3b: genotypes of marker WHA5-22 in males and females from H. suweonensis

Sample	Sex	WHA5-22	
Locality Siheung			
14SUR48	F	228	228
14SUR50	F	228	234
14SUR51	F	231	234
14SUR52	F	228	234
14SUR53	F	228	234
14SIH03	F	228	231
14SIH04	F	228	234
14SIH01	M	228	228
14SIH02	M	228	228
14SUR49	M	228	228
14SUR55	M	228	228
14SUR56	M	228	228
Locality Geumcheon			
13BHV383	M	228	228
13BHV386	M	228	231
13BHV387	M	228	228
13BHV389	M	228	231
13BHV390	M	228	231
13BHV391	M	228	231
13BHV384	M	228	228
13BHV393	M	228	228
13BHV394	M	228	231
13BHV396	M	228	231
13BHV397	M	228	228
13BHV398	M	228	231
13BHV399	M	228	231
13BHV400	M	228	231
13BHV401	M	228	231
13BHV403	M	228	228
13BHV404	M	228	231
13BHV416	M	228	231

Most females are heterozygotes with a specific allele *234*, not found in any males, suggesting a ZW pattern.
F: female, M: male.

File S4: Inference of sex-linkage from adult and family data

File S4a: data interpretation

Two lines of evidence support autosomal location of linkage group 1 in *H. sarda*, *H. savignyi* and *H. japonica*. First, males and females did not carry sex-specific alleles in these species, and their genotypes did not suggest any pattern of heterogamety (File S4c). Statistically, this translated into an absence of male-female differentiation (i.e. non-significant F_{ST}), as reported (File S4b). Second, inheritance of alleles from parents to offspring was not associated to sex (File S4b), providing direct evidence that LG1 does not lie within the sex-chromosomes in these species.

In *H. felixarabica*, male genotypes and allele frequencies were not consistent with an XY system (no pattern of male heterogamety; File S4c), but an hypothetical ZW system cannot be ruled out without female samples, not available here.

In *H. meridionalis*, marker *Ha*-T45 displays a perfect XY pattern. At this locus, every 20 males were genotyped *209*/*212*, and every 20 females were genotyped *209*/*209* (File S3). From combinatorial statistics, the probability that this occurs by chance, assuming autosomal localization, is obtained as the ratio of $\frac{20!}{20!\,0!}2^{20}$ (number of combinations of 20 copies of allele *212* among 20 males, one copy each) over $\frac{80!}{20!\,60!}$ (number of combinations of these 20 copies among 80 copies of genes), which amounts to $p = 2.97\times10^{-13}$. The obvious parsimonious explanation is that allele *212* is fixed on the Y, and that allele *209* is fixed on the X. Departure from autosomal expectations at LG1 was also depicted by the significant male-female F_{ST} (File S4b).

In *H. suweonensis*, 5 out of the 7 females from locality Siheung were heterozygotes at locus WHA5-22, harboring one copy of allele *234*, which was not present in any of the 5 males from the same population, and neither from the 18 males sampled at locality Geumcheon (apart from Siheung by <50km). In Siheung, the probability that such sex bias arose by chance corresponds to the ratio $\frac{7!}{5!\,2!}2^5$ (number of combinations of 5 copies of allele *234* among 7 females, maximum one copy each) over $\frac{24!}{5!\,19!}$ (number of combinations of these five copies among 24 copies of genes), which amounts to $p = 0.016$. Consequently, females are significantly differentiated from males based on this marker (Table S4b). The most parsimonious explanation is that LG1 constitutes a ZW pair of sex chromosomes in this taxon, with WHA5-22 allele *234* segregating on the W, as otherwise suggested from cytogenetics (Yu & Lee 1990).

File S4b: Summary statistics used to document sex-linkage

Taxon	**Fisher's exact test**	♀-♂ **FST**
Hyla sarda	NS in 11 tested families	0.006^{NS}
Hyla savignyi	NS in 2 tested families	0.0143^{NS}
Hyla meridionalis	-	**0.1764***
Hyla japonica	NS in 5 tested families	0.0006^{NS} (Seocheon) 0.0121^{NS} (Andong)
Hyla suweonensis	-	**0.3242*** (Siheung)

^{NS}: non-significant; *: $p<0.05$, highlighted in bold. Fisher's exact test: test of sex-specific allele inheritance in families with sexed offspring.

File S4c: Sex-specific allele frequencies

H. sarda

Ha-T52			*Ha*-T51			WHA5-22			*SmarcB1*			*Ha*-H108			*Ha*-T11			*Ha*-T45			*Ha*-A103		
	♀	♂		♀	♂		♀	♂		♀	♂		♀	♂		♀	♂		♀	♂		♀	♂
352	0.229	0.229	*372*	0.396	0.458	*222*	0.021	0.042	*242*	0.458	0.542	*203*	0.125	0.229	*329*	0.15	0.17	*209*	0.042	0	*178*	0.125	0.229
371	0.375	0.292	*374*	0.438	0.479	*225*	0	0.063	*243*	0.542	0.458	*221*	0	0.042	*332*	0.854	0.833	*215*	0.958	1	*191*	0.479	0.563
373	0.396	0.479	*376*	0.042	0	*228*	0.146	0.146				*230*	0.271	0.375							*193*	0	0.021
			378	0.125	0.063	*231*	0.833	0.75				*237*	0.604	0.354							*195*	0.354	0.146
																					197	0.021	0.042
																					199	0.021	0

H. savignyi

Ha-T52			*Ha*-T51			WHA5-22			*SmarcB1*			*Ha*-T45			*Ha*-A103		
	♀	♂		♀	♂		♀	♂		♀	♂		♀	♂		♀	♂
367	0.5	0.361	*374*	1	0.806	*234*	0	0.056	*246*	0.056	0.056	*206*	0.056	0.139	*186*	0.083	0.139
372	0.5	0.639	*376*	0	0.194	*236*	0.111	0.222	*247*	0.167	0.194	*212*	0.944	0.861	*188*	0.083	0.194
						239	0.861	0.722	*248*	0.778	0.75				*190*	0.278	0.222
						242	0.028	0							*192*	0.167	0.194
															194	0.111	0.111
															198	0.278	0.139

H. felixarabica

Ha-T52		*Ha*-T51		WHA5-22		*Ha*-M2		*SmarcB1*	
	♂		♂		♂		♂		♂
356	0.1	*374*	0.70	*214*	0.15	*108*	0.05	*241*	0.95
374	0.9	*376*	0.05	*217*	0.2	*121*	0.95	*244*	0.05
		378	0.25	*219*	0.05				
				222	0.6				

H. meridionalis

Ha-T52			*SmarcB1*			*Ha*-T11			*Ha*-T45		
	♀	♂		♀	♂		♀	♂		♀	♂
356	0.175	0.2	*239*	0.75	0.733	*330*	0.5	0.775	***209***	**1**	**0.5**
365	0.825	0.8	*240*	0.25	0.267	*333*	0.475	0.2	***212***	**0**	**0.5**
						336	0.025	0.025			

H. japonica (Seocheon)

Ha-T52			*Ha*-T51			WHA5-22			*SmarcB1*			*Ha*-T11		
	♀	♂		♀	♂		♀	♂		♀	♂		♀	♂
355	0.313	0.136	*374*	0.63	0.35	*219*	0.00	0.05	*220*	0.00	0.14	*325*	0.11	0.00
356	0.313	0.500	*375*	0	0.05	*222*	0.111	0.091	*222*	1	0.864	*328*	0.056	0.136
357	0.188	0.136	*376*	0.063	0.15	*225*	0.389	0.455				*331*	0.778	0.864
358	0.125	0.227	*377*	0	0.05	*228*	0.5	0.409				*334*	0.056	0
360	0.06	0.00	*378*	0.125	0.2									
			379	0.063	0.1									
			380	0	0.05									
			383	0	0.05									
			387	0.063	0									
			389	0.063	0									

H. japonica (Andong)

Ha-T52			*Ha*-T51			WHA5-22			*SmarcB1*			*Ha*-T11			*Ha*-T45		
	♀	♂		♀	♂		♀	♂		♀	♂		♀	♂		♀	♂
355	0	0.214	*374*	0.25	0.23	*219*	0.00	0.04	*218*	0.00	0.04	*325*	0.38	0.04	*209*	0	0.036
356	0.625	0.357	*375*	0.375	0.154	*222*	0.00	0.036	*220*	0	0.036	*328*	0	0.143	*212*	1	0.964
357	0.375	0.25	*376*	0.25	0.192	*225*	0.875	0.607	*222*	1	0.929	*331*	0.625	0.821			
358	0	0.107	*378*	0	0.269	*228*	0.125	0.286									
359	0.00	0.071	*379*	0.125	0.077	*231*	0	0.036									
			381	0	0.038												
			383	0	0.038												

H. suweonensis

WHA5-22			
	Siheung		Geumcheon
	♀	♂	♂
228	0.5	1	0.67
231	0.14	0	0.33
234	**0.36**	**0**	**0**

CHAPTER VII

STRONG BETWEEN-MALE VARIATION OF XY RECOMBINATION IN *HYLA* TREE FROGS

Christophe Dufresnes and Nicolas Perrin

Dufresnes C, Perrin N. Strong between-male variation of XY recombination in *Hyla* tree frogs.

Strong between-male variation of XY recombination in *Hyla* tree frogs

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ABSTRACT

Recombination between sex chromosomes conditions their evolutionary fate. The arrest of recombination between mammals and birds' sex chromosomes drove them into degeneration. In contrast, low rates of X-Y recombination maintained sex chromosome homomorphic in tree frogs, but the dynamics of this process are still obscure. Here we report strong heterogeneity of male recombination at the sex chromosomes of Palearctic *Hyla* tree frogs: only few individuals produced the observed XY recombinants, whereas recombination is completely suppressed in most males. This phenotypic variation may reflect some balanced selection on XY recombination resulting from the counteracting effects of sex-antagonistic selection (favoring full linkage) and genetic load (favoring recombination). Interestingly, the same pattern holds in *Hyla* species where this chromosome pair is not sex-linked, suggesting conserved mechanisms. As discussed for the European tree frog *H. arborea*, the documented variance will be important to consider in our comprehension of evolutionary stable sex chromosome recombination in future empirical and theoretical studies.

INTRODUCTION

Recombination between sex chromosomes is the key factor driving their evolutionary fate. Sex-chromosome dimorphism, as found in mammals and birds, is the result of a long process initiated by the arrest of recombination between gametologs in the heterogametic sex (XY males, ZW females). Classical models of sex-chromosome evolution predict that, soon after the appearance of the sex-determining locus, the suppression of XY recombination constitutes a crucial first step to promote full linkage between this locus and neighboring sex-antagonistic genes, so that male- and female-beneficial alleles are only transmitted to son and daughters respectively (Rice 1987, 1996). This arrest of recombination has far-reaching consequences for the non-recombining gametolog: unable to purge deleterious mutations that tend to get fixed under the combined effects of enhanced drift, selective sweeps, background selection and Müller's ratchet, Y chromosomes starts to progressively degenerate, by losing gene functions, accumulating repetitive elements, and ultimately facing irreversible structural changes (reviewed by Charlesworth & Charlesworth 2000).

In other lineages however, recent empirical studies imply that XY recombination arrest is not a necessary step (Stöck et al. 2011, 2013, Dufresnes et al. 2014b).

Theory suggests that non-zero evolutionary stable rates of XY recombination can be selected in populations (Grossen et al. 2012), as a balance between sex-antagonistic selection (which should favor absolute linkage) and genetic load (which should favor some purging recombination). These stable rates can remain quite conserved throughout radiations: Dufresnes et al. (in prep) outlined a phylogenetic inertia of extremely low recombination at one particular chromosome pair among several closely-related species of tree frogs (*Hyla* sp), regardless of its function as a sex-chromosome or as an autosome. Furthermore, superimposed on this long-term inertia, rates of XY recombination may show fine-scale variation between conspecific populations, and evolve extremely rapidly (Dufresnes et al. 2014b).

Given the important evolutionary consequences and strong selective forces driving XY recombination, characterizing the variance underlying this trait becomes an important question. Such information may inform on the mechanisms underlying this process in natural populations, and lay the ground work for further empirical and theoretical analyses. In the context of frequent sex-chromosome turnovers, as faced by many species systems with homomorphic sex chromosomes (Bachtrog et al. 2014), one appealing aspect is to contrast the dynamics of recombination in related species where the same pair of chromosomes is either used as autosomes or as sex chromosomes.

Western-Palearctic tree frogs (*Hyla* sp.), which diversified over the last 10My, provides a unique opportunity for such analyses. Recombination between sex chromosomes was evidenced in this group (Stöck et al. 2011, Dufresnes et al. 2014b, in prep). One linkage group, referred to as LG1 (Dufresnes et al. 2014a), is of particular interest because it maps to the sex chromosomes of several taxa, with male heterogamety (XY), but is autosomal in others (Dufresnes et al. in prep). Despite these differences in function, sibship analyses showed

that males from all species investigated share similar patterns of recombination at LG1, involving extremely rare or absent crossovers in the central part of the chromosome, and increased rates at the periphery (Dufresnes et al. 2014b, in prep; illustrated in Figure 1).

Here we dissect within-population variation of male recombination rates at this linkage group (LG1) in four *Hyla* species, where LG1 is either sex-linked or autosomal, based on the sibship data published by Dufresnes et al. (2014b, in prep). We report replicate patterns of strong between-males variation in recombination, with only a small proportion of males yielding most of the recombinants in each species, and discuss evolutionary implications.

METHODS

We considered sibship data of *Hyla* linkage group 1 generated by Dufresnes et al. (2014b) for *H. arborea* (19 families from two biogeographic regions encompassing glacial refugia) and Dufresnes et al. (in prep) for *H. sarda* (18 families), *H. savignyi* (17 families) and *H. meridionalis* (5 families), all using the same set of microsatellite markers encompassing most of the chromosome. This linkage group is sex-linked in *H. arborea* and *H. meridionalis* but not in *H. sarda* and *H. savignyi*. Nonetheless, LG1 male recombination is similarly reduced in all four species (Dufresnes et al. in prep, Figure 1). In comparison to the original datasets, here we only included families with at least 20 offspring, originating from regions where at least one event of male recombination was documented, and informative for at least one combination of potentially recombining loci (i.e. shown to experience crossovers in the species/region under focus). For *H. arborea*, families from the two biogeographic regions were pooled and analyzed altogether as these did not differ significantly in recombination rates (Dufresnes et al. 2014b).

In order to test whether recombination rates differ between males, we computed, for each family, the proportion of recombinants expected if male recombination was homogeneous within populations. Given the low amount of male recombination within each species (equivalent to <12 cM), the expected number of recombinants n_e in a family can be directly inferred from the recombination fraction r and the total number of offspring genotyped n_{tot}, as $r = \frac{n_e}{n_{tot}}$, where r can be averaged from the recombination distance D (as $r = \frac{D}{100}$), provided by the linkage maps of Dufresnes et al. (2014b, in prep). For each family, n_e was computed considering the distance D between informative markers, and so that would have been potentially detectable from the data. Observed numbers of recombinants n_o were directly counted from the data. Expected and observed numbers of recombinants were compared for each species using χ^2 tests of homogeneity, as $\chi^2 = \sum_i \frac{(n_{oi}-n_{ei})^2}{n_{ei}}$, where n_{oi} and n_{ei} respectively represent the numbers of observed and expected recombinants of each family i.

RESULTS AND DISCUSSION

We documented strong between-male variation in recombination for all *Hyla* species investigated (Figure 1). Observed rates followed a bimodal distribution: most males featured null or low recombination fractions ($r < 0.1$) with a few males contributing most recombinants ($r > 0.1$). Assuming homogeneous recombination among individuals, based on the population averages, most males should have featured intermediate recombination rates ($0.05 < r < 0.1$). Accordingly, observed values significantly departed from these expected rates for 3 out of 4 species, and over all species (Table 1).

The strong heterogeneity of male recombination at LG1 in *Hyla* is consistent with some balanced phenotypic variation, involving two main recombining phenotypes: a frequent variant yielding few or no recombinants, and a rare variant yielding many recombinants. Given that evolution ary stable rates of XY

species	*Nf*	χ^2	*p*
H. arborea	19	20.1	0.330
H. sarda	18	32.9	**0.012**
H. savignyi	17	28.4	**0.028**
H. meridionalis	5	15.2	**0.004**
overall	59	96.6	**0.001**

TABLE 1: departure from male recombination homogeneity at *Hyla*'s linkage group 1. *Nf*: number of families, *p*: p-value (highlighted when < 0.05). *H. arborea* data combine families from two biogeographic regions (Adriatic coast, Southern Balkans), that do not significantly differ in recombination rates (Dufresnes et al. in prep).

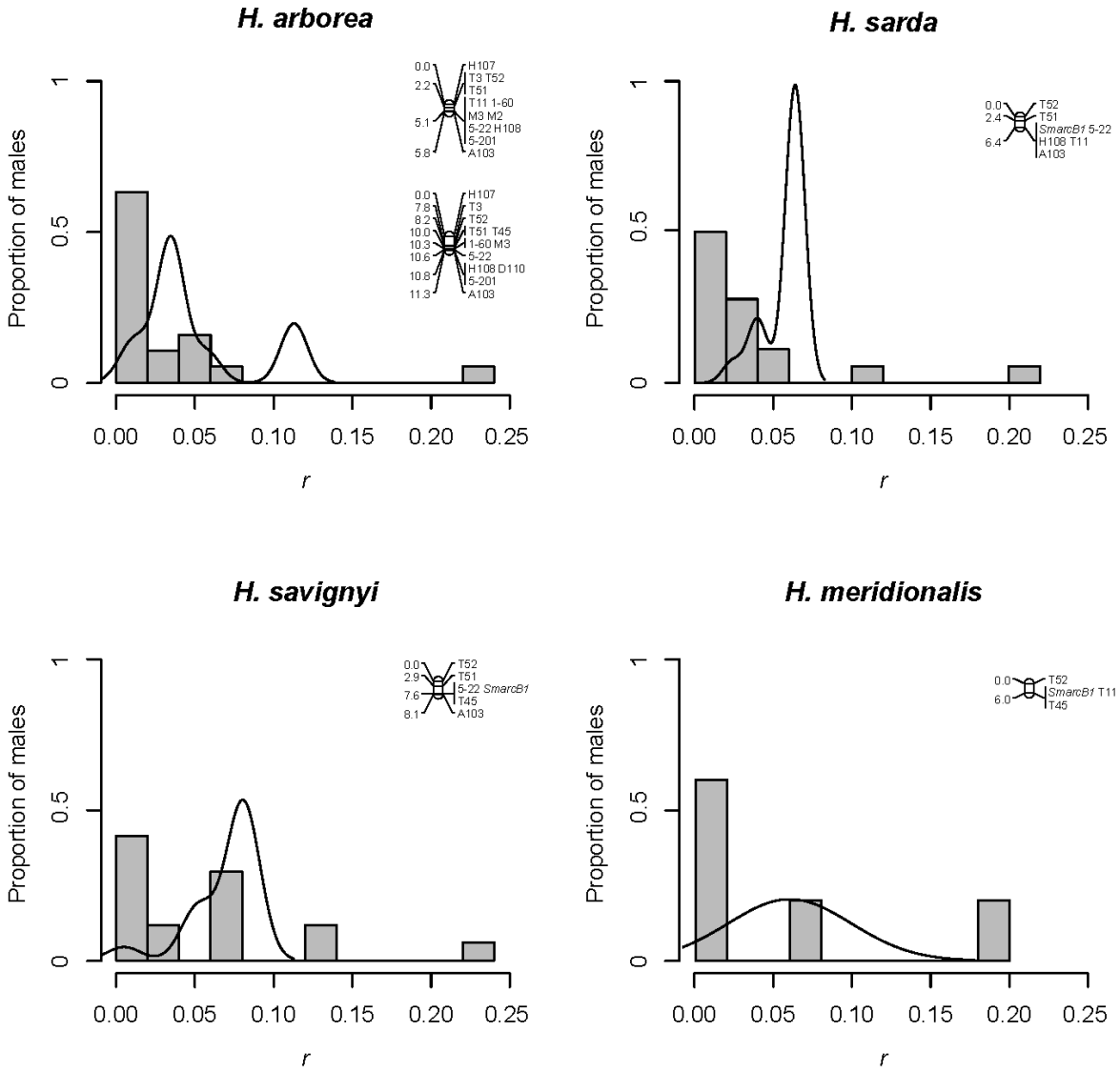

FIGURE 1: Recombination variation between LG1-recombining males within each species. Barplots show observed data. Solid lines show kernel densities of expected data (given marker informativeness within families) assuming homogeneous rates within populations. Note that for *H. arborea*, the expected distribution is bimodal because one third of the families were informative for the most-recombining peripheral markers (right mode), whereas the other two thirds were only informative for the less-recombining central portion (left mode). Male LG1 linkage maps are shown, adapted from Dufresnes et al. (2014b, in prep; for *H. arborea*, upper map: Southern Balkans, lower map: Adriatic coast). *r*: recombination fraction.

recombination stem from two counteracting forces (sex-antagonistic selection *versus* genetic load; although other forms of selection may contribute, Otto 2014), it is tempting to interpret the maintenance of this polymorphism as a response to a balanced selection driven by these forces. In other systems, recombination is known to respond quickly to selection (Charlesworth & Charlesworth 1985, Brooks 1988). On the other hand, under this hypothesis it is surprising that a very same pattern of variation is also shared by species where this linkage group is autosomal (*H. sarda*, *H. savignyi*), and where no such selection should be *a priori* involved.

Although the origin of this polymorphism remains unclear, it seems very conserved among the *Hyla* lineages investigated, suggesting some phylogenetic inertia. This result is in line with Dufresnes et al. (in prep), which proposed that recombination patterns at LG1 were fine-tuned and conserved among species following its specialization as a recurrent XY sex-chromosome pair. This phenotypic variance might have a genetic signature; although multiple genetic factors were shown to control recombination (Murdoch et al. 2010), one particular gene *Prdm9* seems to disproportionally account for within- and between-population variance in mammals (Baudat et al. 2013); sex-specific recombination rates were also shown to be directly associated with sequence variation at the *RNF212* gene (Kong et al. 2008). The heterogeneity reported here thus provides opportunities to test such candidate genes that potentially contributes to this trait. Characterizing the genetic architecture of XY recombination in tree frogs may in turn inform on the underlying evolutionary processes maintaining this polymorphism throughout the radiation.

More specifically, our results provide novel insights regarding the evolution of sex-chromosomes and XY recombination in *H. arborea*. In this species, male recombination, and consequently X-Y differentiation, remarkably differ between closely-related populations: ancestral populations from glacial refugia feature nonzero male recombination (and low X-Y differentiation) whereas recently-diverged post-glacial populations show virtually absent male recombination (and strong X-Y differentiation) (Dufresnes et al. 2014b). This biogeographic pattern can be explained in the light of the phenotypic variation documented in refugial populations of *H. arborea*: whereas these maintained both the recombining and non-recombining variants, only the latter may have contributed to the post-glacial recolonization, the rare recombining variant being lost by expansion-associated drift.

Empirical knowledge of the variance underlying XY recombination will be important to consider in future evolutionary research, and particularly for implementing theoretical models of sex-chromosome evolution in simulation studies.

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PART 3

HOMOMORPHIC SEX CHROMOSOMES AND SPECIATION

CHAPTER VIII

STRONGER TRANSFERABILITY BUT LOWER VARIABILITY IN TRANSCRIPTOMIC- THAN IN ANONYMOUS MICROSATELLITES: EVIDENCE FROM HYLID FROGS

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Stronger transferability but lower variability in transcriptomic- than in anonymous microsatellites: evidence from Hylid frogs

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Abstract

A simple way to quickly optimize microsatellites in nonmodel organisms is to reuse loci available in closely related taxa; however, this approach can be limited by the stochastic and low cross-amplification success experienced in some groups (e.g. amphibians). An efficient alternative is to develop loci from transcriptome sequences. Transcriptomic microsatellites have been found to vary in their levels of cross-species amplification and variability, but this has to date never been tested in amphibians. Here, we compare the patterns of cross-amplification and levels of polymorphism of 18 published anonymous microsatellites isolated from genomic DNA vs. 17 loci derived from a transcriptome, across nine species of tree frogs (*Hyla arborea* and *Hyla cinerea* group). We established a clear negative relationship between divergence time and amplification success, which was much steeper for anonymous than transcriptomic markers, with half-lives (time at which 50% of the markers still amplify) of 1.1 and 37 My, respectively. Transcriptomic markers are significantly less polymorphic than anonymous loci, but remain variable across diverged taxa. We conclude that the exploitation of amphibian transcriptomes for developing microsatellites seems an optimal approach for multispecies surveys (e.g. analyses of hybrid zones, comparative linkage mapping), whereas anonymous microsatellites may be more informative for fine-scale analyses of intraspecific variation. Moreover, our results confirm the pattern that microsatellite cross-amplification is greatly variable among amphibians and should be assessed independently within target lineages. Finally, we provide a bank of microsatellites for Palaearctic tree frogs (so far only available for *H. arborea*), which will be useful for conservation and evolutionary studies in this radiation.

Keywords: amphibian, EST, *Hyla*, population genetics, transcriptome

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Introduction

Microsatellites are the most popular molecular tools available on the population genetics market, even though they are now increasingly challenged by other types of markers (e.g. genotyping by sequencing) arising from next-generation sequencing (NGS) approaches. In animal research, they are particularly used for landscape (e.g. Dubey *et al.* 2009) and conservation genetics (e.g. Beebee 2005; Luquet *et al.* 2011), analyses of fine-scale population structure (e.g. Dufresnes *et al.* 2013), parentage and kinship analyses (Purcell & Chapuisat 2013), as well as evolutionary studies (e.g. Dufresnes *et al.* 2011b). Although the improvements in enrichment methods (reviewed by Zane *et al.* 2002) combined with the application of NGS (e.g. Jennings *et al.* 2011; Prunier *et al.* 2012) have greatly facilitated the isolation and characterization of microsatellites for target species, the *de novo* development of markers still represents a substantial investment. Therefore, the reuse of cross-amplifying loci in congeneric taxa remains a money- and time-saving option (e.g. Dufresnes *et al.* 2011a). Cross-amplification is also a prerequisite for multispecies studies, such as analyses of hybrid zones (e.g. Colliard *et al.* 2010) and comparative linkage mapping (e.g. Stöck *et al.* 2011), and ways to enhance the utility of markers across more species have been proposed (e.g. developing primers from consensus sequences, Dawson *et al.* 2010, 2013; Jan *et al.* 2012). However, reusing markers might be tedious in some groups, such as amphibians, where cross-amplification success is unpredictable and unexpectedly low (e.g. 21% in ranid frogs, Primmer & Merilä 2002; 7% in bufonid toads, Rowe *et al.* 2000), presumably because of their genome size and complexity (Garner 2002; Hendrix *et al.* 2010), the relatively low number of potentially amplifying loci (PALs, Drechsler *et al.* 2013) and of the underestimated divergence times within

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taxonomic groups (Primmer *et al.* 2005). Genetic distance seems to be a good predictor of amplification success (Primmer *et al.* 2005), but the strength of this relationship varies greatly among species groups (e.g. Hendrix *et al.* 2010).

An alternative way to quickly obtain markers usable in different lineages at low cost is to develop microsatellites from transcriptomes (e.g. using expressed sequenced tags, EST; e.g. Frenkel *et al.* 2012; Wang *et al.* 2012; Du *et al.* 2013; Yuan *et al.* 2013). This approach has the advantage that, providing a reference genome of any species with sufficiently conserved synteny, markers can be mapped and selected to be distributed across the whole genome (e.g. Du *et al.* 2013). In addition, most transcriptome-derived loci are situated in coding regions (e.g. Du *et al.* 2013); they should be more conserved than randomly located anonymous markers, and cross-amplify across deeper diverged lineages. On the other hand, lower levels of polymorphism might be expected for the exact same reasons, which can limit their utility for fine-scale population genetics. Dawson *et al.* (2010, 2013) have shown that transcriptomic microsatellites designed with conserved primers (i.e. between distant model organisms) cross-amplify better than anonymous markers in birds. These assumptions have yet never been tested in amphibians, where microsatellite development and optimization are particularly challenging.

With at least eight species distributed around the Mediterranean Basin and a well-resolved phylogeny (Stöck *et al.* 2008, 2012), Western Palaearctic tree frogs (*Hyla arborea* species complex) are well suited for the task. Microsatellite markers optimized from enriched libraries have been long available for the nominal taxon *H. arborea* (Table 1), and we (Brelsford *et al.* 2013; Dufresnes *et al.* 2013) have recently developed new polymorphic loci from the transcriptome of a Swiss male of this species. To set up a calibration between amplification success and taxa divergence for anonymous and transcriptome-derived microsatellites, we compare their cross-amplification patterns in *H. arborea* and eight close relatives (six from the Western Palaearctic, one from the Eastern Palaearctic and one from the Nearctic). We further compare their levels of polymorphism across and within taxa. Moreover, our study provides a bank of microsatellites for Palaearctic tree frogs, which will be valuable for future conservation and evolutionary research on this group.

Materials and methods

DNA sampling and extraction

DNA samples were gathered from *H. arborea* [Vaud, Switzerland (CH); Crete, Greece: *H. arborea kretensis*], *H. molleri* (Madrid province, Spain), *H. orientalis* (Thrace, Greece), *H. intermedia* (Ticino, Switzerland; new taxon 2 cf. Stöck *et al.* 2012), *H. sarda* (Corsica, France), *H. savignyi* (Paphos district, Cyprus), *H. meridionalis* (Camargue, France), *H. japonica* (Hiroshima, Japan) and *H. cinerea* (Georgia, USA). Detailed sampling information is available in Table S1. Genomic DNA was extracted from noninvasive buccal swabs (live adults; Broquet *et al.* 2007) or ethanol-preserved tissues (tadpoles) with the Qiagen DNeasy Tissue Kit or the Qiagen BioSprint robotic workstation.

Microsatellite bank and PCR amplification

We included 18 anonymous microsatellites (noncoding) and 17 from a transcriptome of *H. arborea* (Table 1 and S2). Among the latter, four were newly isolated in this study, following the same procedure as Brelsford *et al.* (2013). All loci were originally developed from Western European *H. arborea* individuals (Arens *et al.* 2000: the Netherlands; other work: Western Switzerland) which belong to the same phylogeographic group (Dufresnes *et al.* 2013). All markers were mapped (BLASTN and TBLASTX) to the *Xenopus tropicalis* genome (assembly 7.1, http://xenbase.org) to determine their position and whether they are coding. Because sex-linked markers may have skewed patterns of polymorphism (Berset-Brändli *et al.* 2007), we selected loci expected to be autosomal, at least in *H. arborea* (Berset-Brändli *et al.* 2008b; Brelsford *et al.* 2013) and most likely in *H. molleri*, *H. intermedia* and *H. orientalis*, which share the same sex chromosomes (Stöck *et al.* 2011, 2013). To do so, we excluded published markers from the sex linkage group (linkage group 1 in Berset-Brändli *et al.* 2008b) or that blast to *X. tropicalis* scaffold 1 (except three confirmed autosomal markers from this scaffold, see Table 1), which is mostly homologous to *H. arborea* linkage group 1 (Brelsford *et al.* 2013).

All but two microsatellites were amplified in 10 μL multiplex PCRs (named A–D: anonymous, and F–I: transcriptomic, following Dufresnes *et al.* 2013; Table S2), with 3 μL of DNA (10–100 ng), 3× Qiagen Multiplex Master Mix and primers (concentration as in Table S2). Multiplex PCR conditions were as follows: 95 °C for 15′ (initial denaturation); 35 cycles of 94 °C for 30″ (denaturation), 58 °C for 1′30″ (annealing), 72 °C for 1′ (elongation); 60 °C for 30′ (final elongation). Markers *Ha*-H116 and *Ha*-A139 were first amplified separately with the exact same procedure to test for cross-amplification, but we then used the following alternative PCR protocols for subsequent genotyping (Berset-Brändli *et al.* 2008b). For *Ha*-H116, PCR templates (10 μL) contained 2.5 μL of DNA, 1× Qiagen PCR buffer (with 1.5 mM of $MgCl_2$), 0.2 mM of dNTPs, 0.5 μM of each primer and 0.25 units of

Table 1 Cross-amplification patterns of 35 microsatellites in 10 taxa of Hylid tree frogs

Microsatellite name	References	Product size range	*H. arborea* (W-Europe) $n = 30$	*H. arborea kretensis* $n = 30$	*H. intermedia* $n = 20$	*H. orientalis* $n = 16$	*H. molleri* $n = 23$	*H. sarda* $n = 22$	*H. savigny* $n = 15$	*H. meridionalis* $n = 16$	*H. japonica* $n = 11$	*H. cinerea* $n = 5$
Markers developed from transcriptome												
Ha-T32	[2]	300–323	+	+	+	+	–	+	+	+	+	+
Ha-T41	[2]	258–304	+	+	+	+	+	+	+	+	+	+
Ha-T49	[2]	124–172	+	+	+	+	+	+	+	+	–	–
Ha-T50	[3]	113–151	+	+	+	+	+	+	+	+	*	+
Ha-T53	[1]	347–378	+	+	+	+	+	+	+	*	–	–
Ha-T54	[1]	200–208	+	+	+	+	+	+	–	+	–	–
Ha-T55	[1]	302–320	+	+	+	+	+	+	+	*	+	+
Ha-T56	[3]	227–254	*	+	+	+	+	*	–	–	–	+
Ha-T58	[3]	240–258	+	+	+	+	+	+	–	–	–	–
Ha-T60	[3]	271–294	+	+	+	+	+	+	+	+	+	+
Ha-T61	[1]	234–240	+	+	+	+	+	+	+	+	–	–
Ha-T63	[3]	202–216	+	+	+	+	+	+	+	+	+	+
Ha-T64	[3]	196–221	+	+	+	+	+	+	+	+	+	+
Ha-T66	[3]	100–121	+	+	+	+	+	+	+	+	*	+
Ha-T67	[3]	266–298	+	+	+	+	+	+	+	+	–	–
Ha-T68	[3]	348–362	+	+	+	+	+	–	+	+	–	+
Ha-T69	[3]	268–287	+	+	+	+	+	+	+	+	–	–
Anonymous markers												
Ha-A11	[4]	105–143	+	*	–	–	+	–	–	–	–	–
Ha-A110	[5]	184–198	+	*	–	*	+	–	+	–	–	–
Ha-A119	[4]	234–252	+	*	–	*	–	–	*	–	–	–
Ha-A127	[4]	247–281	+	+	–	*	*	–	–	–	–	–
Ha-A130	[4]	138–147	+	*	–	–	–	–	–	–	–	–
Ha-A136	[5]	148–166	+*	*	–	–	–	–	–	–	–	–
Ha-A139	[5]	271–280	+	–	–	–	–	–	–	–	–	–
Ha-B12	[4]	109–172	+	+	*	*	–	–	–	–	–	–
Ha-B5R3	[4]	223–247	+*	+	–	–	–	–	–	–	–	–
Ha-D104	[5]	240–264	+	+	–	–	–	–	–	–	–	–
Ha-D115	[4]	154–218	+	+	–	–	+	–	–	–	–	–
Ha-D3R3	[4]	159–172	+	–	–	–	–	–	–	–	–	–
Ha-E2	[4]	144–161	+	+*	+	–	–	+	+	–	–	–
Ha-H116	[5]	191–248	+	*	*	+	+	–	+	+	–	–
WHA1-103	[6]	196–257	+	+	+	+	*	+	–	–	–	*
WHA1-20	[6]	120–199	+	+	–	–	–	–	–	–	–	–
WHA1-25	[6]	83–141	+	–	+	+	+	–	+	–	–	–
WHA1-67	[6]	181–243	+	+*	–	+	+	+	+	+	–	–

+, perfectly amplifying ($p_0 = 0$); +*, amplifying but low proportion of null alleles detected ($p_0 < 0.2$); *, partially amplifying ($p_0 > 0.2$); –, not amplifying ($p_0 = 1$).
References: [1]this study, [2]Brelsford *et al.* 2013; [3]Dufresnes *et al.* 2013; [4]Berset-Brändli *et al.* 2008a; [5]Berset-Brändli *et al.* 2008b; [6]Arens *et al.* 2000.

Qiagen Taq. For *Ha*-A139, PCRs were also carried out in 10 μL, with 1 μL of DNA, 1× Qiagen PCR buffer (with 1.5 mM of $MgCl_2$), 0.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.5 μM of each primer, 1× Qiagen Q-solution and 0.3 units of Qiagen Taq. PCR conditions were identical for both markers: 94 °C for 3′ (initial denaturation); 45 cycles of 94 °C for 45″ (denaturation), 58 °C for 45″ (annealing), 72 °C for 1′ (elongation); and 72 °C for 5′ (final elongation). *Ha*-H116 and *Ha*-A139 were pooled and genotyped along with multiplexes A and B, respectively. All amplicons were subsequently analysed on an ABI-3100 sequencer, and allele sizes scored using the size standards ROX-350 (multiplexes A–D) or ROX-500 (multiplexes F–I; GENEMAPPER 4.0, Applied Biosystems, Inc.). To avoid scoring nonspecific loci in congeneric species, we only scored alleles close to the source species range (i.e. no more than 30 bp between the source and the target species ranges) and with similar chromatographic profiles. Markers that would not amplify were repeated separately. If no PCR product could be obtained after the repeat, it would be declared nonamplifying.

Population genetics analyses

We computed the number of alleles (k) and heterozygosity (H_0) and tested Hardy–Weinberg equilibrium within taxa for each locus in FSTAT 2.9.3 (Goudet 1995) when at least 10 individuals amplified. The frequencies of null alleles (p_0) were estimated with MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004; when all samples amplified), or from the proportion of nonamplifying individuals (p_0^2; when only a few samples amplified), and we performed corrections when necessary. For each marker, we computed allelic frequencies within taxa/populations after corrections (FSTAT).

Amplification success and polymorphism versus genetic distance

For each population, the amplification success was calculated as the mean proportion of amplifying alleles per marker ($1-p_0$, e.g. 1 for a perfectly amplifying locus, 0.8 for a locus with 20% of null alleles, etc.). We also computed the percentage of usable loci (polymorphic and properly amplifying, i.e. less than 20% of null alleles, $p_0 < 0.2$) and the mean proportion of null alleles (p_0) in amplifying markers ($p_0 > 0$). As a proxy to genetic distance, we took advantage of the mitochondrial dating from Stöck *et al.* (2012), for Western Palaearctic species, Dufresnes *et al.* (2013), for the southern *H. arborea* haplogroup occurring in Crete and Schmidt *et al.* (2005), for the deepest-diverged *H. cinerea* and *H. japonica*. To cast our results among previous work, we related *cytochrome-b* sequence divergence to amplification patterns of anonymous microsatellites in tree frogs (this study), toads (Rowe *et al.* 2000), salamanders (Hendrix *et al.* 2010) and newts (Krupa *et al.* 2002; Garner *et al.* 2003), as Primmer *et al.* (2005) did for ranid frogs. We used published *cyt-b* sequences (Table S3), aligned with SEAVIEW (Gouy *et al.* 2010) and analysed in DNASP (Librado & Rozas 2009). Some taxa from Hendrix *et al.* (2010) were not included due to little sequence overlap between available *cyt-b* (<250 bp). Relationships were assessed with linear regressions on untransformed (% of *cyt-b* divergence) or log-transformed data (divergence time, see Results), separately for anonymous and transcriptomic loci.

Influence of the primer design and PCR conditions

PCR amplification can also depend on several technical features, such as amplicon length, deviation from optimal annealing temperature, concentration of $MgCl_2$ and whether priming sites are coding (i.e. potentially more conserved). When applicable, we tested whether these conditions differed between our two marker sets (ANOVAS) and whether they had affected the degree of cross-amplification (i.e. covariates in ANCOVAS with divergence time). Furthermore, we compare these characteristics between our and previous studies on amphibians (ANOVAS), using available published data (compiled in Table S4). To this purpose, we calculated the melting temperature (T_m) of each primer with PRIMER3PLUS (Untergasser *et al.* 2007) and their optimal annealing temperature ($T_{opt} = T_m - 2$) and computed the deviation from the PCR annealing temperature used (T_a), noted $\Delta T_a - T_{opt}$.

Polymorphism of transcriptomic and anonymous markers

For properly amplifying markers ($p_0 < 0.2$) and after correction for null alleles, we compared k and logit-transformed H_0 (Warton & Hui 2011) between transcriptomic (ta) and anonymous (an) loci with generalized linear mixed models (GLMM, R Development Core Team 2011, package lme4) including marker type as a fixed factor, and species and markers as random factors. To explore what features influence variability within each marker type, we ran additional models to predict k and H_0 from the number of tandem repeats, and the coding/noncoding nature of the sequence (the latter only for transcriptomic loci). Significance was tested by removing variables through a backward model selection procedure. Moreover, we also assessed whether the number (ANOVA) and type (dinucleotide, trinucleotide, etc., χ^2 test) of tandem repeats significantly differ between sets.

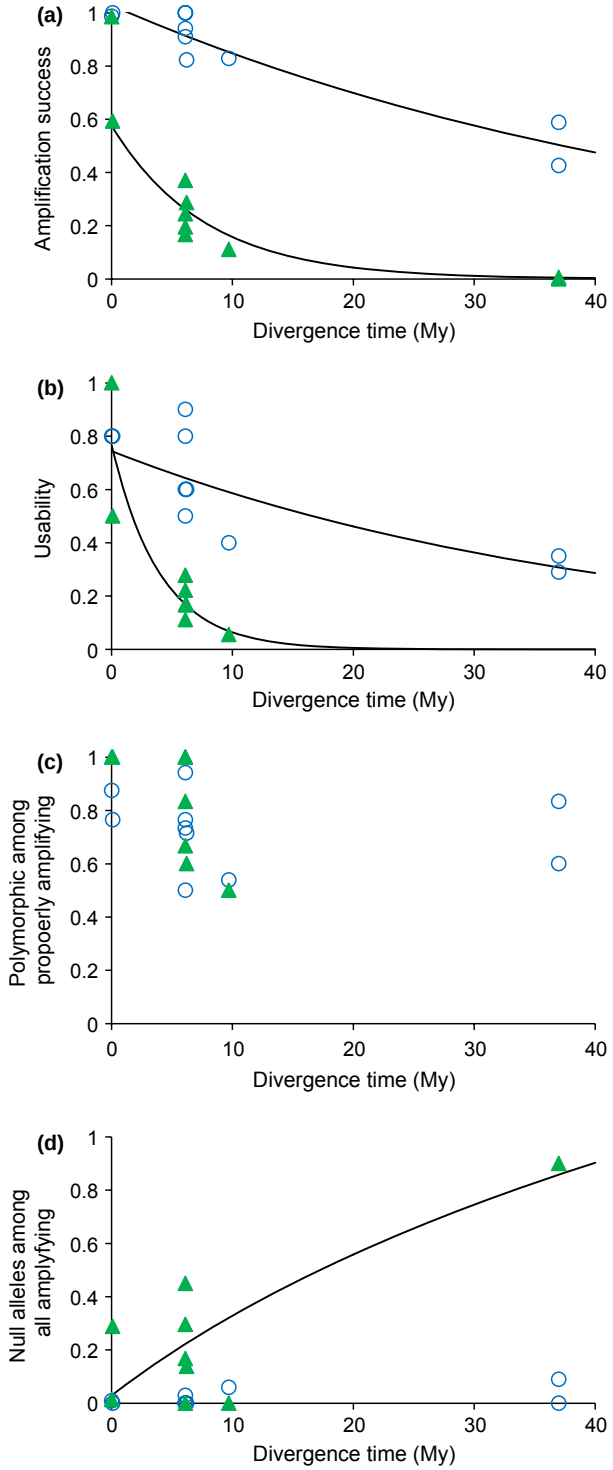

Fig. 1 Amplification success (a), proportion of usable loci among total (b) and among properly amplifying ($p_0 < 0.2$, c), and proportion of null alleles in amplifying loci (d) vs. divergence time for transcriptomic (blue circles) and anonymous markers (green triangles). Regression curves are displayed when significant ($P < 0.05$). (a) Amplification success (mean proportion of amplifying alleles per locus $1-p_0$): for transcriptomic: $y = e^{-0.0193x+0.0277}$, $F_{1,8} = 61.9$, $P < 0.001$, $R^2 = 0.89$; for anonymous: $y = e^{-0.1297x-0.5511}$, $F_{1,7} = 130.7$, $P < 0.001$, $R^2 = 0.95$). (b) Proportion of polymorphic and properly amplifying ($p_0 < 0.2$) markers among total: for transcriptomic: $y = e^{-0.0239x-0.2956}$, $F_{1,8} = 18.9$, $P = 0.003$, $R^2 = 0.70$; for anonymous: $y = e^{-0.2480x-0.2664}$, $F_{1,6} = 36.6$, $P < 0.001$, $R^2 = 0.86$. (c) Proportion of polymorphic loci among properly amplifying ($p_0 < 0.2$): for transcriptomic: $y = e^{-0.0019x-0.3169}$, $F_{1,8} = 0.13$, $P = 0.73$, $R^2 = 0.02$; for anonymous: $y = e^{-0.0572x+0.0651}$, $F_{1,6} = 5.3$, $P = 0.06$, $R^2 = 0.47$. (d) Mean proportion of null alleles among all amplifying markers ($p_0 > 0$): for transcriptomic: $y = \ln(0.0292x - 4.6674)$, $F_{1,8} = 2.6$, $P = 0.15$, $R^2 = 0.24$; for anonymous: $y = \ln(0.0359x + 1.0301)$, $F_{1,7} = 20.6$, $P = 0.003$, $R^2 = 0.75$.

Results

Marker homology in X. tropicalis and population genetics analyses

No anonymous markers could be successfully aligned to the *X. tropicalis* genome, but homologous sequences of transcriptomic loci were found (Table 1). Table S5 summarizes microsatellite variability, allele ranges, results of tests for departure from HW equilibrium and estimation of null alleles within each taxon. A few markers did not meet HW expectations in some taxa, which were, in most cases, suggested to be associated with the presence of null alleles (Table S5). Allelic frequencies are provided in Table S6.

Amplification success versus genetic distance

The amplification success (Fig. 1a) and the number of usable markers (Fig. 1b) decreased exponentially with divergence time for both types of markers, respectively, by 6.7- and 10.4-fold faster for anonymous loci. Estimated from the regressions (log-transformed data), the half-lives of our microsatellite sets (time for which the proportion of amplifying loci is 50%) were approximately 1.1 My for anonymous and 37 My for transcriptomic. Considering only usable loci, these values correspond to 1.7 My and 16.6 My, respectively. Furthermore, we could not significantly relate the proportion of polymorphic loci among properly amplifying to divergence time (Fig. 1c), although it substantially decreased for anonymous markers. The proportion of null alleles (p_0) among amplifying anonymous makers increased with divergence, a trend which was not shared by transcriptomic loci (Fig. 1d).

Sequence divergence (*cyt-b*) was a good predictor for the amplification success of anonymous markers in tree frogs and other amphibians (Fig. 2), and the relationship varied significantly between species groups (ANCOVA, $F_{4,38} = 6.91$, *P*-value = 0.0003).

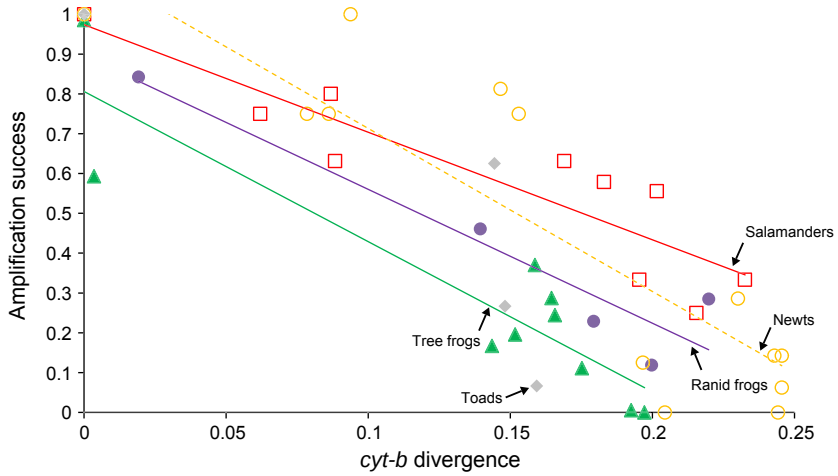

Fig. 2 Amplification success of microsatellite anonymous markers versus *cyt-b* divergence in tree frogs, ranid frogs, bufonid toads, newts and salamanders. Regression curves are displayed when significant. For tree frogs: $y = -3.77x + 0.806$, $F_{1,8} = 39.8$, $P < 0.001$, $R^2 = 0.83$; for ranid frogs, see Figure 4 in Primmer *et al.* 2005; for toads: $y = -4.71x + 1.02$, $F_{1,2} = 6.0$, $P = 0.13$, $R^2 = 0.75$; for newts: $y = -4.09x + 1.12$, $F_{1,13} = 61.1$, *P*-value < 0.001, $R^2 = 0.82$; for salamanders: $y = -2.7x + 0.97$, $F_{1,8} = 35.5$, *P*-value < 0.001, $R^2 = 0.82$.

Table 2 Comparison of the main characteristics between transcriptomic and anonymous loci, and their influence on cross-amplification and variability

	Transcriptomic (n = 17)	Anonymous (n = 18)	
Marker characteristics			
Amplicon length	246.1 (±72.3)	191.9 (±51.6)	ANOVA, $F_{1,33} = 6.6$, **$P = 0.015$**
$\Delta T_a - T_{opt}$	0.3 (±0.2)	3.2 (±2.1)	ANOVA, $F_{1,33} = 32.5$, **$P < 0.001$**
Number of repeats	9.7 (±5.6)	17.7 (±6.2)	ANOVA, $F_{1,33} = 15.9$, **$P < 0.001$**
Type of repeats	di-: 35%, tri-: 53%, other: 12%	di-: 72%, tri-: 6%, tetra-: 22%	$\chi^2 = 15.0$, **$P = 0.005$**
Number of alleles k	2.94 (±2.12)	4.96 (±3.62)	GLMM, $\chi^2 = 6.4$, **$P = 0.011$**
Heterozygosity H_0	0.34 (±0.28)	0.49 (±0.29)	GLMM, $\chi^2 = 4.6$, **$P = 0.033$**
Factors affecting cross-amplification (ANCOVA)			
Divergence time	$F_{1,148} = 47.8$, **$P < 0.001$**	$F_{1,148} = 25.8$, **$P < 0.001$**	
Amplicon length	$F_{1,148} = 0.1$, $P = 0.72$	$F_{1,148} = 0.2$, $P = 0.66$	
$\Delta T_a - T_{opt}$	$F_{1,148} = 1.5$, $P = 0.22$	$F_{1,148} = 0.03$, $P = 0.85$	
Number of coding priming regions	$F_{1,148} = 1.5$, $P = 0.22$	–	
Factors affecting k (GLMM)			
Number of repeats	$\chi^2 = 0.1$, $P = 0.71$	$\chi^2 = 3.9$, **$P = 0.048$**	
Coding nature of markers	$\chi^2 = 0.1$, $P = 0.80$	–	
Interaction	$\chi^2 = 1.7$, $P = 0.19$	–	
Factors affecting H_0 (GLMM)			
Number of repeats	$\chi^2 = 0.2$, $P = 0.63$	$\chi^2 = 1.5$, $P = 0.22$	
Coding nature of markers	$\chi^2 = 0.0$, $P = 1.00$	–	
Interaction	$\chi^2 = 3.1$, $P = 0.08$	–	

Significant *P*-values are marked in bold.

Influence of the primers design and PCR conditions

Anonymous microsatellite amplicons were on average smaller, and their annealing temperatures less optimal than transcriptomic (Table 2). However, none of these factors affected the degree of cross-amplification, and neither did the coding/noncoding nature of primers for transcriptomic loci (Table 2). Note that all our markers were originally amplified with the same concentration of $MgCl_2$ (1.8 mM), which therefore could not explain differences in cross-amplification either. Finally, differences in $MgCl_2$ concentration (salamanders: 1.5 or 1.8 mM; ranid frogs: 1.5 mM; newts: 1.5 mM for all but one marker; tree frogs: 1.8 mM; toads: 2 mM) and deviations from optimal annealing temperature (not significantly different between studies $F_{3,46} = 2.7$, *P*-value = 0.055) would hardly explain the variation in cross-amplification rates reported among amphibians (Table S4).

Polymorphism of transcriptomic and anonymous markers

The number of alleles (k) and observed heterozygosities (H_0, logit-transformed) were both affected by the type of

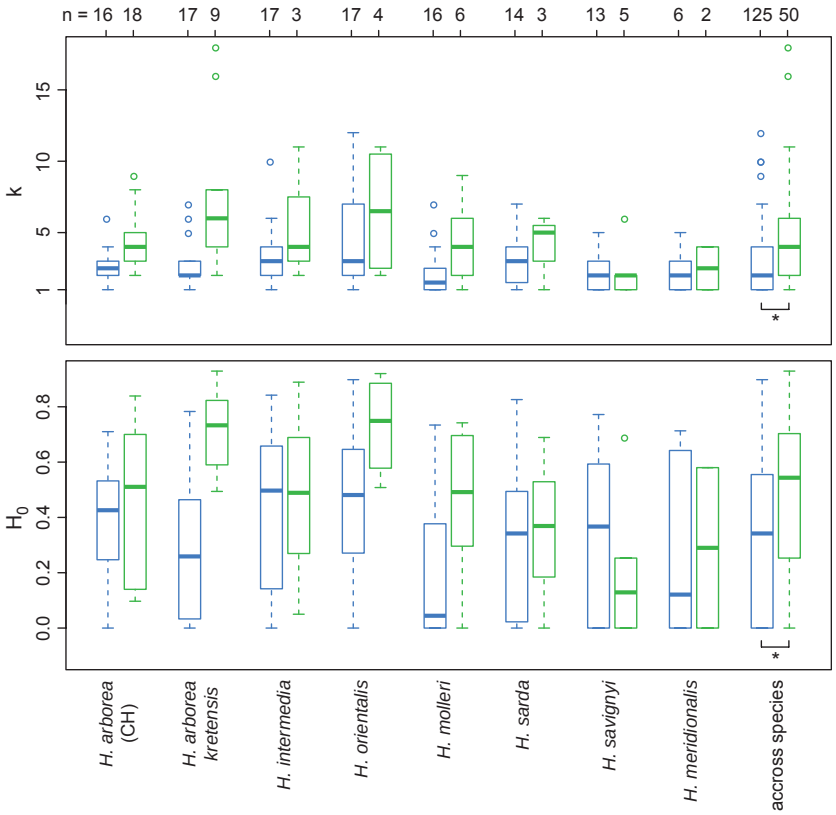

Fig. 3 Comparison of the numbers of alleles (k) and observed heterozygosities (H_0) between transcriptomic (blue) and anonymous markers (green) within and across *Hyla* taxa. Sample sizes (numbers of properly amplifying markers) are mentioned on the top. Significance of GLMMs is indicated (*: $P < 0.05$). See text and Table 2 for details.

markers, being significantly lower for transcriptomic than anonymous markers across species (Table 2, Fig. 3). Transcriptomic microsatellites possessed fewer repeats, and the proportion of each repeat type also differed between sets (Table 2). The former positively influenced the number of alleles (but not heterozygosity) in anonymous markers. For transcriptomic loci, neither the number of microsatellite repeats, whether markers are coding/noncoding, nor their interaction had a significant effect (Table 2). Note that because of the distribution of the data (i.e. one repeat type was prominent within each set, table 2), it was not possible to use the repeat type as a variable in the GLMMs.

Discussion

It is well known that the amplification success and polymorphism of microsatellite loci decrease with the genetic distance from the source species, as shown in birds (Primmer *et al.* 2005; Dawson *et al.* 2010, 2013), mammals (Moore *et al.* 1991), fishes (Carreras-Carbonell *et al.* 2008), reptiles (e.g. Glenn *et al.* 1996) and amphibians, including salamanders (e.g. Hendrix *et al.* 2010) and ranid frogs (Primmer *et al.* 2005; Nair *et al.* 2012). Our results for hylid tree frogs conform to this general pattern, which seems to hold across amphibians. Moreover, the comparison with other radiations illustrates that this relationship differs substantially between taxonomic groups: half of the microsatellites still amplified after 8% to 18% of *cyt-b* divergence (calculated from the regressions, Fig. 2). With a half-life of only 1.1 My, cross-amplification rates in tree frogs are thus among the lowest documented in amphibians. In contrast, Hendrix *et al.* (2010) reported that 65% of loci still worked between clades separated by 30 My in salamanders. The considerable differences in genome size (C-values) found between and within amphibian lineages were suggested to account for these large disparities (Garner 2002; Hendrix *et al.* 2010). Within *Hyla* tree frogs, however, poor cross-amplification might only stem from the fast evolution of flanking regions (Balloux *et al.* 1998), given that species feature similar C-values (e.g. on average 4.8 for *H. arborea*, 4.7 for *H. japonica*, www.genomesize.com). In addition, most of the anonymous loci developed for *H. arborea* belong to the same family of repetitive elements (possibly retrotransposons, A. Horn, unpublished) and may thus have comparable evolutionary rates. It is unlikely that this pattern stems from technical differences between our and previous studies, at least for the key parameters we accounted for (primer design, $MgCl_2$ concentration).

Whereas optimizing anonymous markers for other species might thus be an unwarranted endeavour, loci isolated from transcriptomes appear to last much

longer through evolutionary times. In our case, more than 80% of these microsatellites still amplified across the Circum-Mediterranean radiation (diverged some 10 Mya, Stöck *et al.* 2012), suggesting strong conservation of priming sites (the key factor of cross-amplification, Dawson *et al.* 2013; although whether priming regions are coding did not affect transferability in *Hyla*) and/or high marker sequence similarity between species (Küpper *et al.* 2008). In addition, there was no evidence of decrease in variability with genetic distance, and microsatellite utility was only a function of amplification success. In contrast, Dawson *et al.* (2010, 2013) documented a decrease in polymorphism with genetic distance, but they focused on much higher divergences (including passerines and nonpasserine birds). Again, our results were not affected by methodological features, because the same PCR conditions were used for all markers, and deviations from optimal primer annealing temperature, although better for transcriptomic primers, were not related to the degree of cross-amplification. Other attempts to cross-amplify transcriptomic markers similarly resulted in high levels of cross-species transferability (e.g. Qu *et al.* 2012; Du *et al.* 2013; Yuan *et al.* 2013), better than for anonymous markers (Dawson *et al.* 2010, 2013). At first glance, exploiting transcriptomes thus appears to be a method of choice to quickly obtain large sets of markers compatible in congeneric species.

On the other hand, the conserved nature of transcripts also reflected on the intraspecific level of variability: transcriptome-derived markers displayed significantly lower number of alleles and heterozygosity than markers developed from enriched libraries. Reports for new transcriptomic microsatellite development accordingly featured rather low locus diversity in the source species (mean number of allele per locus, e.g. Triwitayakorn *et al.* 2011: 3.9; Frenkel *et al.* 2012: from 1 to 6 depending on the population; Wang *et al.* 2012: 2.4; Liu *et al.* 2013: 4.1; Du *et al.* 2013: 2.7; Yuan *et al.* 2013: 1.4). This might in part stem from a methodological bias in favour of highly variable loci (with more tandem repeats) when developing anonymous markers, that have so far not been applied to transcriptomic markers (which in addition may be located in more conserved regions of the genome). Indeed, in *Hyla*, the numbers of microsatellite repeats in the source individual were significantly lower for loci developed from the transcriptome (which also differed by repeat type), a feature known to impact marker variability (Balloux *et al.* 1998), as highlighted in our anonymous loci. Additionally, potential selection acting on transcriptomic loci could be a concern for analyses of neutral genetic diversity, especially for tri- and hexanucleotides, commonly found in coding regions (Du *et al.* 2013), but we did not detect any difference in variability between coding and noncoding markers, and the few significant departures from Hardy–Weinberg equilibrium (4 cases of 98) most likely resulted from the occurrence of nonamplifying alleles (Table S5). Rather than direct selection, indirect effects via Hill–Robertson interference (hitchhiking and background selection) might contribute to this reduced variation.

Therefore, microsatellites isolated from transcriptomes have to be chosen carefully for fine-scale analyses of population variability. In addition, several studies reported HW deviations in EST-derived marker sets (e.g. Kong & Li 2008; Smee *et al.* 2013), and only a subset of the microsatellites characterized from a transcriptome are actually usable for sound population genetics (e.g. Kong & Li 2008: 9 of 15; Qu *et al.* 2012: 41 of 46). One way to improve the amount of usable markers would be to design consensus primers with distant model organisms (e.g. using available genomes, Dawson *et al.* 2010, 2013; Jan *et al.* 2012), which should reduce the probability of null alleles and maintain polymorphism in cross-amplifying species (providing sufficient variability of loci in the sources, Dawson *et al.* 2013). Once a proper set has been optimized, their high transferability across taxa makes them ideal candidate markers for multispecies surveys, such as analyses of hybrid zones, particularly in our case given that many loci displayed diagnostic differences in allelic frequencies (Table S6).

The European tree frog *H. arborea* has been a model species in the field of population genetics for more than a decade, and microsatellites were intensively used to address various questions related to conservation (e.g. Broquet *et al.* 2010; Luquet *et al.* 2011) and landscape genetics (e.g. Dubey *et al.* 2009), mating systems (e.g. Broquet *et al.* 2009; Jaquiéry *et al.* 2010), phylogeography (Dufresnes *et al.* 2013) and sex chromosome evolution (e.g. Berset-Brändli *et al.* 2007, 2008b; Dufresnes *et al.* 2011b; Stöck *et al.* 2011). The establishment of a comprehensive microsatellite set compatible in Western Palaearctic tree frogs (plus some Eastern Palaearctic and Nearctic taxa) significantly expands the possibilities for research to the entire radiation, where no markers have been available so far. It will be particularly relevant for multilevel analyses of population structure and reproductive isolation, as several lineages form secondary contact zones and seem to harbour cryptic diversity (e.g. *H. intermedia*, *H. orientalis*, *H. meridionalis*, Stöck *et al.* 2012). Finally, it will allow genetic-based conservation assessments for the regionally threatened tree frogs populations found across many countries (AmphibiaWeb: amphibiaweb.org).

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C.D., A.B. and N.P. designed the project; A.B. contributed the transcriptomic markers, further optimized by C.D; C.D. conducted laboratory work; Data were analysed by C.D. and P.B. C.D. drafted the manuscript.

Data accessibility

Microsatellite genotypes are available in a supplementary table archived in Dryad doi:10.5061/dryad.0fh44. GenBank accession numbers for the four newly developed markers are provided in Table S2.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Detailed sampling information.

Table S2 GenBank accession numbers and details on marker features, primer sequences and PCR protocols.

Table S3 GenBank accession numbers of *cyt-b* sequences used to compute mitochondrial divergence in tree frogs (856 alignable bp), toads (270 alignable bp), newts (778 alignable bp) and salamanders (645 alignable bp).

Table S4 Primer design and PCR conditions of previous cross-amplification studies in amphibians.

Table S5 Amplification success (frequency of amplifying alleles $1-p_0$), allele range and number (k), observed heterozygosity (H_0) and F_{is} for each marker and taxon.

Table S6 Within-taxa allelic frequencies for each locus.

CHAPTER IX

FIRST-GENERATION LINKAGE MAP FOR THE EUROPEAN TREE FROG (*HYLA ARBOREA*) WITH UTILITY IN CONGENERIC SPECIES

Christophe Dufresnes, Alan Brelsford and Nicolas Perrin

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SHORT REPORT Open Access

First-generation linkage map for the European tree frog (*Hyla arborea*) with utility in congeneric species

Christophe Dufresnes*, Alan Brelsford and Nicolas Perrin

Abstract

Background: Western Palearctic tree frogs (*Hyla arborea* group) represent a strong potential for evolutionary and conservation genetic research, so far underexploited due to limited molecular resources. New microsatellite markers have recently been developed for *Hyla arborea*, with high cross-species utility across the entire circum-Mediterranean radiation. Here we conduct sibship analyses to map available markers for use in future population genetic applications.

Findings: We characterized eight linkage groups, including one sex-linked, all showing drastically reduced recombination in males compared to females, as previously documented in this species. Mapping of the new 15 markers to the ~200 My diverged *Xenopus tropicalis* genome suggests a generally conserved synteny with only one confirmed major chromosome rearrangement.

Conclusions: The new microsatellites are representative of several chromosomes of *H. arborea* that are likely to be conserved across closely-related species. Our linkage map provides an important resource for genetic research in European Hylids, notably for studies of speciation, genome evolution and conservation.

Keywords: Conservation, Heterochiasmy, Hylid frogs, Microsatellites, Population genetics, Recombination, Transcriptome

Background

Genetic maps based on linkage disequilibrium are powerful tools to address many aspects regarding the evolution of animal genomes like QTL mapping, recombination and chromosome synteny [1]. Moreover, they provide valuable resources for the use of molecular markers in population genetics. European tree frogs (*Hyla arborea* group) have become a model system in this field and were intensively studied in contexts of conservation (e.g. [2]), phylogeography (e.g. [3]), as well as mating systems (e.g. [4]) and sex-chromosome evolution (e.g. [5]). This radiation forms a genetically-rich group, including at least eight species distributed across the Mediterranean Basin, some with deep intraspecific divergences [6]. Nevertheless, despite high potential for research, so far most work has been restricted to the single *H. arborea*, for long the only taxon for which microsatellite markers and their linkage map were available [7]. Indeed, most of these markers turned out to be unusable in congeneric species [8].

To overcome this issue, we have recently developed a new set of EST-derived microsatellite loci with high cross-species utility, thus extending opportunities for research to the entire circum-Mediterranean radiation [8]. Here we present a first-generation linkage map for *H. arborea* combining these new microsatellites with those earlier published, to produce a useful resource for future evolutionary, ecological and conservation genetic applications. As most EST-derived markers could be aligned on the *Xenopus tropicalis* genome [8], we took advantage of this new map to document patterns of synteny of our linkage groups between *Hyla* and *Xenopus*.

Methods

Tree frog families (parents + tadpoles) were obtained from controlled crosses, as described [9]. Families were

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chosen from the Balkan Peninsula, where populations display the highest amounts of genetic diversity [3]. Nine families originated from Krk island, Croatia (45.1704°, 14.6229°), one near Karlovac, Croatia (45.5435°, 15.5729°), one near Progar, Serbia (44.7422°, 20.1381°) and one from the Neusiedlersee region, Austria (47.9261°, 16.8634°), for a total of 12 families (n = 352 tadpoles, from 24 to 30 per family). DNA from parents (non-invasive buccal swabs, [10]) and offspring (ethanol fixed tadpoles) was extracted using the Qiagen Robotic Workstation. Our study was approved by the relevant Institutional Animal Care and Use Committee (IACUC), namely the Service de la Consommation et des Affaires Vétérinaires du Canton de Vaud (Epalinges, Switzerland; authorization N°1798) and sampling was conducted under collecting permits (N°532-08-01-01 issued by the Nature Protection Directorate of the Croatian Ministry of Culture; N°353-01-29 issued by the Ministry of Environment and Spatial Planning of the Republic of Serbia); research was carried out in compliance with the Convention on Biological Diversity (CBD) and Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES); no field-captured animals were harmed and the majority of offspring obtained from the crosses (>80% of each clutch) was released to their ponds of origin.

We genotyped 43 microsatellites polymorphic in at least one parent, including 23 loci mapped by Berset-Brändli et al. [7] (based on Swiss *H. arborea* families) and 20 markers developed since [3,5,8,11]. All but one marker (*Ha*-H116) were amplified in nine multiplex PCRs (Additional file 1: Table S1), following Dufresnes et al. [8]. PCRs were carried out in 10 μL, including 3 μL of DNA (10-100 ng), 3× Qiagen Multiplex Master Mix, and primers (concentrations: Additional file 1: Table S1). Thermal conditions were as follow: 95°C for 15', 35 × (94°C for 30", 58°C for 1'30", 72°C for 1'), 60°C for 30'. Locus *Ha*-H116 was amplified separately in a 10 μL PCR containing 2.5 μL of DNA (10-100 ng), 1× Qiagen PCR buffer (with 1.5 mM of $MgCl_2$), 0.2 mM of dNTPs, 0.5 μM of each primer and 0.25 units of Qiagen Taq. Conditions consisted of 94°C for 3', 45 × (94°C for 45", 58°C for 45", 72°C for 1'), 72°C for 5'. PCR products of *Ha*-H116 were pooled and genotyped along with multiplex A. All amplicons were analyzed on an ABI-3100 sequencer with size standards Rox-350 (multiplex A-D) or Rox-500 (multiplex E-I). Alleles were scored with Genemapper 4.0 (Applied Biosystems, Inc.). Additional file 1: Table S1 provides detailed marker information. In complement to our microsatellite dataset, we included genotypes from a SNP within the gene *Fryl*, shown to be sex-linked in *H. arborea* (methods: [11]).

We used CRI-MAP [12] to estimate linkage and recombination rates through calculations of LOD scores (function *twopoint*), determine the most likely order of loci (functions *all* and *flips*) and calculate sex-specific genetic distances (function *build*). The graphical representation of the map was produced with MAPCHART [13]. We compared recombination rates between Balkanic (this study) and Swiss populations [7] by paired Wilcoxon signed-rank tests, considering only combinations between neighboring informative loci to avoid pseudo-replication.

Results and discussion

We identified eight linkage groups (Figure 1), potentially representing eight out of the 12 chromosomes of *H. arborea* [14]. LG1 to LG6 corresponds to the six groups reported by Berset-Brändli et al. [7], with LG1 being the sex chromosomes. Two new linkage groups could be identified (LG7 and LG8). Six loci displayed no significant linkage disequilibrium, possibly because of low informativeness and/or because they represent some of the four remaining chromosomes: the same WHA1-25, *Ha*-A127, *Ha*-B5R3 remained similarly unlinked in the Swiss families [7]. However, it is not excluded that several linkage groups map to the same chromosome.

We documented drastically reduced recombination rates in males compared to females across most of the genome, including the sex-linkage group, in accordance with Berset-Brändli et al. [7]. Male recombination seems even under complete arrest for some segments (e.g. LG4: *Ha*-T50 – *Ha*-T66, LG5: *Ha*-A11 – *Ha*-T67). Our autosomal maps were on average 13.7 times longer in females than in males, which is close to what these authors reported for Switzerland (14.3). As they developed, this extreme pattern is in line with the pleiotropic model of Haldane and Huxley [15-17], according to which recombination is repressed over the whole genome in the heterogametic sex (males in *H. arborea*), as a way to prevent recombination between homomorphic sex-chromosomes. Interestingly, however, one linkage group (LG8) does not feature such strong male-biased heterochiasmy, perhaps indicating different mechanisms which would deserve further investigation with additional markers. Moreover, recombination rates did not significantly differ between the two regions, neither in females (Wilcoxon signed rank test, p = 0.26) nor in males (p = 0.87), at least for the few combination of informative loci common to both studies (n = 9 and 12 for females and males respectively). Rates of recombination are provided in Additional file 2: Table S2.

Only one major chromosome rearrangement was apparent since the divergence from *Xenopus* (~200 Mya): *H. arborea*'s LG4 is homologous to both *X. tropicalis*'s scaffold 1 (*Ha*-T32, *Ha*-T41 and *Ha*-T49) and scaffold 2 (*Ha*-T50, *Ha*-T66). Reciprocally, most of *X. tropicalis*'s scaffold 1 is syntenic with *Hyla*'s sex chromosomes (LG1, as shown by [11]), and to this conserved LG4 segment. In contrast, from our data other linkage groups featured no signs of rearrangements: markers lying within different *Xenopus* scaffolds either belong to different *Hyla* linkage groups, or

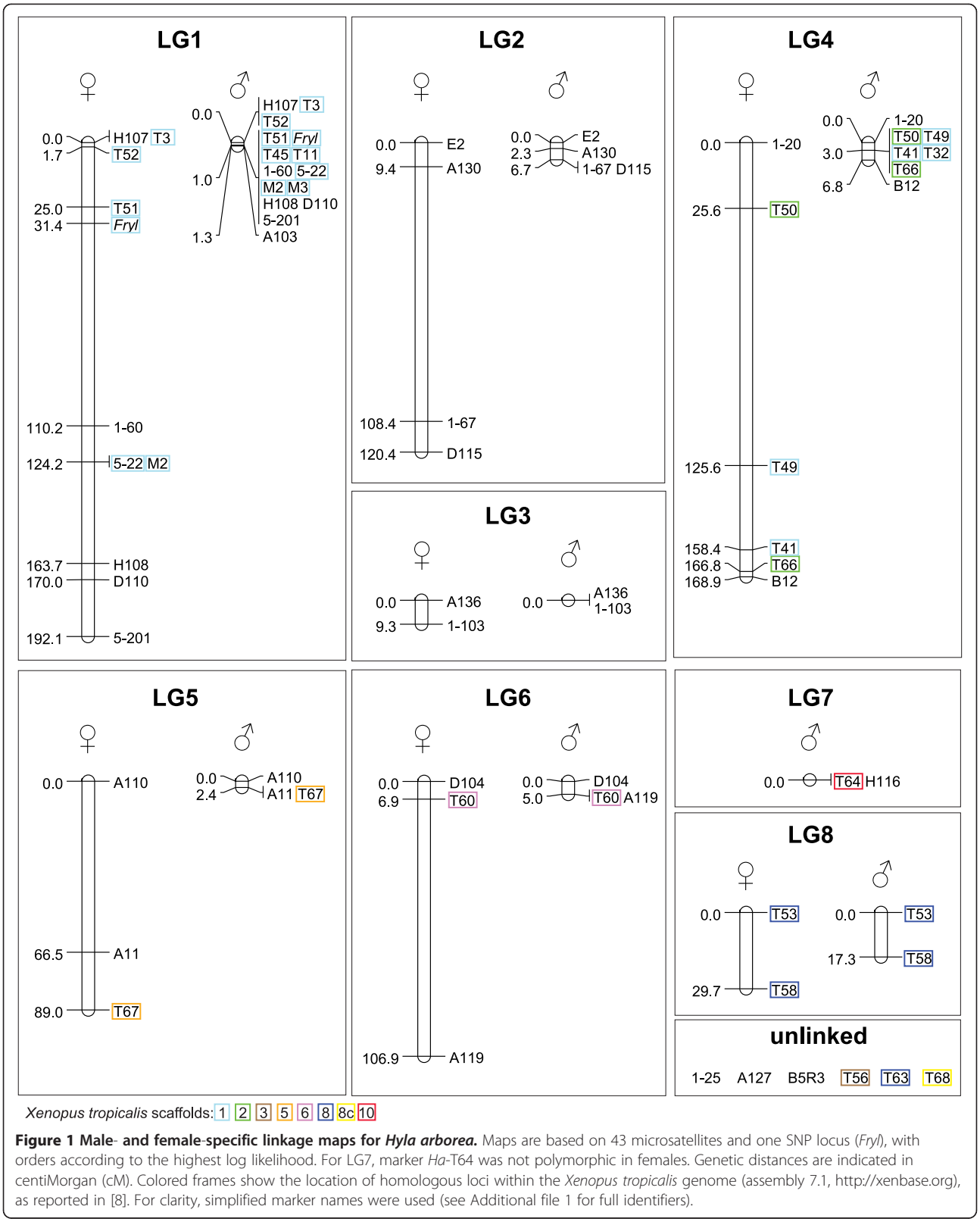

Figure 1 Male- and female-specific linkage maps for *Hyla arborea*. Maps are based on 43 microsatellites and one SNP locus (*Fryl*), with orders according to the highest log likelihood. For LG7, marker *Ha*-T64 was not polymorphic in females. Genetic distances are indicated in centiMorgan (cM). Colored frames show the location of homologous loci within the *Xenopus tropicalis* genome (assembly 7.1, http://xenbase.org), as reported in [8]. For clarity, simplified marker names were used (see Additional file 1 for full identifiers).

remained unlinked. Accordingly, LG8 includes two markers from the same *Xenopus* scaffold 8. Note that a third marker from this scaffold (*Ha*-T63) remained unlinked, but its low informativeness with other LG8 loci (polymorphic only in one female) prevents firm conclusions. Although the coverage of some chromosomes is weak (i.e. synteny is supported by a few loci per LG), it is thus likely that the same linkage groups hold between *H. arborea* and its Western Palearctic congenerics (diverged over the last 10 My, [6]), and our map should also be suitable for these taxa. High-density linkage mapping using genotyping-by-sequencing (e.g. [18]) will give a much higher resolution for documenting patterns of sex-specific recombination across *H. arborea* chromosomes, and for detecting events of rearrangements across anuran frogs.

The characterization and mapping of new microsatellite markers compatible across Western Palearctic *Hyla* tree frogs (plus some Eastern Palearctic and Nearctic taxa, [8]) significantly expands possibilities for genetic surveys in this group. In particular, their high transferability and known relative genomic localization make them ideal assets for speciation studies, i.e. for analyzing levels of hybridization in secondary contact zones and screening for patterns of differential introgression over the genome. These advantages will also allow multispecies comparative linkage mapping to understand the evolution of sex chromosomes and recombination in this group [5]. Finally, this marker set will be useful to unravel the cryptic diversity suspected in several understudied taxa (e.g. *H. orientalis*, *H. savignyi*, *H. meridionalis* [6]), as well as for other applications related to the conservation of these emblematic species, threatened in many regions and countries.

Availability of supporting data

Microsatellite genotypes are archived in Dryad (http://doi.org/10.5061/dryad.16pj3). doi:10.5061/dryad.16pj3.

Additional files

Additional file 1: Table S1. Information on the microsatellites used in this study.

Additional file 2: Table S2. Sex-specific recombination rates in *Hyla arborea* estimated from Balkanic (this study) and Swiss populations [7].

Abbreviations

Mya: Million years ago; LG: linkage group.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors designed the study. CD performed fieldwork, labwork and analyses. The manuscript was drafted by CD and improved by AB and NP. All authors read and approved the final manuscript.

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CHAPTER X

LARGE-X EFFECTS DESPITE SEX-CHROMOSOME HOMOMORPHY IN EUROPEAN TREE FROGS

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* equal contribution

Dufresnes C, Brelsford A, Crnobrnja Isailović J, Tzankov N, Lymberakis P, Perrin N. Large-X effects despite sex-chromosome homomorphy in European tree frogs.

Large X-effects despite sex-chromosome homomorphy in European tree frogs

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ABSTRACT

Sex chromosomes have been assigned a key role in driving speciation, which may however be mediated by their degree of differentiation. Most support yet come from species with degenerated sex chromosomes. Here we document restricted introgression at sex-linked compared to autosomal loci in a natural hybrid zone between two lineages of tree frogs (*Hyla arborea* and *Hyla orientalis*) with undifferentiated XY chromosomes. Genome-wide introgression was low (~20 km) across two independent transects, suggesting advanced reproductive isolation, but cline widths were significantly narrower for sex-chromosome by 6 km, consistent with a large X-effect. Our results imply that non-degenerated sex chromosomes may also effectively affect reproductive isolation, with sex-linked hybrid incompatibilities rising from faster male or faster heterogametic sex evolution.

INTRODUCTION

The mechanisms by which reproductive isolation accumulates between incipient species constitute a central issue in the study of speciation. Two empirical 'rules of speciation', Haldane's rule and Coyne's rule (or large-X effects), both assign a key role to sex chromosomes in the building of intrinsic postzygotic isolation (i.e. hybrid infertility or inviability). From Haldane's rule, when a sex is absent, rare, or sterile in an interspecific cross, this is usually the heterogametic sex (Haldane 1922). Haldane's rule is obeyed by the vast majority of animal taxa studied in this respect (Schilthuizen et al. 2011), and appears to also apply to plants (Brothers & Delph 2010). Coyne's rule (or large X-effect) refers to the disproportionally high impact of X or Z chromosomes in driving hybrid dysfunctions, compared to autosomes (Turelli & Moyle 2007). QTL mapping and backcross analyses in *Drosophila* have provided compelling evidence that genetic factors with the largest effects on hybrid sterility tend to be X-linked (Coyne & Orr 2004, Masly & Presgraves 2007, Presgraves 2008, Qvarnström & Bailey 2009).

Several alternative models have been proposed to account for both of these empirical rules. i) The '*dominance model*' notes that, if the Dobzhanski-Muller incompatibilities that impact hybrid fitness are partially recessive, they are more likely to be expressed when involving genes on the hemizygous X (or Z) chromosomes (Muller 1940, Turelli & Orr 1995). This model predicts Haldane's effects in both XY and ZW systems, but only for species with degenerated / silenced Y or W chromosomes. ii) The '*faster-X*' theory holds that genetic changes accumulate faster on the X or Z chromosome than on autosomes, due to hemizygous exposure to selection in the heterogametic sex (Charlesworth et al. 1987). This effect has the potential to contribute to both large X-effects and Haldane's rule, but also specifically applies to systems with degenerated / silenced Y or W chromosomes. iii) The '*faster-male*' theory holds that sexual selection drives especially rapid evolution of male-expressed genes, inducing more rapid divergence of genes involved in male than in female reproduction (Wu & Davies 1993, Wu et al. 1996). This may explain why hybrid male sterility evolves before hybrid female sterility, and thus account for sterility aspects of Haldane's rule, but only in XY systems. This model makes no assumption regarding the degeneracy of Y or W chromosomes. Finally, iv) the '*faster heterogametic sex*' model assigns a role to epistatic interactions between X and Y (or Z and W) chromosomes. Epistasis may result from a history of genomic conflicts, such as the control of meiotic-drive X chromosomes by Y-linked genes

(e.g. Frank 1991, Hurst & Pomiankowski 1991, Tao & Hartl 2003, McDermott & Noor 2010). Note that X-Y hybrid incompatibilities may also result from the requirement of complementary alleles from conspecific gametologs for proper differentiation of the heterogametic sex. This model does not either make any assumption regarding the degeneracy of X and Y chromosomes.

These alternative theories are not mutually exclusive: both Haldane's and Coyne's rules are likely underlain by a diversity of mechanisms. The relative contribution of the four models in driving speciation thus becomes an important empirical question. One way of evaluating it is through a comparison of F1-hybrid sterility or viability between systems with homomorphic versus heteromorphic sex chromosomes. In *Anopheles* mosquitoes, where X and Y chromosomes are highly heteromorphic, hybrid males suffer from strongly depressed sterility and viability, while in *Aedes* mosquitoes, where X and Y chromosomes are homomorphic, hybrid males only suffer from limited sterility (Presgraves & Orr 1998). This suggests that dominance- and/or faster X-effects are required to account for the inviability aspects of Haldane's rule, but that faster male- and/or faster heterogametic sex effects also contribute to fertility aspects. In *Xenopus* frogs, where sex chromosomes are homomorphic, hybrid fertility is more depressed in ZZ males than in ZW females, running against Haldane's rule; by sex-reversing frogs, Malone & Michalak (2008) showed hybrid male sterility to be determined by the phenotypic sex (male versus female), not the genotypic sex (ZZ versus ZW), hence providing strong support for faster-male effects (but also showing that the genes involved in male sterility are mostly autosomal). A recent meta-analysis by Lima (2014) corroborates the view that postzygotic incompatibilities expressed in F1 hybrids are heavier in species with heteromorphic than with homomorphic sex chromosomes, assigning an important role to the dominance- and/or faster-X models.

An alternative way of evaluating the relative contributions of these selective forces to speciation is through the study of hybrid zones, which constitute natural laboratories to characterize the genetic basis of reproductive barriers. As genomic regions responsible for pre- and postzygotic isolation are selectively filtered, screening for differential introgression patterns over the genome (e.g. through cline fitting techniques) offers a powerful way to outline genes or genomic segments involved in speciation processes (Payseur 2010). As the dominance- and faster-X models assume degenerated Y or W chromosomes, they only predict restricted introgression for heteromorphic sex chromosomes. The faster-male model predicts no restriction to sex-chromosome introgression in ZW systems; in XY systems, a restriction is only expected if the genes responsible for male sterility are preferentially located on sex chromosomes (an assumption with little empirical support; Qvarnström & Bailey 2009). The faster-heterogametic sex model, finally, predicts restricted sex-chromosome introgression in both XY and ZW systems, independently of the degeneracy of sex chromosomes.

Until now, patterns of sex-chromosome introgression have been mostly documented in systems with differentiated sex chromosomes, such as mammals (Payseur et al. 2004, Teeter et al. 2008), birds (Saetre et al. 2003, Carling & Brumfield 2008, Storchova et al. 2010, Elgvin et al. 2011) and insects (Haggen & Scriber 1989). All these studies have found important restrictions to the introgression of sex chromosomes, confirming their crucial role in driving speciation. However, such studies have limited power to disentangle alternative models, except that faster-male effects can be ruled out in female-heterogametic systems such as birds. Very few studies have investigated the introgression of sex-linked genes in species with homomorphic sex chromosomes. The only ones we are aware of are investigations on female-heterogametic *Populus* trees, where introgression rates did not differ between sex chromosomes and autosomes, based both on experimental crosses (Macaya-Sanz et al. 2011) and on analyses of natural hybrid zones (Stölting et al. 2013). This contrasts sharply with systems involving heteromorphic sex chromosomes, suggesting a strong role to the dominance- and/or faster X model in preventing sex-chromosome introgression.

Here we investigate the patterns of introgression across a hybrid zone between two species of European tree frogs, *Hyla arborea* and *H. orientalis*. The former is distributed from Southern Balkans to North-Western Europe, and the latter from Asia minor to North-Eastern Europe. Their contact zone runs from North-Eastern Greece to Central Balkans along the Carpathian chain, and upper north across lowland Poland along the Vistule river (Stöck et al. 2012). Although the two lineages diverged in Mio-Pliocene times (5 Mya), they are morphologically so similar that their specific status has been questioned (Speybroeck et al. 2010). Both lineages inherited from their common ancestor the same pair of XY chromosomes, maintained homomorphic through occasional X-Y recombination (Stöck et al. 2011, 2013; Guerrero et al 2012). Post-glacial populations of *H. arborea*

in NW Europe show complete arrest of XY recombination in males and some X-Y differentiation in terms of allelic frequencies, with male-specific alleles fixed on Y haplotypes, testifying to the absence of recombination in their recent history; in contrast, refugial populations from the Southern Balkans display occasional XY recombination in males, and no noticeable X-Y differentiation (Dufresnes et al. in press a). No similar information is available from *H. orientalis*. We performed a dense sampling of populations along two transects across the contact zone, one in northeastern Greece and one in southeastern Serbia. Individuals were genotyped for one mitochondrial gene as well as a series of autosomal and sex-linked microsatellite markers, and cline analyses were performed to compare these distinct sets of markers for patterns of introgression. Because *Hyla* sex chromosomes are homomorphic, we do not expect their introgression to be restricted by dominance- or faster X-effects. Thus, if sex chromosomes indeed show limited introgression, this may only stem from faster heterogametic sex- and/or faster-male effects. The latter in particular is expected to play a significant role in lowering male hybrid fitness (because sexual selection is likely to be strong in these lek-breeding species), but this should only limit sex-chromosome introgression if sexually selected genes map predominantly to the sex chromosomes.

METHODS

DNA SAMPLING AND EXTRACTION

Tree frogs were captured on the field from 97 localities (n = 588 individuals) distributed across Northern Greece, Southern Serbia, Kosovo region, Bulgaria and Western Turkey, corresponding to the southern parapatric ranges of the two species (Stöck et al. 2012). Two contact zones were specifically targeted through fine-scale transects: west-eastward across southern Serbia (loc. 13-26); west-eastward along the northeastern Greek coast (loc. 54-78). Details on sampling localities can be found in Table S1. DNA was sampled from non-invasive buccal swabs (live adults, Broquet et al. 2007) and from ethanol-preserved tissues (tadpoles) and extracted using the Qiagen robotic workstation or the Qiagen DNeasy Blood & Tissue kit.

MITOTYPING AND MICROSATELLITE GENOTYPING

We designed a mitotyping procedure by enzyme restriction of the mitochondrial *cytochrome-b* (*cyt-b*) based on published sequences from both species (sampled in the areas of contact; Genbank JX182103-06, JX182264-66) screened for restriction sites with the NEB cutter online tool (http://tools.neb.com/NEBcutter2/index.php).
We selected enzyme *MseI* which distinctively cuts *cyt-b* haplotypes in a species specific way, yielding four segments for *H. arborea*'s (~360, 300, 200 and 100bp) and three for *H. orientalis*'s (~700, 200 and 50 bp). A total of 578 individuals was mitotyped as follow: (1) *cyt-b* (~950bp) was amplified in 10µL PCRs (methods: Dufresnes et al. 2013); (2) PCR products were enzymatically digested for 2 hours at 37°C in 6 µL reactions containing 2µL of PCR product, 0.07µL of *MseI* (New England Biolabs), 0.06µL of BSA and 0.4 µL of NEB buffer #4, following the manufacturer's recommendations; (3) mitotyping profiles were visualized and scored on an 1.5% agarose gel after ~45' of migration at 110V.
We genotyped 582 individuals for 17 autosomal and 6 sex-linked microsatellite loci (listed in Table S2), cross-amplifying in both species and featuring interspecific polymorphism (Dufresnes et al. 2014). All markers were amplified in multiplexes and amplicons ran on an ABI 3100 genetic analyzer and scored with Genemapper 4.0 (Applied Biosystem) following protocols from Dufresnes et al. (in press b).

POPULATION GENETIC ANALYSES

In order to accurately locate and document patterns of introgression between *H. arborea* and *orientalis*, we conducted a series of analyses to characterize the genetic structure of tree frogs throughout the study area. First, we performed Bayesian clustering of individual microsatellite genotypes into Hardy-Weinberg groups using STRUCTURE (Pritchard et al. 2000). We used the admixture model without prior on sample origin, and tested from one to 11 groups (K) with 10 replicate runs per K, each run consisting of 100'000 iterations following a burn-in of 10'000. The most-likely number of groups was determined by the Evanno method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl & vonHodt 2011). Replicates were combined using CLUMPP (Jakobsson & Rosenberg 2007) and graphical displays of individual probabilities of assignment (barplots) were obtained with DISTRUCT (Rosenberg 2004).

Second, we decomposed genetic variation by a Principal Component Analysis (PCA) on microsatellite genotypes (*adegenet* R package, Jombart et al. 2008a). To get insights into the spatial structure, we performed a spatial Principal Component Analysis (sPCA) on population allelic frequencies (Jombart et al. 2008b; implemented in *adegenet*). This multivariate analysis summarizes both geographic (spatial proximity) and genetic

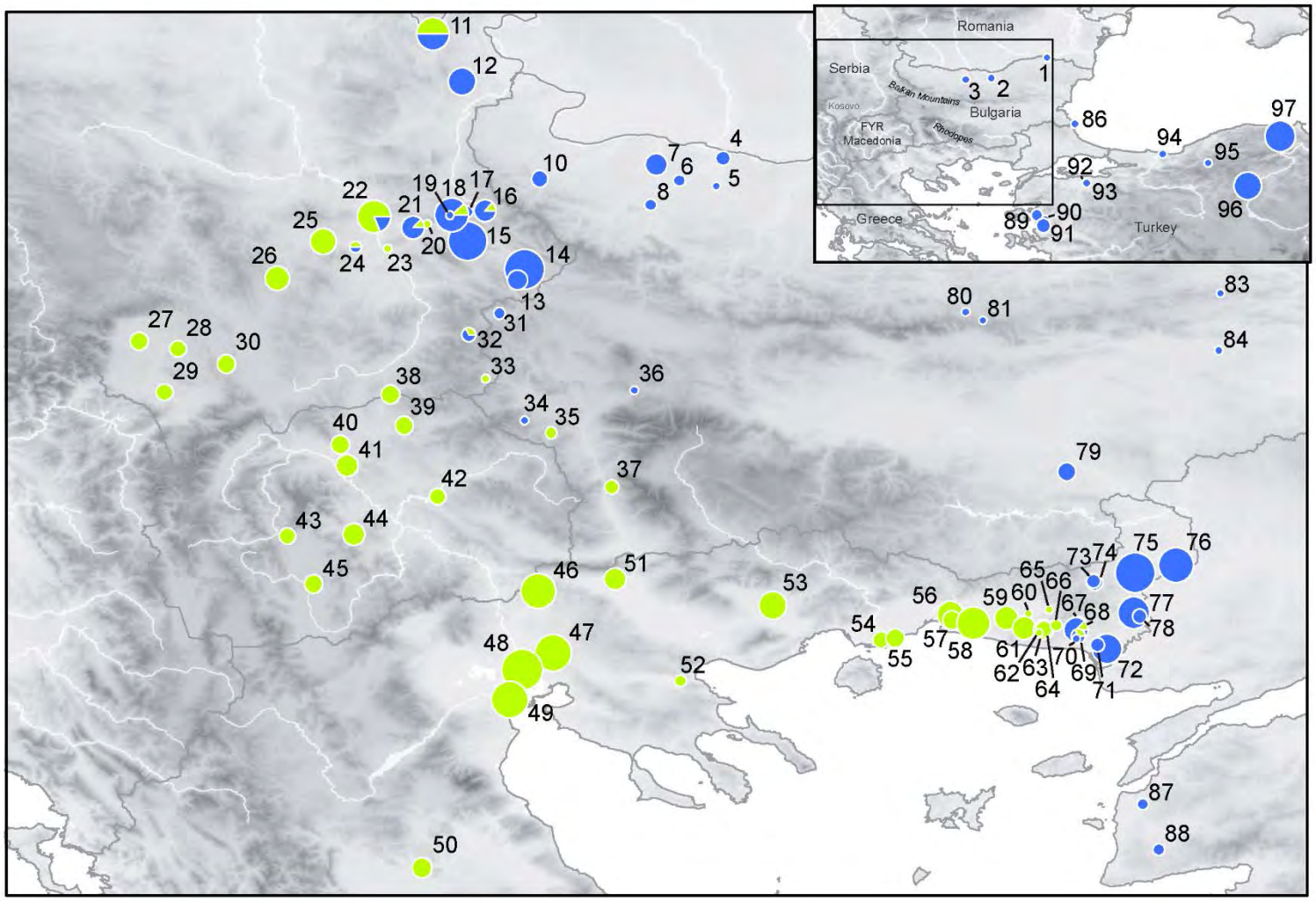

FIGURE 1: Distribution of *H. arborea* (green) and *H. orientalis* (blue) mtDNA haplotypes. Pie charts are proportional to sample size. Zooms on transects are provided in Figure 3.

(allele frequencies) information into principal components to detect and test for patterns of structuration. We conducted the sPCA using an edited Gabriel graph as a spatial connection network, and interpolated the obtained population scores to the entire geographic space of our study area.

CLINE ANALYSES OF CONTACT ZONES

To conduct cline analyses, we first converted each microsatellite locus to a two-allele system using the *Introgress* R package (Gompert and Buerkle 2010), and calculated the frequency of *H. orientalis* composite alleles for each locus in each population using a custom R script. For each population on the two transects, we calculated the distance between that population and the easternmost population of the transect (sites 17 and 35 for Serbia and Greece respectively) using Google Earth (https://earth.google.com). We then fit four-parameter sigmoid clines to the population allele frequencies using the *hzar* R package (Derryberry et al. 2014), in which the cline for each locus is defined by its center, width, and estimated allele frequency in each of the parental species. Log-transformed cline widths were compared between sex-linked and autosomal markers, as well as between the two transects using a generalized linear model (GLM).

RESULTS

GENETIC STRUCTURE

The distributions of the two mitochondrial haplotypes were delineated by the Balkan and Rhodope Mountains, where several parapatric populations occur (Figure 1). Over the study area, *H. arborea* mtDNA was found across Kosovo, southern Serbia, FYR Macedonia and northern Greece, where it reaches its most eastern range in Thrace. It is also present at the extreme southwestern end of Bulgaria, namely at two stations upstream the Struma valley (loc. 35, 37). Reciprocally, *H. orientalis* mtDNA extends from Turkey over most Bulgaria, and meets *H. arborea* in Thrace and southeastern Serbia.

Bayesian clustering of nuclear markers by STRUCTURE unequivocally suggested two groups ($K = 2$, $\Delta K = 2362.8$; second best solution: $K = 6$, $\Delta K = 5.4$), corresponding to the respective gene pools of the two species (Figure 2). The geographic distribution of these STRUCTURE groups intimately matches mitochondrial data, with intermediate probability of assignments in parapatric populations likely resulting from genetic introgression (loc. 11-12, 18-24, 33-34, 60-70). Moreover, close inspection of fine-scale transects suggests sharp geographic transitions between nuclear and mitochondrial gene pools over a few tens of kilometers (Figure 3).

Accordingly, the most informative component of the PCA was attributed to the differentiation between lineages (first axis, 10.3% of the total variance, Figure S1), showing that some individuals sampled in the contact zones features signs of admixture (i.e. intermediate scores on this axis). The analysis further depicted high intraspecific diversity in both species, particularly in Turkish *H. orientalis* populations (axis 2, 1.8% of the total variance, Figure S1), and partly associated with some subtle geographic structuring in *H. arborea* (over a NW-SE gradient; axis 3, 1.7% of the total variance, Figure S1). The spatial PCA recovered a significant pattern of global structure (i.e. positive spatial autocorrelation, $\lambda 1$; G-test, $p = 0.003$) but no local structure (i.e. negative spatial autocorrelation; L-test, $p=1.0$) (Figure S2a). The corresponding sPCA scores nicely illustrate the level of *H. arborea*/*orientalis* admixture in parapatric populations (Figure S2b) and allowed accurate delineation of species ranges across the area (Figure S2c).

CLINE ANALYSES

Cline analyses of geographic transects yielded replicate patterns of narrow transitions between the two species (Figure 4a). Average cline widths for nuclear loci were 19 km and 20 km in SE-Serbia and NE-Greece respectively. Mitochondrial clines slightly contrast, being wider in SE-Serbia (35 km), whereas restricted to 4 km and shifted eastward by 10 km in NE-Greece (Table S2). Cline centers fall within relatively unsuitable tree frog habitats, namely the urban area of Serbia's third city Nìs and the dry reliefs of Rhodopes' southeastern tip in Greece (Figure 3).

From both transects cline widths appear generally narrower for sex-linked compared to autosomal loci (Figure 4b). Clines expand over ~14 km for sex-linked versus ~20 km for autosomal markers in SE-Serbia, respectively over ~16 km versus ~22 km in NE-Greece (Table S2), hence a 6 km difference in both cases. Combining data in a GLM confirmed this trend: cline widths significantly differ between sex-linked and autosomal loci ($p = 0.027$), but not between transects ($p = 0.14$).

Microsatellites were well-informative about species, featuring diagnostic alleles (i.e. distinctive fixed or frequent alleles between taxa) and thus enabled accurate cline estimations: the range of species-specific allelic frequencies spanned over 0.7 on average (Table S2).

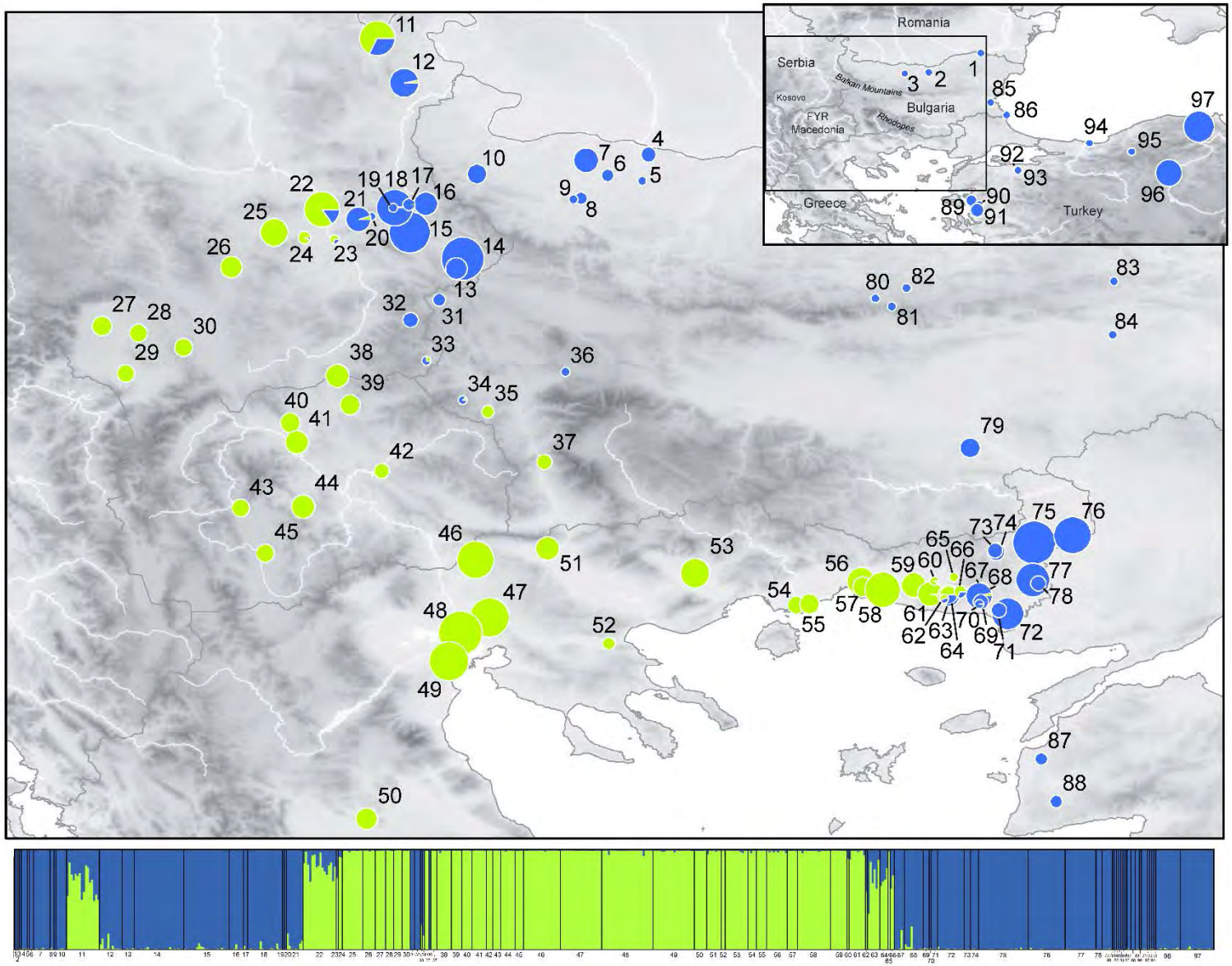

FIGURE 2: Bayesian clustering assignments of individual microsatellite genotypes (barplots) and mean probability assignment of each population (map) into two groups (STRUCTURE, K = 2). Pie charts are proportional to sample size. Zooms on transects are provided in Figure 3.

DISCUSSION

REPRODUCTIVE ISOLATION BETWEEN HYLA *LINEAGES*

The two *Hyla* lineages under study have clearly reached an advanced level of reproductive isolation. Nuclear and mitochondrial markers provided clear-cut and consistent results regarding the geographical delimitation of two highly distinct genetic entities, and our dense sampling allowed delimitation of species ranges at a fine-scale resolution. Introgression between the two gene pools appears very limited, with patterns of spatial structure testifying to sharp transitions across the contact zone in both transects. The nuclear markers provided more consistent estimates of cline width (19 and 20 km respectively) than the mitochondrial *cyt-b* gene (4 and 35 km), as expected from the lower sampling variance (nuclear markers also produced more variable and less consistent estimates when considered individually).

Such narrow clines cannot result from neutral effects only. In the absence of selection, cline width (w) can be predicted from a diffusion model as a function of dispersal distance (σ) and time since contact (t), as $w = 2.51\sigma\sqrt{t}$ (Barton & Gale 1993). Assuming an average dispersal distance of 1.5 kilometer (Vos et al. 2000), cline widths would exceed our nuclear estimates (20 km) in 30 generations (i.e. ~90 years). Thus, observed cline widths are clearly limited by selection against hybrids. From a taxonomic point of view, these results support the independent history and specific status of the eastern tree frog *H. orientalis*, and claim for its recognition as a full species.

Timeframes of speciation can be estimated through correlations between divergence time and hybridizability in natural populations (e.g. Singhal & Moritz 2013; Beysard & Heckel 2014). In European green toads, lineages with ~1.9 My divergence display relatively free introgression across secondary contact zones (Dufresnes et al. 2014b), while others with ~2.6 My divergence have already reached almost complete isolation (Colliard et al. 2010). Our results thus fit with the relatively old split between *H. arborea* and *H. orientalis* (~5 My), and parallel the nearly absence of gene flow between *H. arborea* and the Italian *H. intermedia* (Verardi et al. 2009), a sister species to *H. orientalis*, with the same divergence time to *H. arborea* (Stöck et al. 2012).

Phylogeographic analyses show that *H. arborea* populations survived Quaternary glaciations in the Southern Balkans, and *H. orientalis* in circum-Black Sea refugia (Stöck et al 2012; Dufresnes et al. 2013). These two species might thus have a long history of contact in our study area, during which selection against hybrids may have reinforced assortative mating (Lemmon & Kirkpatrick 2006), e.g. through the fine-tuning

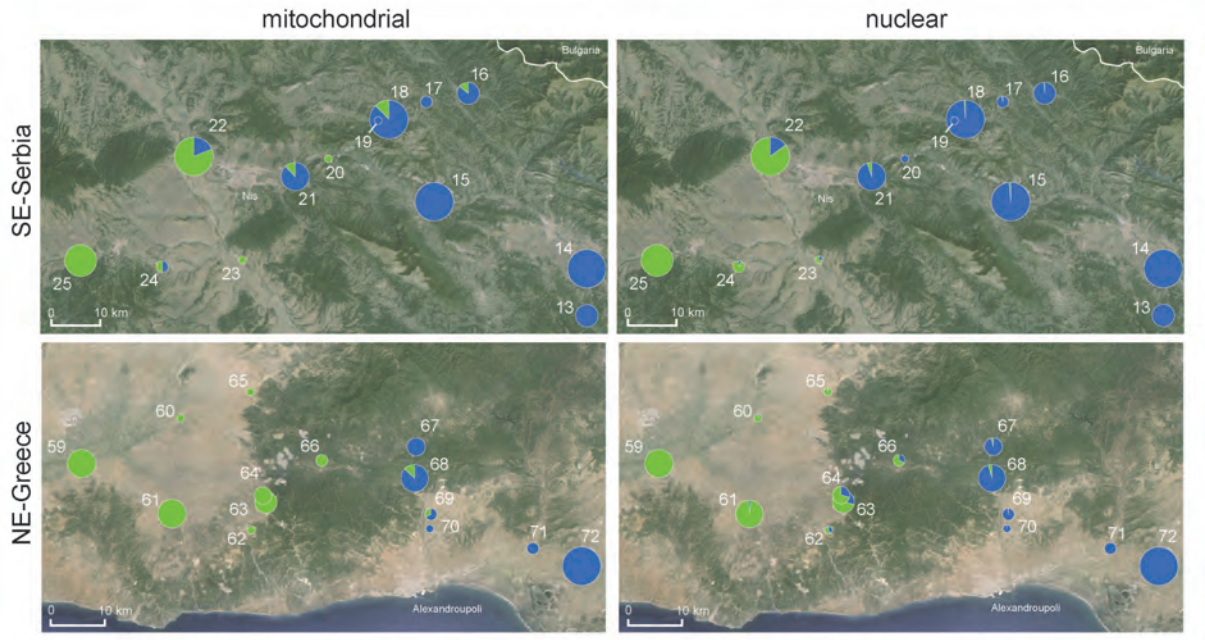

FIGURE 3: Distribution of mtDNA haplotypes and nuclear clusters (STRUCTURE) over SE-Serbian and NE-Greek hybrid zones. green: *H. arborea*; blue: *H .orientalis*.

of mating calls (Höbel & Gerhardt 2003). Limited possibilities for dispersal might further hinder genetic admixture: the Balkan and Rhodope Mountains are largely inhospitable for tree frogs, which are restricted to valleys and may migrate sporadically between catchment areas. As our transects exemplify, species boundaries are delineated by specific geographic features likely to constrain dispersal (Figure 3): the urban area of Nìs in SE-Serbia, and dry pre-Rhodopean hills in NE-Greece, where suitable breeding sites appear scattered and disconnected (CD and AB pers. obs.). More generally, eastern-European mountain chains (the Balkans and Carpathians) are known phylogeographic barriers for Western Palearctic vertebrates and constitute a suture zone (Hewitt 2011). Analyses of the northern part of this tree-frog contact zone, particularly in Poland where the two species came in parapatry much more recently (after the last glacial, < 15'000 years) and which presents no obvious barrier to dispersal, complemented by range-wide bio-acoustic surveys, should provide relevant insights on the mechanisms and time-scale of reinforcement.

Restricted sex-chromosome introgression

From our cline analyses, sex-linked loci show less introgression than autosomal loci, providing clear evidence for large X-effects (possibly better referred to as 'large sex-chromosome effects' in the case of homomorphic sex chromosomes). Cline widths for sex chromosomes are decreased by ~6 km (i.e. some 30%) relative to autosomes. Although significant, the effect is relatively small (R^2 = 0.103). Importantly, given the homomorphy and occasional X-Y recombination that characterize sex chromosomes in tree frogs, this restriction cannot be explained by the dominance- and/or faster-X models, which assume degenerated or silenced Y chromosomes.

The two alternatives, namely the faster-heterogametic sex and faster-male models, make no assumptions regarding the degeneracy of Y chromosomes, and are thus better candidates to explain the trend documented here. Male frogs are expected to be under strong sexual selection due to female mate choice, and the more so in lek-breeding species such as tree frogs; clear evidence for faster-male effects on hybrid male sterility has been gathered from studies on *Xenopus* (Malone & Michalak 2008). However, this should only translate into limited sex-chromosome introgression if the genes involved in male sterility preferentially map to the sex chromosomes, an assumption that has received little empirical evidence so far; the faster-male effects documented in *Xenopus* frogs are clearly controlled by autosomal genes (Malone & Michalak 2008). Testing among these two alternatives is bound to be difficult in the case of

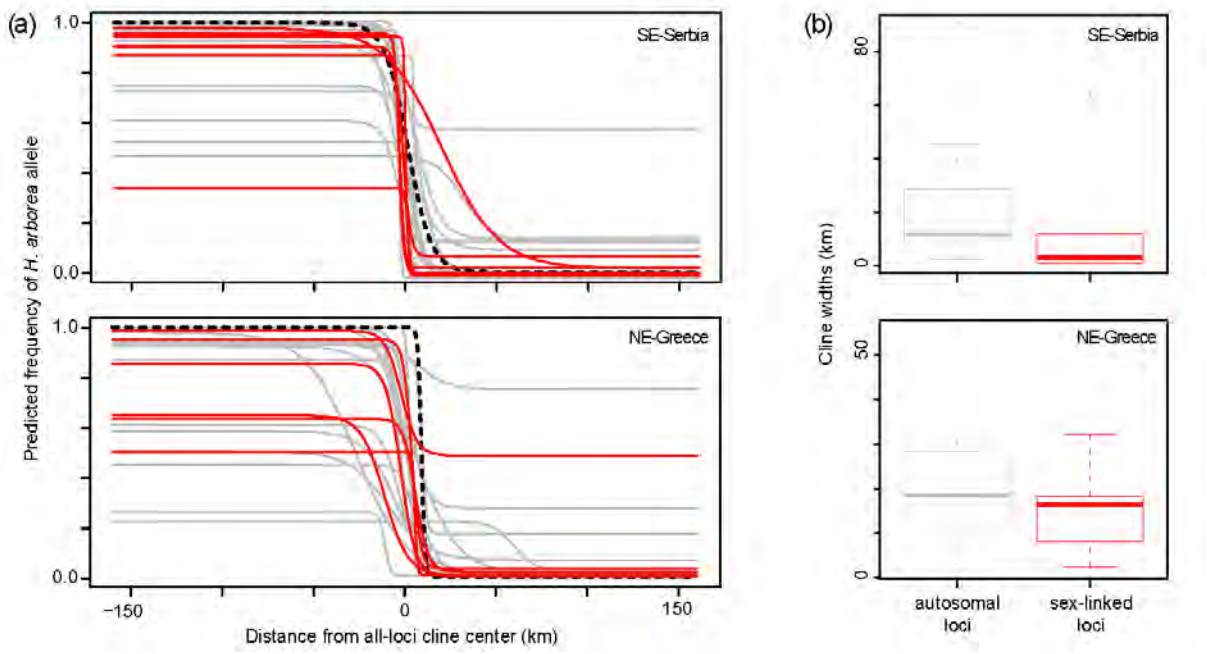

FIGURE 4: Cline analyses over both transects. (a) Allele frequency clines built from individual mitochondrial (dashed line), autosomal (grey lines) and sex-linked (red lines) loci over both transects; (b) Comparison of cline widths between autosomal and sex-linked markers.

Hyla, because males are the heterogametic sex. In principle, the two models might induce different introgression levels for X and Y chromosomes: faster-heterogametic sex effects imply interactions between X and Y chromosomes, whereas faster-male effects do not necessarily involve genes on the X or on the Y. Unfortunately, X and Y-linked markers could not be phased from our data, because X-Y recombination is too frequent in *H. arborea* refugial populations to allow significant differentiation at neutral markers (Dufresnes et al. in press a). Phasing might be feasible in the northern part of the contact zone (Poland), where *H. arborea* X and Y haplotypes are clearly differentiated (Dufresnes et al. in press). However, differential X and Y introgression is also bound to depend on demographic parameters such as local population sizes and sex-specific dispersal rate (Currat et al., 2008; Petit & Excoffier, 2009; Payseur 2010). Little is known about dispersal habits of tree frogs; breeding dispersal seems higher for males than for females (Voss et al. 2000), but sex-specific dispersal distances are poorly documented. Shifted mitochondrial clines may also reflect sex-biased dispersal (Toews & Brelsford 2012); however, the contrasted mtDNA shapes between our two transects rather suggest the action of drift. More generally, we found substantial among-locus variation in cline estimates (Figure 4, Table S2), which stems from drift rather than from differential selection acting on individual markers, since outliers were not the same between transects.

A more promising avenue for testing among these two models might consist in complementing cline analyses with measurements of gene expression: the faster-male model predicts that genes with male-biased expression should display both larger dN/dS values and narrower clines. In addition, gene location might provide further information on the relative importance of faster-male- and faster-heterogametic sex effects: after controlling for male-biased expression, residual effects of sex-linkage on introgression might indicate additional faster-heterogametic sex effects.

Our study, the first of its kind in an animal system with homomorphic sex chromosomes, thus provide two important results: 1) it gives evidence for a sex-chromosome effect on speciation independent of Y degeneracy, which allows to exclude a role for the dominance- and faster X-models; 2) it shows this effect to be modest, implying that the large effects found in systems with differentiated sex chromosomes might largely stem from dominance- and faster X-models.

Seeking direct evidence for Haldane's rule through experimental crosses in *Hyla* might also contribute useful information on the relative importance of these different speciation mechanisms. Systems with homomorphic sex chromosomes apparently provide rather mixed support for this rule, which seem moreover to depend on the patterns of heterogamety. Whereas male-heterogametic systems seem to comply, as documented e.g. in newts (Arntzen et al. 2009) and teleost fishes (Tech 2006, Russell & Magurran 2006, Mendelson et al. 2007, Crow et al. 2007, Kitano et al. 2009; but see Bolnick & Near 2005), female-heterogametic systems provide limited or no support, as documented e.g. in *Xenopus* (Malone et al. 2007) and Bufonid toads (Malone & Fontenot 2008). Sibship analyzes of hybridizing poplars (*Populus alba* and *P. tremula*) with undifferentiated ZW sex chromosomes (Yin et al. 2008), provided no support either for lower female hybrid fitness (Macaya-Sanz et al. 2011). If Haldane's rule mostly arises from dominance- and faster-male effects (Schilthuizen et al. 2011), it should indeed not apply to ZW systems with homomorphic sex chromosomes.

The diversity of sex-determining systems in amphibian radiations makes them valuable frameworks to dissect the evolutionary forces involved in reproductive isolation. In particular, W-Palearctic tree frogs can tell us more on the timeframe of these forces, as they feature numerous contact zones between lineages of varying levels of divergence (Stöck et al. 2012). Qvarnström & Bailey (2009) predicted that sex chromosomes should become increasingly influent as speciation progresses. Here we have shown that sex-linked loci are relatively sheltered from introgression in the *H. arborea*/*orientalis* system, a witness of the latest stages of speciation. Our study stresses for comparative analyses with more nascent lineages, and raises opportunities for next-generation sequencing to pinpoint the genomic regions responsible for sexual isolation.

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Table S1: List of sampling localities and sample sizes.

ID	Country	Locality	Latitude	Longitude	male	female	immature / larvae	unknown	TOTAL
1	Bulgaria	Ajdemir	44.09	27.15	1	-	-	-	**1**
2	Bulgaria	Aleksandrovo	43.44	25.40	1	-	-	-	**1**
3	Bulgaria	near Pleven	43.40	24.60	-	-	1	-	**1**
4	Bulgaria	Harlets	43.71	23.84	-	-	3	-	**3**
5	Bulgaria	Altimir	43.55	23.80	1	-	-	-	**1**
6	Bulgaria	Mihajlovo	43.58	23.59	2	-	-	-	**2**
7	Bulgaria	Valchedram	43.68	23.45	-	-	8	-	**8**
8	Bulgaria	v. Dobrusha	43.44	23.42	2	-	-	-	**2**
9	Bulgaria	Pastrina hill	43.43	23.37	1	-	-	-	**1**
10	Bulgaria	Borovitsa	43.59	22.77	-	-	5	-	**5**
11	Serbia	Djerdap	44.44	22.15	-	-	16	-	**16**
12	Serbia	Stevanske Livade	44.16	22.32	11	-	-	-	**11**
13	Serbia	Vlasi	43.00	22.64	6	-	-	-	**6**
14	Serbia	Sukovo	43.06	22.68	23	1	-	-	**24**
15	Serbia	Bela Palanka	43.23	22.35	22	-	-	-	**22**
16	Serbia	Kalna/Y40	43.40	22.45	2	-	5	-	**7**
17	Serbia	Jalovik Izvor	43.40	22.34	2	-	-	-	**2**
18	Serbia	Lozan	43.38	22.25	13	4	-	-	**17**
19	Serbia	Okruglica	43.38	22.24	1	-	-	-	**1**
20	Serbia	Ostrovica	43.32	22.11	1	-	-	-	**1**
21	Serbia	Prozek	43.31	22.03	8	-	-	-	**8**
22	Serbia	Tripale	43.37	21.80	16	-	-	-	**16**
23	Serbia	Cecena	43.18	21.88	1	-	-	-	**1**
24	Serbia	Zitoradja	43.19	21.69	-	-	2	-	**2**
25	Serbia	Donja Toponica	43.23	21.50	9	1	-	-	**10**
26	Serbia	Prepolac	43.01	21.23	-	-	9	-	**9**
27	Kosovo	Ljesane	42.64	20.42	-	-	5	-	**5**
28	Kosovo	Iglarevo	42.60	20.65	-	-	4	-	**4**
29	Kosovo	Xërze	42.34	20.57	-	-	4	-	**4**
30	Kosovo	Banjica	42.51	20.94	-	-	5	-	**5**
31	Bulgaria	v. Yarlovtsi	42.80	22.53	1	1	-	-	**2**
32	Serbia	Vlasina	42.68	22.36	3	-	-	-	**3**
33	Serbia	Bosilegrad	42.42	22.45	1	-	-	-	**1**
34	Bulgaria	Novo selo, Osogovo Mountain	42.18	22.68	-	-	-	1	**1**
35	Bulgaria	Tishanovo, Osogovo Mountain	42.11	22.84	-	-	-	2	**2**
36	Bulgaria	Belchin, Verila	42.35	23.32	-	-	-	1	**1**
37	Bulgaria	Stara Kresna,Pirin	41.79	23.19	1	-	-	2	**3**
38	Serbia	Prohor Pcjinska	42.33	21.90	6	1	-	-	**7**
39	FYR Macedonia	Rugince	42.15	21.98	-	-	5	-	**5**
40	FYR Macedonia	Orlanci	42.04	21.60	-	-	5	-	**5**
41	FYR Macedonia	R'zanicino	41.91	21.64	-	-	7	-	**7**

42	FYR Macedonia	Stip	41.73	22.17	-	-	4	-	**4**
43	FYR Macedonia	Debreste	41.50	21.29	-	-	4	-	**4**
44	FYR Macedonia	Izvor	41.51	21.68	-	-	7	-	**7**
45	FYR Macedonia	Loznami	41.22	21.45	-	-	5	-	**5**
46	Greece	Doirani	41.18	22.76	18	-	-	-	**18**
47	Greece	Pikrolimni	40.82	22.85	20	-	-	-	**20**
48	Greece	Chalkidona	40.72	22.67	18	7	-	-	**25**
49	Greece	Aliakmonas	40.55	22.59	20	-	-	-	**20**
50	Greece	Kerameia	39.56	22.08	-	-	6	-	**6**
51	Greece	Megalochori	41.25	23.21	6	1	-	-	**7**
52	Greece	Volvi	40.66	23.59	1	1	-	-	**2**
53	Greece	Drama	41.10	24.13	11	-	-	-	**11**
54	Greece	Nestos delta, W-side	40.90	24.77	4	-	-	-	**4**
55	Greece	Nestos delta, E-side	40.91	24.85	5	-	-	-	**5**
56	Greece	Vistonida	41.04	25.17	10	-	-	-	**10**
57	Greece	Fanari	41.01	25.18	4	1	-	-	**5**
58	Greece	Listos	40.99	25.31	14	2	-	-	**16**
59	Greece	Venna	41.02	25.50	8	-	-	-	**8**
60	Greece	Tsifliki	41.05	25.63	1	-	-	-	**1**
61	Greece	Krovyli	40.97	25.61	8	-	-	-	**8**
62	Greece	Avra	40.94	25.69	1	-	-	-	**1**
63	Greece	Sykorrachi	40.96	25.72	6	-	-	-	**6**
64	Greece	Sykorrachi	40.96	25.72	4	-	-	-	**4**
65	Greece	Ipio	41.07	25.75	1	-	-	-	**1**
66	Greece	Kirki	40.98	25.79	2	-	-	-	**2**
67	Greece	Aysimi	40.98	25.92	5	-	-	-	**5**
68	Greece	Avas Army Base	40.95	25.91	9	-	-	-	**9**
69	Greece	Amfitriti	40.92	25.92	3	-	-	-	**3**
70	Greece	Amfitriti	40.90	25.91	1	-	-	-	**1**
71	Greece	Evros delta, small river	40.87	26.03	3	-	-	-	**3**
72	Greece	Evros delta, fields	40.84	26.09	13	-	-	-	**13**
73	Greece	Mega Dereio	41.24	26.01	3	-	-	-	**3**
74	Greece	Mega Dereio	41.23	26.02	4	-	-	-	**4**
75	Greece	Protokklisio	41.29	26.25	24	-	-	-	**24**
76	Greece	Didymotique	41.33	26.49	18	-	-	-	**18**
77	Greece	Fylakto	41.05	26.25	15	-	-	-	**15**
78	Greece	Tychero	41.03	26.28	3	-	-	-	**3**
79	Bulgaria	Ostar Kamak	41.88	25.85	-	-	5	-	**5**
80	Bulgaria	dam Smirnenski, near Gabrovo	42.81	25.26	-	-	1	-	**1**
81	Bulgaria	Potok	42.76	25.36	1	-	-	-	**1**
82	Bulgaria	Triyavna	42.88	25.46	-	-	-	1	**1**
83	Bulgaria	Straldza	42.92	26.75	-	-	-	1	**1**
84	Bulgaria	Mochuritsa river	42.59	26.74	-	-	-	1	**1**
85	Bulgaria	Poda	42.44	27.47	1	-	-	-	**1**
86	Bulgaria	Silistar	42.02	28.01	1	-	-	-	**1**
87	Turkey	Ezine	39.93	26.30	-	-	2	-	**2**
88	Turkey	Ezine	39.67	26.39	-	-	2	-	**2**
89	Turkey	Dikili	39.18	26.83	-	-	2	-	**2**

90	Turkey	Bergama	39.04	27.09	-	-	1	-	**1**
91	Turkey	Aliağa	38.85	27.02	-	-	3	-	**3**
92	Turkey	Karacabey	40.20	28.35	-	-	-	1	**1**
93	Turkey	Karacabey	40.17	28.39	-	-	1	-	**1**
94	Turkey	Karasu	41.08	30.76	-	-	1	-	**1**
95	Turkey	Gerede	40.80	32.17	-	-	1	-	**1**
96	Turkey	Kalecik	40.09	33.41	-	-	12	-	**12**
97	Turkey	Taskopru	41.63	34.42	-	-	16	-	**16**

Table S2: List of genetic markers and their cline estimates. Center shows deviation from the all-loci cline center. p gives ranges of estimated allele frequencies in parental species.

			SE-Serbia cline			NE-Greece cline		
Locus	**location**	**Ref.**	**center**	**width**	**p**	**center**	**width**	**p**
cyt-b	mtDNA	-	+1.3	35.1	0.00-0.97	+10.2	4.1	0.00-1.00
Ha-T3	sex chromosome	1	-1.4	11.7	0.10-0.89	+4.2	8.1	0.06-0.97
Ha-T52	sex chromosome	1	-4.9	2.3	0.02-0.97	-0.3	18.2	0.18-1.00
Ha-T11	sex chromosome	1	-3.1	1.0	0.64-0.98	+7.3	2.4	0.51-0.99
Ha-T51	sex chromosome	1	+3.1	0.8	0.06-0.99	+3.0	17.3	0.34-0.98
WHA5-22	sex chromosome	2	-3.8	3.5	0.15-0.99	-11.5	32.1	0.33-0.99
Ha-H108	sex chromosome	3	+22.3	63.3	0.06-0.97	-0.3	15.3	0.02-0.53
Ha-T32	autosome	1	-1.3	9.3	0.02-0.99	+5.8	6.8	0.00-0.99
Ha-T49	autosome	1	-4.2	31.7	0.40-0.99	-0.2	11.9	0.39-0.97
Ha-T41	autosome	1	+43.1	90.2	0.49-0.87	+64.4	31.9	0.77-0.98
Ha-T64	autosome	4	-6.5	28.6	0.01-0.99	+1.6	19.9	0.06-1.00
Ha-T69	autosome	4	-1.4	10.1	0.08-0.83	-9.4	1.9	0.71-0.99
Ha-T54	autosome	5	-0.3	13.1	0.01-1.00	+2.8	12.5	0.03-1.00
Ha-T55	autosome	5	-4.7	9.1	0.01-0.38	+2.0	31.3	0.07-0.25
Ha-T58	autosome	4	-1.4	45.4	0.06-0.91	+6.0	40.4	0.07-0.96
Ha-T50	autosome	4	+1.6	3.5	0.12-0.87	+8.6	7.6	0.14-0.80
Ha-T56	autosome	4	+2.7	30.2	0.00-0.85	+4.2	15.2	0.00-0.76
Ha-T66	autosome	4	+1.4	10.7	0.28-0.97	+7.3	13.4	0.52-1.00
Ha-T60	autosome	4	+2.3	16.1	0.01-0.96	+2.7	18.4	0.00-1.00
Ha-T61	autosome	5	-4.2	11.5	0.03-1.00	-25.0	55.6	0.02-1.00
Ha-T68	autosome	4	-0.7	17.3	0.26-0.97	-7.4	27.0	0.57-0.91
Ha-T63	autosome	4	+0.4	3.8	0.55-0.93	-2.4	28.4	0.42-0.97
Ha-T67	autosome	4	-1.6	11.5	0.00-0.95	-0.3	25.0	0.01-1.00
WHA1-103	autosome	2	-3.7	2.6	0.03-1.00	-0.9	18.4	0.00-1.00

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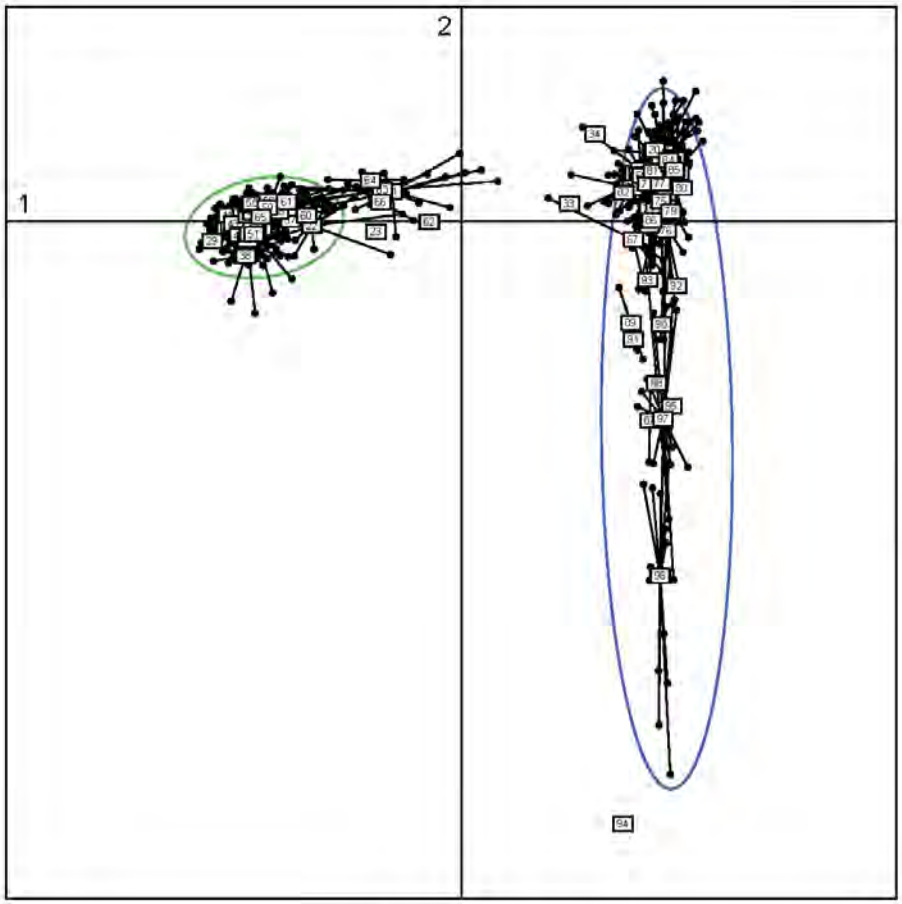
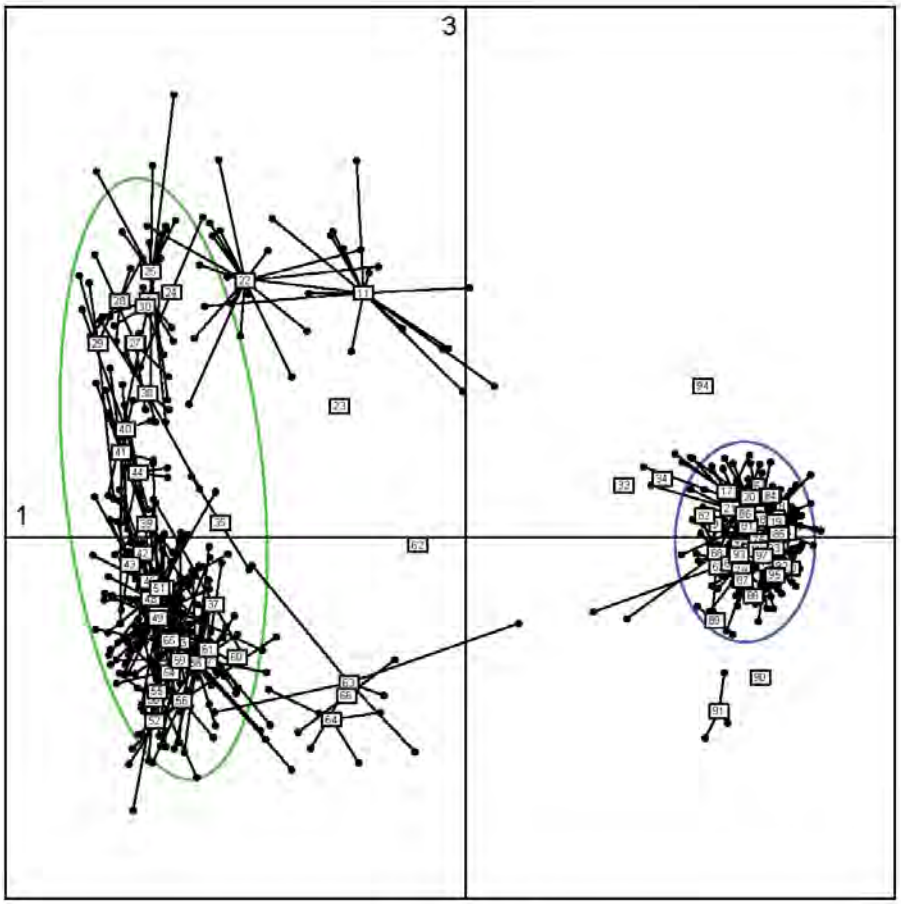
Figure S1: First axes of the principal component analysis on individual microsatellite genotypes. Dots represent individuals, linked to populations (labels). Elipses show the main *H. arborea* (green) and *H. orientalis* (blue) gene pools.

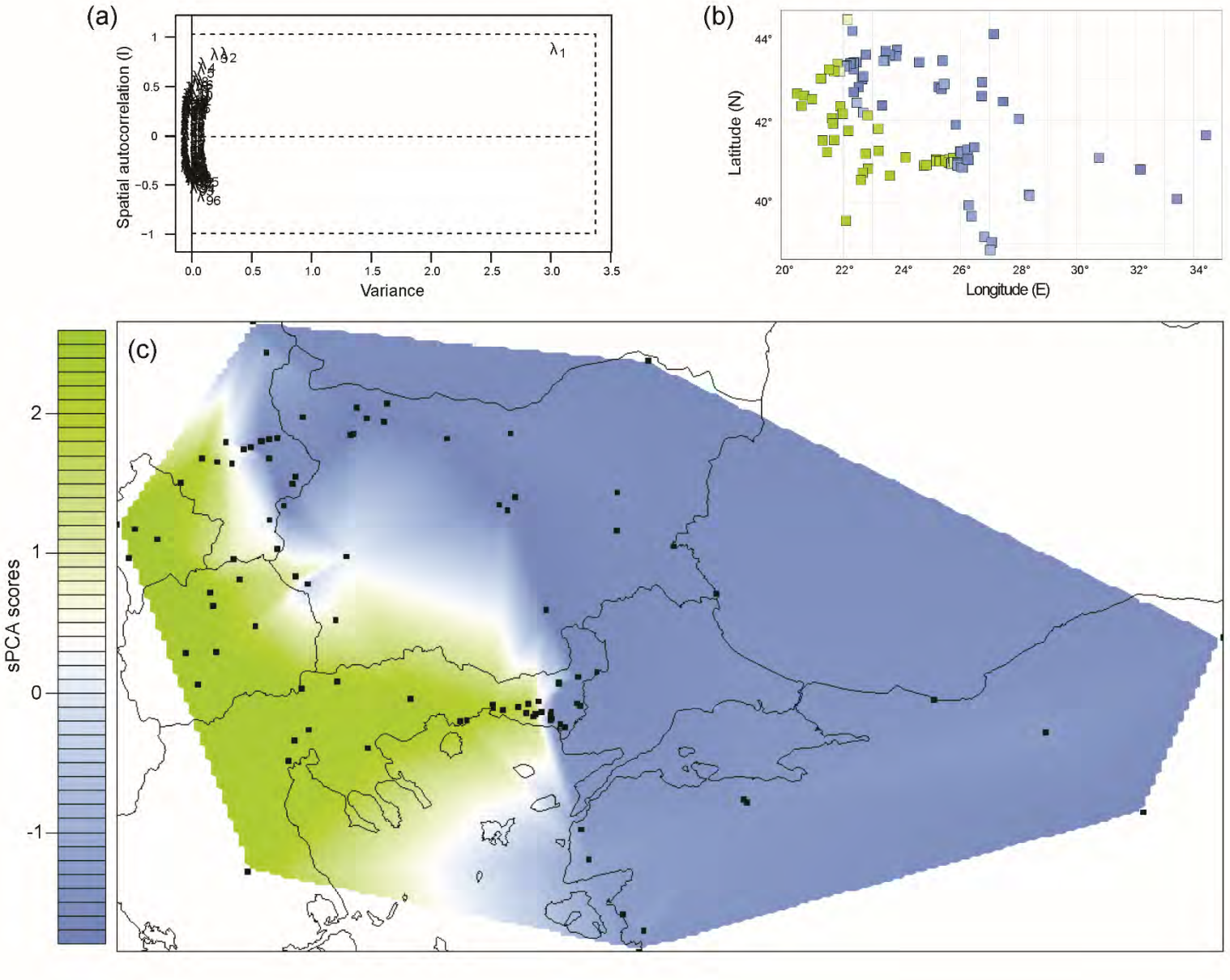


Axis 1 / Axis 2



Axis 1 / Axis 3

Figure S2: spatial Principal Component Analysis (sPCA) on population allelic frequencies. (a) Decomposition of eigen-values; the first dimension catches most of the variance, and testifies for a single global structure (λ1). (b) First dimension's sPCA scores (colors) of each population (squares) plotted in the geographic space. (c) Interpolated map of sPCA scores throughout the study area; black lines show country borders.



CONCLUSIONS AND PERSPECTIVES

As developed in the introduction, two main explanations were proposed to account for the prevalence of homomorphic sex chromosomes in many vertebrates, invertebrates and plants: occasional XY recombination and frequent transitions of sex chromosomes. These completely different processes either renew or replace the senescing gametolog, and both can be triggered to by the Y genetic load (Grossen et al. 2012, Blaser et al. 2014). Using *Hyla* tree frogs as a model system, we have empirically demonstrated that, not only both models checked out in the wild, they can even co-occur within single radiations. Based on fine-scale phylogeographic frameworks and multi-level comparisons (from individuals to radiations), we were able to characterize some underlying mechanisms of these models and make inferences on how they evolve across natural populations in space and time. Figure 1 summarizes some of the main pieces of the puzzle.

THE EVOLUTION OF XY RECOMBINATION

It is universally accepted that recombination arrest is a necessary step in the evolution of sex chromosomes, in order to keep sex-antagonistic genes and the sex-determining locus under full linkage (Charlesworth & Charlesworth 2000). In the first part of this work (**Chapter I-III**), we have debunked this view, by showing that sex-chromosome maintenance was still possible despite low but continuous male recombination. Our results also solved the paradox posed by the lack of detectable XY recombination in previously studied tree frog populations despite high XY sequence similarity (Stöck et al. 2011, Guerrero et al. 2012), which, as we showed, was explained by male recombination differences between populations relating to their biogeographic history. Recent homologous transitions within the youngest European *Hyla* lineages could be rejected based on shared trans-species variation of the sex-determining gene *DMRT1* (**Chapter V**).

While it avoids Y decay, XY recombination should conflict with sex-antagonistic genes: as advantageous combinations are broken down by crossovers, recombining males must produce a proportion of "intersex" unfit offspring, which will be selected against in populations. As shown in **Chapter VII**, tree frogs may canalize this cost through a form of balanced selection on recombination, maintaining a few little-recombining males among a majority of non-recombining individuals. It will be important to implement such variation in theoretical models to test this hypothesis. This mechanism differs from XY recombination through sex-reversal, which rather yield rare burst of recombinants (Perrin 2009). While we did sample one sex-reversed tree frog in the wild (**Chapter III**), this process might be too rare and stochastic in tree frogs (i.e. perhaps because they are less ESD-sensitive compared to other cold-blooded vertebrates) to insure evolutionary stable rates of Y purging.

The direct and indirect evidences for XY recombination and their consequences on sex chromosome (un)differentiation provided by our studies cast very well along recent work on cold-blooded vertebrates, which recently argued for such processes (Stöck et al. 2011, 2013a). From our results, seemingly young sex-chromosomes can carry old sex-determining genes, like *DMRT1* in the case of tree frogs (**Chapter V**), which also determines sex in other deeply diverged vertebrate lineages (Graves & Peichel 2010). XY recombination may thus account for the sex-chromosome homomorphy found in many groups of amphibians, fishes and reptiles (Perrin 2009). Why did these organisms overcome the cost of XY recombination while the sex chromosomes of other lineages (e.g. mammals, birds) stopped recombining a long time ago remains an open question. Mammals and birds evolved dosage compensation to cope with mutational load of the Y/W. Endothermic vertebrates are also less sensitive to environmental effects, offering little scope to temperature-induced sex reversal. Low XY recombination rates might be a more stable evolutionary strategy in organisms producing hundreds of offspring per generation (i.e. hundreds of potential recombinants, among which a few had purged deleterious mutations without losing advantageous SA combinations, thus being fitter than the population average), but on the other hand may be inappropriate in less-fecund organisms, like K-strategists (i.e. where the probability of having some XY-recombinants fitter than the population average is nearly null). In additions, groups with instable sex determining systems (i.e. enduring frequent transitions) may be more flexible to recombine sex chromosomes as these may have less time to recruit many SA genes (but see next section). Testing these hypotheses will benefit from theoretical modelling, as well as better empirical data on how and in what density SA genes accumulate on sex-chromosomes.

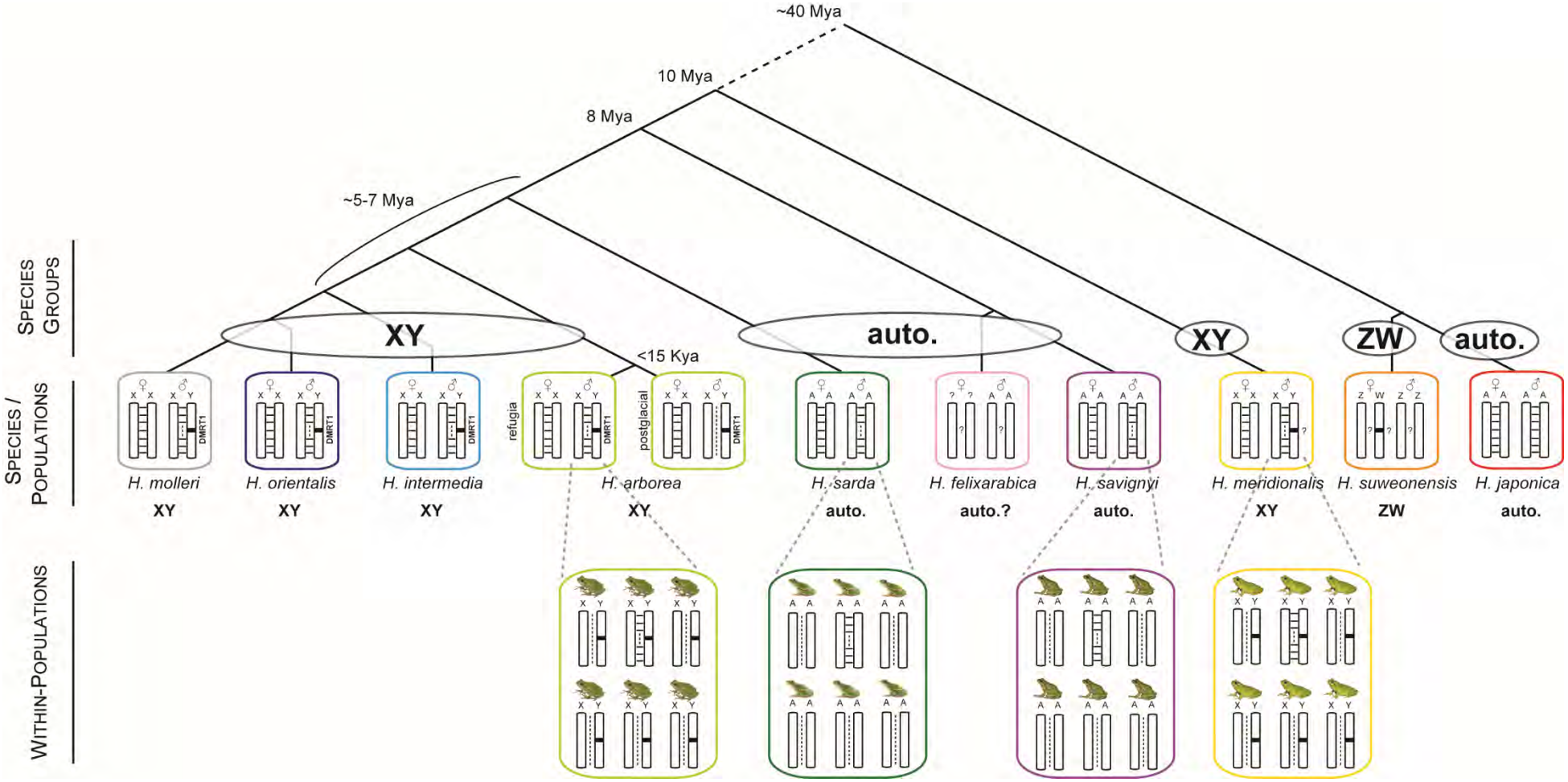


FIGURE 1: Sex-linkage and recombination patterns at linkage group 1 (LG1) in Palearctic tree frogs. Sex-linkage (Chapter VI), sex-determining gene (black bars, labelled when known c.f. Chapter V), average recombination rates (inferred from crosses and patterns of sex-chromosome differentiation; Chapter VI), and within-population variation in male recombination (Chapter VII). For *H. arborea*, differences between refugial and post-glacial populations are shown (Chapters II-III).

Furthermore, our results raise interesting insights on the evolution of recombination. An array of genetic and environmental factors can affect recombination rates (discussed in **Chapter III**). As we show, XY recombination can be rapidly evolving: in *H. arborea*, recombination between sex chromosomes completely stopped in less than 5'000 generations, causing their rapid differentiation (**Chapter II and III**). This rapid evolution, and assuming that within-population variation in recombination may be shaped by a response to selection (**Chapter VII**), pleads for a simple genetic architecture of this trait, and provide a nice framework to screen candidate genes known to affect recombination in other systems (e.g. *RNF212*, *Prdm9*, Kong et al. 2008, Baudat et al. 2013). On the top of these fine-scale variations, the similitudes shared by diverged *Hyla* species in terms of low male recombination rates at linkage group 1, independent from sex-linkage (**Chapter VI, VII**), support a generally conserved inertia, indicative of chromosome-specific regulation, which proximate mechanisms remain unknown.

European tree frogs provide a fascinating system for the study of sex chromosome differentiation. Over the whole chromosome, less than 50 Kb encompassing *DMRT1* remained differentiated between X and Y over the last five million years (**Chapter V**). Therefore, as outlined in **Chapter III**, the geographic cline of XY recombination and differentiation across *H. arborea* populations offers a unique opportunity to study the first steps of sex-chromosome evolution *in statu nascenti* e.g. by contrasting genomic X-Y (or male-female) divergences across the chromosome (i.e. from *DMRT1* to telomeres) in different populations along the cline. It would be interesting to conduct similar surveys in the closely-related *H. orientalis*, which in addition to its phylogenetic relatedness to *H. arborea*, also share many similar biogeographic features (i.e. large distributions encompassing southern refugia and postglacial populations), and thus makes an ideal replicate system to test whether the same patterns hold across species.

THE DYNAMICS OF SEX-CHROMOSOME TURNOVERS

Sex-chromosome turnovers have been reported in several taxonomic groups, for instance in ranid frogs (Miura 2007) and stickleback fishes (Ross et al. 2009). However, so far, little was known regarding the dynamics and temporal scale of these frequent transitions. We addressed this matter in **Chapter VI**, by documenting how one linkage group (LG1) was used and re-used as a sex-chromosome across lineages of gradual divergences. While we could show that transitions could be frequent even within short evolutionary periods (i.e. we evidenced at least two transitions over the last 10 My), one interesting feature was the ability of this linkage group, which carries the sex-determining gene *DMRT1* (**Chapter V**), to have been independently co-opted for sex in diverged lineages. This finding empirically

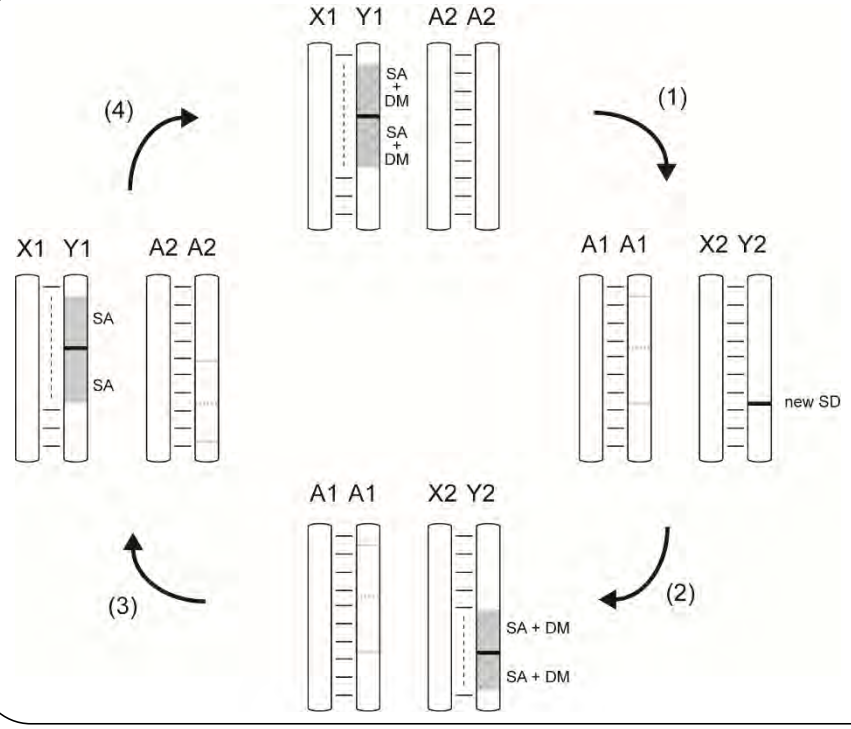


FIGURE 2: Hot potato model of sex-chromosome transitions (Blaser et al. 2014). Genetic load induces turnovers towards a new sex chromosome (1), which in turn specializes (through recombination arrest and SA genes) but ineluctably accumulates deleterious mutations (2). This triggers another transition, favored towards the chromosome pair that already carries SA genes (3). This pair further self-reinforces its specialization before the next turnovers (4), and so on.

corroborates the rising idea that some chromosomes makes better sex chromosomes than others, precisely because they contain genes that can occupy the top position in the sex-determining cascade (Graves & Peichel 2010, O'Meally et al. 2012). It is hardly random that *Hyla*'s LG1 is also mostly homologous to the sex chromosomes of other unrelated anuran amphibians, as well as to the bird Z chromosome (**Appendix**). Therefore, the widely accepted view that sex-determination systems endure rapid changes in cold-blooded vertebrates may also consider that these changes may often not be random, but follow some replicate patterns. Following Volff et al.'s metaphor, the same few "ephemeral dictators" may regularly come back to power for determining sex. Interestingly, this specialization in *Hyla* seems also characterized by fine-tuned recombination rates, i.e. phylogenetic inertia of extremely low male recombination at a linkage group that is frequently used for XY sex determination. In contrast, the seemingly distinct dynamics in Asian species, where different heterogametic systems occur in closely-related species and where recombination seems restricted in both sexes is intriguing, and stresses out for extending the survey to other Asian and North-American relatives. Finding the sex-determining genes in diverged *Hyla* species as well as characterizing the sex –determining systems of lineages were LG1 is autosomal will be crucial to further understand these dynamics of biased turnovers.

The potential specialization and re-use of European *Hyla* linkage group 1 for sex evokes the "hot-potato" model proposed by Blaser et al. (2014). This model balances genetic load of sex-linked deleterious mutation with effects of autosomal sex-antagonistic genes to account for endless biased transitions towards recurrent sex chromosomes (illustrated in Figure 2). However, in *Hyla* turnovers occur on a background of XY recombination that already purges deleterious mutations on a regular basis (**Chapter VI**). Then, in our system the importance of genetic load for triggering turnovers might be negligible compared to other forces, like sex-antagonistic or sex-ratio selection, which could be theoretically explored through simulations implementing both turnovers and XY recombination models.

HOMOMORPHIC SEX CHROMOSOMES AND SPECIATION

In **Chapter X**, we showed that the sex chromosomes of *H. arborea* and *H. orientalis* are more protected from hybridizing introgression than their autosomes, and thus disproportionally affect reproductive isolation even if there are not differentiated. Since hybrid incompatibilities should be expressed in the same way throughout the genome in *Hyla*, this result implies that sex chromosomes non-randomly accumulate pre- and/or post-zygotic incompatibilities. It is of fundamental importance for speciation research as dominance effects, not at play here, are usually assumed to be the most contributing factors. In contrast, in *Hyla* the importance of sex-linked loci for reproductive isolation rather stems from the predominance of X-Y incompatibilities, and/or the non-random build up a male-expressed genes (which should evolve and diverge faster than the rest of the genome). Sex chromosomes offers opportunities for sex-antagonistic selection and lack of recombination that may attract genes coding for male-specific traits, as well as co-adapted gene complexes involved in pre-mating isolation (e.g. species recognition systems). As such, the fact that *Hyla* frogs feature specialized sex chromosomes (c.f. **Chapter VI** and the previous section) may thus feed back to their genetics of speciation. Specialized sex chromosomes are expected to accumulate SA genes during their cyclic sex-determining reigns (Blaser et al. 2014, Figure 2), in turn making them less prone to introgression?

According to Qvanström & Bailey (2009), the importance of sex chromosomes in speciation should gradually increase as speciation progresses. It will thus be interesting to similarly investigate contact zones between younger *Hyla* lineages to test for replicate patterns. Moreover, the tree frog radiation allows future comparison between species pairs differing in their sex-determining system (i.e. *H. orientalis* and *H. savignyi*, which come into contact in Asia Minor, **Chapter IV**). More generally, our results open the way to future speciation studies in other systems with homomorphic sex chromosomes, so far largely unexploited in this context.

IMPLICATIONS FOR CONSERVATION

Beyond their interests for evolutionary biology, our results also have applied implications for biogeography and conservation biology of tree frogs, some which can be more generally extended. European *Hyla* populations are threatened in many regions and countries (**Chapter I**). In **Chapter VIII** and **IX** we provided and characterized a new set of microsatellite markers suitable for future conservation genetics applications in all Western-Palearctic tree frog lineages.

The cryptic intraspecific diversity and independent phylogenetic histories of taxa that are yet not validated as full species (exposed in **Chapter IV**) claim for new taxonomic considerations, and consequently more specific

protections (Speybroeck et al. 2010). This is particularly true for *H. orientalis* (considered as a subspecies of *H. arborea*) for which we demonstrated strong reproductive isolation (**Chapter X**). Genetic work should be accompanied by descriptions of lineages candidate for specific statuses, e.g. *H. meridionalis* n.t. 1, as recently done for *H. felixarabica*.

In addition, several lessons for conservation strategies can be drawn from the range-wide phylogeographic analyses of *H. arborea* (**Chapter I**). First, our multi-locus approach allowed to compare the main methods employed to design management units, notably showing that criteria for Evolutionarily Significant Units (ESU) may miss important divergences in young diversifications. Second, we identified an overlooked glacial sub-refugium in the Balkan Peninsula (i.e. the North East coast of the Adriatic Sea) that probably sheltered cryptic diversity in other understudied vertebrates. Third, and probably of most importance, we highlighted a negative trend between refugial diversity and the degree of threats: genetically rich refugial populations raise no concern whereas most genetically depressed populations of post-glacial ranges are threatened. This pattern, which is shared among most European amphibians (Dufresnes & Perrin in press), suggest an increased fixation load and reduced adaptive potential in populations formerly bottlenecked by the founding effects of recolonization. These intrinsic lack of variability may thus interact with and magnify the effects of extrinsic pressures, like pollutants and habitat fragmentation, stressing out for the implementation of new biogeographic criteria for status assessment of regional and national Red-Lists.

APPENDIX

HOMOLOGOUS SEX CHROMOSOMES IN THREE DEEPLY-DIVERGENT ANURAN SPECIES

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HOMOLOGOUS SEX CHROMOSOMES IN THREE DEEPLY DIVERGENT ANURAN SPECIES

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Comparative genomic studies are revealing that, in sharp contrast with the strong stability found in birds and mammals, sex determination mechanisms are surprisingly labile in cold-blooded vertebrates, with frequent transitions between different pairs of sex chromosomes. It was recently suggested that, in context of this high turnover, some chromosome pairs might be more likely than others to be co-opted as sex chromosomes. Empirical support, however, is still very limited. Here we show that sex-linked markers from three highly divergent groups of anurans map to *Xenopus tropicalis* scaffold 1, a large part of which is homologous to the avian sex chromosome. Accordingly, the bird sex determination gene *DMRT1*, known to play a key role in sex differentiation across many animal lineages, is sex linked in all three groups. Our data provide strong support for the idea that some chromosome pairs are more likely than others to be co-opted as sex chromosomes because they harbor key genes from the sex determination pathway.

KEY WORDS: **Amphibian, *Bufo siculus*, convergent evolution, conserved synteny, *DMRT1*, *Hyla arborea*, *Rana temporaria*, sex chromosome turnover.**

Sex chromosomes have been a focus of evolutionary biology for a long time, but until recently, most research has focused on organisms with well-differentiated sex chromosomes, such as fruit flies, mammals, and birds (Bachtrog et al. 2011). In contrast, sex chromosomes are much less differentiated in most amphibians, reptiles, and fishes. Cold-blooded vertebrates also differ from mammals and birds in displaying a relatively high rate of transition in sex determination systems. The sex-determining locus is often found on nonhomologous chromosomes in closely related species, or even within single species (Charlesworth and Mank 2010). This diversity is at first surprising, given the strong conservation of elements of the sex determination pathway across animals (Raymond et al. 1998), but may be explained by mutations causing different genes to take over the top position in a conserved sex determining cascade (Wilkins 1995; Schartl 2004; Volff et al. 2007; Graves 2013).

Two recent reviews have suggested that some chromosomes might be more likely than others to carry the master sex determination gene, through conservation of an ancestral system of sex determination or the reuse of a small set of genes that can capture the top position in the pathway (Graves and Peichel 2010, O'Meally et al. 2012). Thus far, few empirical examples are available to support this hypothesis: among amniotes, the same chromosome is sex linked in birds, monotremes, and one lizard species, and another chromosome is sex linked in both a turtle and a lizard species (O'Meally et al. 2012). However, neither the snake nor the therian sex chromosomes are known to be sex linked in any other amniote (O'Meally et al. 2012). In fish, eight different chromosomes are sex linked among the 16 cases reviewed by Graves and Peichel (2010). In insects, no homology is evident between the sex chromosomes of Diptera, Lepidoptera, and Coleoptera (Pease and Hahn 2012).

Another aspect of homology in sex determination pertains to the master sex-determining gene itself, rather than the chromosome on which it occurs (e.g., Woram et al. 2003; Yano et al. 2013). The transcription factor *DMRT1* is a prime example of a gene involved in sex determination in deeply divergent taxa (Brunner et al. 2001; Matson and Zarkower 2012; Gamble and Zarkower 2012). *DMRT1* orthologs play key roles in male differentiation in *Drosophila* (*doublesex*) and *Caenorhabditis elegans* (*mab3*; Raymond et al. 1998). *DMRT1* is a strong candidate for the major sex-determining gene in birds (Smith et al. 2009). Its paralogs in medaka fish (*Oryzias latipes*) and African clawed frogs (*Xenopus laevis*) act as dominant determiners of maleness and femaleness, respectively (Matsuda et al. 2002; Nanda et al. 2002; Yoshimoto et al. 2008). *DMRT1* is also associated with polygenic sex determination in zebrafish (Bradley et al. 2011) and has recently been shown to be important for the maintenance of the adult male gonadal phenotype in mice (Matson et al. 2011).

To date, little evidence exists for comparisons of sex chromosomes across amphibians. A sex-determining gene (*DM-W*) has been identified only in *X. laevis* (Yoshimoto et al. 2008), and this gene, a partial duplication of *DMRT1*, is found only in a few closely related polyploid species (Bewick et al. 2011). A single chromosome is associated with sex in four species of the *Hyla arborea* group, based on several anonymous microsatellites and two markers associated with the gene *MED15* (Stöck et al. 2011a, in press). In *Rana rugosa*, four genes have been mapped to the sex chromosome by fluorescence in situ hybridization (Miura et al. 1998; Uno et al. 2008). Finally, a series of allozyme linkage studies on 17 species or populations of ranid frogs (reviewed by Miura 2007) show that sex is associated with five different chromosomes (out of 13), depending on species or population. The recent completion of the first high-quality draft assembly of an amphibian genome (*Xenopus tropicalis*; Hellsten et al. 2010; Wells et al. 2011) presents a highly useful tool for sex chromosome comparisons (e.g., Mácha et al. 2012), although DM-W is absent in this species (Yoshimoto et al. 2008; Bewick et al. 2011) and little information is available on its sex chromosome (Olmstead et al. 2010). Provided that synteny is sufficiently conserved across anurans, sex linkage of orthologous genomic regions may be identified even if different genes are sampled in each species.

Previous work on *Bufo*, *Hyla*, and *Rana* has suggested strong synteny between representative karyotypes of these three anuran families (Miura 1995). More recently, several anonymous sex-linked microsatellite markers have been identified within the *Bufo viridis*, *H. arborea*, and *Rana temporaria* species groups (Berset-Brändli et al. 2006; Berset-Brändli et al. 2008; Matsuba et al. 2008; Cano et al. 2011; Stöck et al. 2011a,b, 2013). The only characterized sex-linked gene in any of these species, *MED15* in *H. arborea* (Niculita-Hirzel et al. 2008), is located on the same scaffold as *DMRT1* in *X. tropicalis* (scaffold 1, assembly 7.1, http://xenbase.org). Here, we use a largely novel set of gene-associated molecular markers to address three questions: (1) Is the rate of chromosomal rearrangement sufficiently low in anurans that synteny is preserved between *X. tropicalis* and distantly related species? (2) If so, can we find homologies between sex chromosomes of deeply divergent taxa? (3) If so, is the candidate sex determination gene *DMRT1* involved in these homologies?

Methods

SAMPLES

Hyla arborea full-sib groups and parental DNA samples were sampled from Čižići, Croatia (six families, 20–30 offspring per family), Progar, Serbia (one family, 30 offspring), and Gefira, Greece (one family, 30 offspring). *Hyla intermedia* families were collected from Piazzogna, Switzerland (two families, 20 offspring per family; Stöck et al. 2011a). For RNA sequencing, a single male *H. arborea* was collected at Lavigny, Switzerland.

The *Bufo* family used in this study resulted from a backcross between a wild-caught *Bufo balearicus* female and a F_1-male resulting from a previous cross between a male *Bufo siculus* and a female *B. balearicus* (Colliard et al. 2010). Offspring from this backcross ($n = 48$) were previously characterized with sex-linked microsatellite markers (Stöck et al. 2013). By design, females had two *balearicus* X chromosomes, and males one *balearicus* X and one *siculus* Y chromosome.

Rana temporaria families originated from four wild populations, at Bex, Lavigny, Meitreile, and Retaud, Switzerland. Seven mating pairs were caught during spring 2011. One clutch was obtained from each couple, and offspring were raised until metamorphosis. A total of 424 offspring (40 tadpoles and 9–41 froglets per family) were characterized with 10 microsatellite markers from linkage group 2 (Rodrigues et al. in press), previously shown to be sex-linked in Fennoscandian populations (Cano et al. 2011).

MARKER DESIGN

In each species group, we identified or developed six to 16 gene-based markers with orthologs on *X. tropicalis* scaffold 1, which is 216 Mbp in length (Table 1). Markers were developed for three genes (*DMRT1*, *FGA*, and *SMARCB1*) in all groups, whereas other genes were tested in a single group. Details of marker design, primers, and PCR conditions are presented in Supplementary Materials and Methods. Briefly, we sequenced and assembled the transcriptome of a single *H. arborea* individual, from which we identified SNPs and microsatellite repeats. We used the transcriptome sequence and public *Rana* and *Xenopus* sequences to design intron-crossing primer pairs for *B. siculus* and *R. temporaria*.

Table 1. Genes tested for sex linkage in *Bufo siculus*, *Hyla arborea* or *intermedia*, and *Rana temporaria*.

Gene abbreviation	Gene name	Microsat name	*X. tropicalis* start position, scaffold 1	Zebra finch chromosome	*Bufo* sex-linked	*Hyla* sex-linked	*Rana* sex-linked
CHD1	Chromodomain helicase DNA binding protein 1		30554621	Z	Yes		
SBNO1	Strawberry notch homolog 1	BFG072	46927127	15			Yes[1]
SMARCB1	SWI/SNF-related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1		54751604	15	Yes	Yes	Yes
MED15	Mediator complex subunit 15	Ha5–22	55139383	15		Yes[2]	
NDRG2	NDRG family member 2		64207215	absent		Yes	
ARL8A	ADP-ribosylation factor-like 8A	Ha-T32	69013841	26		**No**	
CSDE1	Cold shock domain containing E1, RNA-binding	Ha-T49	74074167	26		**No**	
LOC100494802	Hypothetical protein	Ha-T41	80975486	26		**No**	
DOCK8	Dedicator of cytokinesis 8		96078164	Z		Yes	
KANK1	KN motif and ankyrin repeat domains 1 (*ANKRD15*)		96235063	Z	Yes		
DMRT1	Doublesex and mab-3 related transcription factor 1		96303907	Z	Yes	Yes	Yes
VLDLR	Very low density lipoprotein receptor		96940006	Z	Yes		
MAP1B	Microtubule-associated protein 1B		101456644	Z		Yes	
RAD23B	RAD23 homolog B	Ha-T11	105864196	Z		Yes	
REEP6	receptor accessory protein 6	BFG131	127119927	28			Yes[1]
MAU2	MAU2 chromatid cohesion factor homolog	BFG191	127776451	28			Yes[1]
CHERP	Calcium homeostasis endoplasmic reticulum protein	Ha-T45	129080135	28		Yes	
FGA	Fibrinogen alpha chain		170007636	4	Yes	Yes	Yes
MTUS1	Microtubule associated tumor suppressor 1	Ha-T51	181270654	4		Yes	
FRYL	FRY-like		184736403	4		Yes	
KIAA0232	KIAA0232	Ha-T3	195144672	4		Yes	
WDR1	WD repeat domain 1	Ha-T52	195655455	4		Yes	
CRTC1	CREB regulated transcription coactivator 1	BFG172	scaffold 6	28			Yes[1]

[1] Cano et al. (2011) and Rodrigues et al. (in press).
[2] Niculita-Hirzel et al. (2008) and Stöck et al. (2011a).

GENOTYPING AND ANALYSES

We screened all markers for heterozygous genotypes in fathers of available families. We then genotyped the mate and the offspring of these heterozygous males (see Table S1 for genotyping methods). All families had previously been genotyped at anonymous sex-linked microsatellites (C. Dufresnes unpubl. ms.; Rodrigues et al. in press; Stöck et al. 2011a,b, 2013). Finally, we performed a χ^2-test for association between paternally inherited alleles at each gene-based marker and at anonymous sex-linked microsatellites. Because nearly all of the offspring used in this study were tadpoles, for which phenotypic sex could not be determined, we did not test for associations between genotypes and phenotypic sex. When both parents of a cross were heterozygous for the same two alleles, we excluded heterozygous offspring from analysis because the paternally inherited allele could not be inferred.

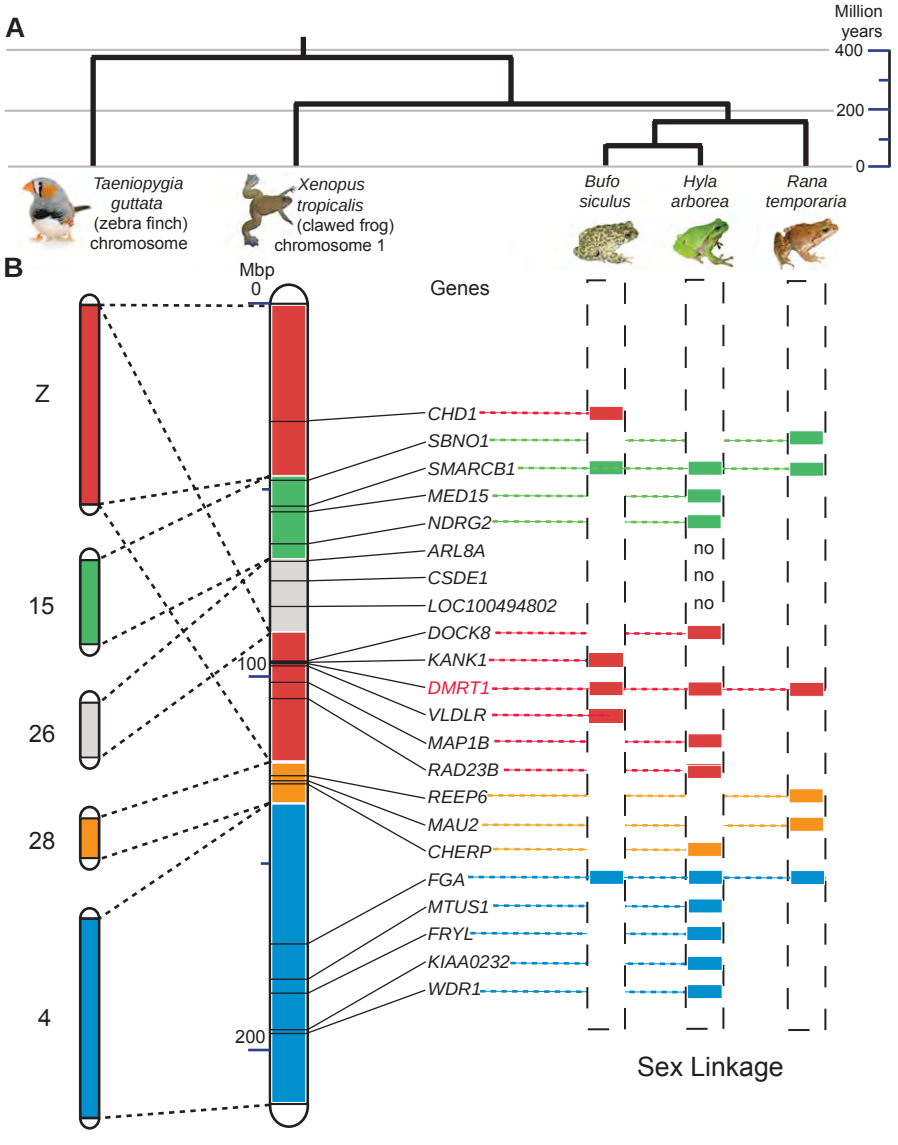

Figure 1. **(A) Relationships among *Bufo siculus*, *Hyla arborea*, *Rana temporaria*, *Xenopus tropicalis*, and *Taeniopygia guttata*, with divergence times taken from http://timetree.org. (B) Physical map of *X. tropicalis* scaffold 1, corresponding avian chromosomes, and genes tested for sex linkage in the *B. viridis*, *H. arborea*, and *R. temporaria* species groups. Sex-linked genes are distributed throughout scaffold 1, except the portion corresponding to zebra finch chromosome 26. See Supplementary Methods for determination of homology between *X. tropicalis* and zebra finch chromosomes.**

Results

For *Hyla*, we obtained 11,034,721 pairs of 100 bp Illumina reads, from which assembly and scaffolding produced 83,923 contigs with total length 45.9 Mbp and N50 700 bp. We identified 423 microsatellite repeats and 11,747 SNPs in the transcriptome. A total of 16 markers found to map to *X. tropicalis* scaffold 1 were tested for sex linkage (Table 1; Fig. 1). Thirteen of these, including *DMRT1*, were highly significantly associated with the genotypes of previously identified anonymous sex-linked markers (Table 2). Three markers found within a small range of *X. tropicalis* scaffold 1 (positions 69–81 Mb) showed no significant sex linkage.

In *Bufo* offspring, all six markers (*CHD1*, *DMRT1*, *FGA*, *KANK1*, *SMARCB1*, *VLDLR*) were perfectly associated with genotypes of the previously tested sex-linked microsatellites (Tables 1, 2; Fig. 1).

In *Rana*, finally, three of four sex-linked microsatellites with BLAST hits to the *X. tropicalis* genome aligned to scaffold 1 (BFG072, BFG131, BFG191; genes *SBNO1*, *REEP6*, *MAU2*) and one to scaffold 6 (BFG172, gene *CRTC1*). We found highly significant associations between genotypes of sex-linked microsatellites and genotypes of SNPs in *DMRT1*, *FGA*, and *SMARCB1* (Table 2).

Table 2. Number of families and offspring genotyped for each gene-based marker. All markers in *Bufo siculus* and *Rana temporaria*, and all but three markers (in bold) in *Hyla arborea/intermedia*, showed highly significant associations with sex-linked microsatellite genotypes. Column *r* denotes frequency of observed recombination between each marker and the anonymous sex-linked microsatellites.

Species	Gene	No. families	No. offspring	χ^2, 1 df	*P*-value	*r*
B. siculus	*CHD1*	1	48	44.0	3.3e−11	0
B. siculus	*DMRT1*	1	48	44.0	3.3e−11	0
B. siculus	*FGA*	1	46	42.0	9.3e−11	0
B. siculus	*KANK1*	1	48	44.0	3.3e−11	0
B. siculus	*SMARCB1*	1	48	44.0	3.3e−11	0
B. siculus	*VLDLR*	1	46	42.0	9.0e−11	0
H. arborea	*ARL8A*	1	30	3.23	**0.072**	>0.27
H. arborea	*CHERP*	1	30	26.1	3.3e−07	0
H. arborea	*CSDE1*	1	30	0.078	**0.78**	0.5
H. arborea	*DMRT1*	3	57	53.1	3.2e−13	0
H. arborea	*DOCK8*	3	56	48.9	2.7e−12	0
H. arborea	*FRYL*	3	41	33.2	8.3e−09	0.017
H. arborea	*KIAA0232*	3	85	81.0	<2.2e−16	0
H. arborea	*LOC100494802*	2	41	1.57	**0.21**	>0.39
H. arborea	*MAP1B*	3	57	52.5	4.4e−13	0
H. arborea	*MTUS1*	2	60	56.1	7.0e−14	0
H. arborea	*NDRG2*	5	96	92.0	<2.2e−16	0
H. arborea	*RAD23B*	2	56	52.1	5.4e−13	0
H. arborea	*WDR1*	2	60	56.1	7.0e−14	0
H. intermedia	*FGA*	2	16	12.3	4.7e−04	0
H. intermedia	*SMARCB1*	3	51	49.0	7.0e−12	0
R. temporaria	*DMRT1*	3	117	101.5	<2.2e−16	0.026
R. temporaria	*FGA*	1	41	37.0	1.2e−09	0
R. temporaria	*SMARCB1*	1	63	51.6	6.9e−13	0.032

Discussion

Our results show extensively conserved synteny across four anuran families (Pipidae, Ranidae, Hylidae, Bufonidae), representing approximately 210 million years of independent evolution (Fig. 1; http://timetree.org). With few exceptions, all markers tested in this study belong to the same linkage group in representatives from all four families. Exceptions include one gene (*CRTC1*) from the same linkage group in *R. temporaria* that maps to scaffold 6 of *X. tropicalis*. In mammalian and avian genome sequences, however, this gene is closely linked to several genes with orthologs on *X. tropicalis* scaffold 1, suggesting that *CRTC1* has been translocated from chromosomes 1 to 6 in a *Xenopus*-specific rearrangement. Similarly, the absence of sex linkage in *H. arborea* for three genes from a 12 Mb region of scaffold 1 (Fig. 1) likely results from a chromosomal rearrangement.

This chromosome turns out to be sex-linked in representatives of three of these families. To our knowledge, this is the first study to document homologous sex chromosomes across multiple amphibian families. Although we cannot fully exclude the possibility that species from the *B. viridis*, *H. arborea*, and *R. temporaria* groups retain an ancestral amphibian sex chromosome pair that remained homomorphic over more than 160 million years, we find it more plausible that this chromosome has more recently evolved sex linkage independently in these three groups. Sex chromosome turnover is known to be high in amphibians (Evans et al. 2012), and transitions have already been documented in Bufonidae (Stöck et al. 2011b) and Ranidae (Miura 2007). Within the genus *Rana*, sex chromosome transitions have occurred multiple times, and chromosome 1 (corresponding to *X. tropicalis* scaffold 1) has been co-opted as the sex chromosome in at least four other species (Miura 2007). Furthermore, differences in sex determination systems among conspecific populations have been documented in at least six cases including *R. temporaria* (Miura 2007; Cano et al. 2011; Rodrigues et al. in press), suggesting a high rate of turnover in this family. Broader sampling, including additional bufonid, hylid, and ranid species as well as representatives of other anuran families, will be necessary to assess the prevalence and rates of transitions of sex linkage of this and other chromosomes.

What feature might predispose this genomic region to repeatedly evolve sex linkage both in amniotes (O'Meally et al. 2012) and in amphibians? The presence of *DMRT1* might be more than a coincidence. This gene appears involved in the male differentiation pathway throughout the whole animal kingdom,

from flies and nematodes to mammals. *DMRT1* or its paralogs determine sex in birds, medaka fish, and African clawed frogs, making it an appealing candidate gene for sex determination in species in which it is sex-linked. Testing if *DMRT1* is the master sex-determining gene in *B. siculus*, *H. arborea*, and *R. temporaria* is a promising avenue for future research. Similarly, the other chromosomes (e.g., 2, 3, 4, and 7 in ranids; Miura 2007) that appear predisposed to capture the sex determination function might harbor other important genes (such as *SOX3* and *AR*; Uno et al. 2008; Oshima et al. 2009) that are known to modulate the expression of sex and participate in the sex determination pathway.

If frequent sex chromosome turnovers are biased toward certain chromosomes, this bias could become a self-reinforcing evolutionary process. Genes with sex-biased expression accumulate disproportionately on sex chromosomes (Rice 1984; Vicoso and Charlesworth 2006; Mank 2009; Bellott et al. 2010), although the rate of gene translocation among chromosomes is low. If a chromosome has often been sex-linked in the past, it may have accumulated genes likely to be involved in sexually antagonistic effects, which could in turn make it more likely to recapture the role of sex chromosome in a turnover event (van Doorn and Kirkpatrick 2007). Importantly, the buildup of deleterious mutations on a non-recombining Y chromosome can trigger a sex-chromosome turnover, where the degenerated Y is lost and replaced by a new male-determining mutation arising on a different chromosome. Simulations show that this process can occur even when counteracted by sexually antagonistic selection (Blaser et al. 2013). This could lead to cyclical sex chromosome turnovers among a limited set of chromosomes with high potential for sexual antagonism. Recombination rate evolution may also predispose turnovers toward chromosomes that have been sex-linked in the past. Five linkage groups in the *R. temporaria* genetic map exhibit reduced recombination in males, and sex linkage has been demonstrated for two of these in different populations (Cano et al. 2011; N. Rodrigues, unpubl. data). Future research should determine whether these five linkage groups correspond to the five chromosomes that are sex-linked in various *Rana* species (Miura 2007), which would show an association between sex-specific recombination rate and propensity to capture the role of sex determination.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Document S1. Supplementary Materials and Methods.

Table S1. Primers, PCR conditions, and restriction enzymes for markers tested.

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MANUSCRIPTS SUBMITTED

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ORAL CONTRIBUTIONS

Dufresnes C, Wassef J, Ghali K, Brelsford A, Stöck M, Lymberakis P, Crnobrnja-Isailovic J, Perrin N. Conservation phylogeography: does historical diversity contribute to regional vulnerability in European tree frogs (*Hyla arborea*)? ***Biology 14* – 2014 – Geneva, Switzerland.**

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Dufresnes C, Wassef J, Ghali K, Brelsford A, Stöck M, Lymberakis P, Crnobrnja-Isailovic J, Perrin N. Late Quaternary fine-scale phylogeography in Europe: Post-glacial recolonization, cryptic genetic structure, and diversity hotspots in the European tree frog (*Hyla arborea*). ***7th World Congress of Herpetology* – 2012 – Vancouver, Canada.**

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