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A molecular phylogeography approach to biological invasions of the New World by parthenogenetic Thiarid snails

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Abstract

The parthenogenetic snail *Melanoides tuberculata*, present in tropical fresh waters of most of the Old World before 1950, has now invaded the Neotropical area. The phylogeography of this snail was studied to evaluate the pathways and number of such invasions. Because of parthenogenetic reproduction, individuals are structured into genetical clones. Within populations from both the original and invaded areas, several morphologically distinct clones (referred to as morphs) often coexist but the amount of genetic divergence among morphs is unknown. Individuals from 27 morphs and 40 populations world-wide were sequenced at two mitochondrial genes (12S and 16S). Our phylogenetic reconstruction suggests that (i) most of the morphological variation observed in the New World predates invasion, (ii) at least six independent introductions have occurred, and (iii) invasive clones are found throughout most of the phylogenetic tree and do not come from a particular region of the area of origin. Two ideas are discussed in the light of these results. The first lies with the specificities of parthenogenesis in an invasion context. While in sexual species, independently introduced populations eventually merge into a single invasive population, in a parthenogenetic species independently introduced clones have distinct invasion dynamics and possibly exclude each other. Second, although repeated invasions in *Melanoides* may have an impact on indigenous molluscan faunas, their most likely effect is the world-wide homogenization of the invasive taxon itself.

Keywords: biological invasions, mtDNA sequences, Thiarid molluscs, phylogeography, *Melanoides tuberculata*, homogenization

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Introduction

During the last century, humans have caused an unprecedented redistribution of many organisms, including plants and animals. Indeed, agriculture and trade exchange have broken down many natural dispersal barriers (Kolar & Lodge 2001). For instance, during the 1994–99 period, American Customs confiscated 4900 mollusc species belonging to 197 different genera from almost 100 countries, in horticultural products, shipping containers, aquarium supplies and other items entering the United States (Robinson 1999). Biological invasions constitute one of the most serious threats to biodiversity (Everett 2000).

One of the main conclusions that has emerged from invasion studies is that the successful establishment of an exotic species is quite improbable and that many failed attempts occur before a successful invasion develops (Williamson 1996). This poses the problem of the predictability of invasions: if they essentially depend on a chain of improbable circumstances and unpredictable chance events then the search for repeatable patterns and general

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'laws of invasion' may be very demanding. On the other hand, the repeated occurrence of invasion under a fixed set of conditions (e.g. along a certain pathway, or by certain types of species) would suggest that such laws exist and are worth studying. Classically, invasions are viewed as unpredictable because they are supposed to rely on one or a few precise episodes during which part of the area of origin happens to be connected to a new, favourable area. For example, studies of spatial spread usually assume that the distribution of an invading species reflects expansion from a single founding event, or at least that repeated introductions can be neglected at a certain scale of time and space (Andow *et al*. 1990). This assumption can be tested using genetic markers to identify the pathways of invasions and to count the number of introductions (see, e.g. Meusnier *et al*. 2001; Downie 2002; Stepien *et al*. 2002): if a single event of colonization occurs, the invading genotypes should come from a particular region, and the overall diversity in the invaded area should be substantially less than that found in the area of origin. Several studies support this view (Schierenbeck *et al*. 1995; Tsutsui *et al*. 2000; Downie 2002). In clonal species, a drastic loss of variation is possible because a single clone can invade an extensive area (e.g. Amsellem *et al*. 2000). Drastic decrease of genetic diversity has also been documented in invasive molluscs. For instance, the invasion of the central East coast of Madagascar by *Biomphalaria pfeifferi* is very recent and is the result of only two microsatellite genotypes (Charbonnel *et al*. 2002). Similarly, the invasion of the Bolivian Altiplano by *Lymnaea truncatula* corresponds to the introduction of a single microsatellite genotype (Meunier *et al*. 2001). However, invasive populations are not always so uniform, and some invasion events are not followed by a reduction in genetic diversity (Bucciarelli *et al*. 2002; Stepien *et al*. 2002).

The freshwater snail *Melanoides tuberculata* is an interesting model for tracing the genealogy of invaders and comparing levels of genetic divergence in native and introduced areas. This tropical species essentially reproduces clonally (Jacob 1957), although some males sporadically appear in natural populations (Livshits *et al*. 1984; Samadi *et al*. 1999). It had been originally described from India (Müller 1774). However, determining its distributional area prior to the increase in human trade (its main vector) a few thousands years ago remains problematic. Plio-Pleistocene fossils have been found in Eastern Africa (Williamson 1981). Glaubrecht (1996), based on a review of the available chorological and fossil evidence, suggests that *Melanoides* colonized Eastern Africa during the Miocene from the Oriental region via land bridges forming in the area of the Arabian Peninsula. Early description of its distributional area included the intertropical belt of the Old World, from Africa to southeastern Asia (Pilsbry & Bequaert 1927; reviewed and discussed in Glaubrecht 1996). It has since then invaded the whole pantropical area, mainly as a result of the trade of aquarium plants (Madsen & Frandsen 1989; Glaubrecht 2000). It was first mentioned in America (Texas) in 1964 (Murray 1964), and this was followed by a rapid expansion. Its current New World distribution ranges from northern Argentina to Florida (Murray 1971; Pointier 1999; Quintana *et al*. 2000). Its expansion in the Australian and Pacific areas is more poorly documented. Riech (1937) recorded it in the Pacific islands from 1937 onwards, but its absence is not certain before this date. Glaubrecht (2000) suggested that *Melanoides*, contrary to earlier assumptions, is not autochthonous in Australia where it was introduced only recently. We will here consider that (i) the New World is the recently invaded area, and (ii) the Old World (Africa and Asia) constitutes the assumed area of origin, as these continents were occupied before 1950. We exclude the Pacific area, whose status is uncertain(Fig. 1). Our main objective is therefore to compare the genetic diversity between the New World and the Old World, to evaluate the number of introductions and putative loss of diversity.

A further important aspect with regard to invasions in *M. tuberculata* is morphological variation. This species exhibits a wide variation of shell ornamentation which allows the definition of discrete morphs (Pointier 1989; Pointier *et al*. 1993; Samadi *et al*. 1999). This variation is heritable and persists through several generations in the laboratory. It has also been shown that each morph essentially corresponds to a single microsatellite genotype, demonstrating that morphs are parthenogenetic lines (Samadi *et al*. 1999). Sixteen different morphs have been observed in different parts of the invaded area (Samadi *et al*. 1999; Pointier 2001), most of which have never been mentioned elsewhere. Unfortunately, most historical records do not provide sufficient detail to identify morphs, and identification is therefore limited to the species name (*Melanoides tuberculata*). Hence, the morphs found in the invaded area could well have stayed unreported elsewhere. The phylogenetic relationships among morphs remain unknown, and a second goal of our study is to fill this gap.

A further goal is to determine whether morph diversity was created after introduction in the invaded area, or whether it results from multiple introductions. There are arguments in favour of both hypotheses. Although parthenogenesis promotes genetic uniformity in the short-term, new morphs can appear as the result of sporadic changes in ploidy and/or rare events of sexual reproduction. At least one such episode has been documented in the recently invaded island of Martinique (West Indies). Two new morphs had been detected in the mid-1990s and microsatellite genotyping suggests that both are products of hybridization between two pre-existing morphs (Samadi *et al*. 1999). On the other hand, two other species of Thiaridae (*Melanoides amabilis* and *Tarebia granifera*, Pointier 1999) have recently invaded some parts of the New World from Asia.

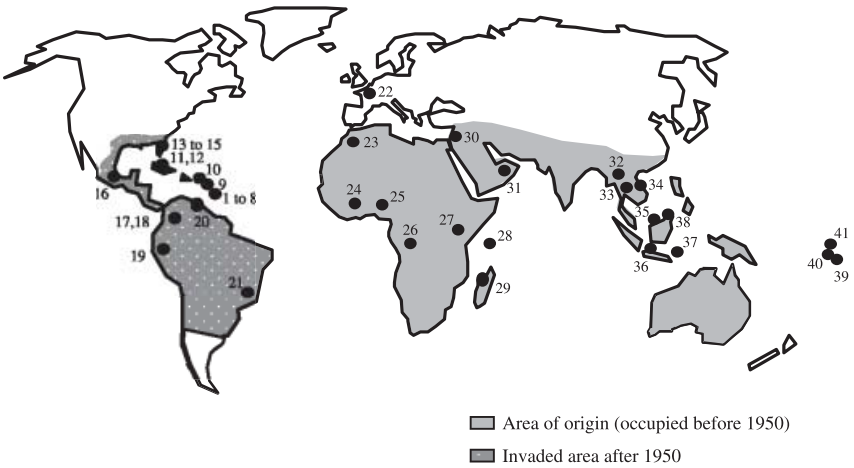

Fig. 1 Location of the populations sampled. Numbers refer to those given in Table 1. The invaded area is America, and the assumed area of origin is the remaining parts of the world except the Polynesian area, whose status is uncertain (see text and Glaubrecht 2000 for details). This map was extrapolated from discrete presence data, and is based on maps in the literature (e.g. Brown 1994), extended using many recently published observations. It is conservative in the sense that the nonshaded area does not contain known sites harbouring natural populations of *Melanoides tuberculata*. On the other hand, some places within the coloured areas may lack *M. tuberculata* because they do not contain suitable habitats. For instance, *Melanoides* is not present throughout desert parts of Australia or Africa. The distribution also suffers from some degree of taxonomic uncertainty. For example, *M. tuberculata* (according to Brown 1994), is absent from most of the Congo and northern Zambezi drainages, where it is replaced by *M. anamola* and *M. victoriae*, respectively. However, Brown mentioned that these species could simply be allopatric forms of *M. tuberculata*.

This suggests that recurrent invasion of *M. tuberculata* morphs is possible.

The present study analyses the distribution of mitochondrial DNA sequences in a world-wide sample of *M. tuberculata*. Phylogenetic reconstructions are used to address several questions about the invasion of the New World by this species. (i) Did most morphs found in the invaded area appear *in situ* from a single common ancestor or were they already differentiated in the area of origin? (ii) If divergence predates invasion, did all the morphs come from a single region or do we have to postulate several independent introductions from different parts of the area of origin? (iii) Should *M. tuberculata* be considered as a single invading taxon?

Materials and methods

Biological model and sampling

Melanoides tuberculata is a tropical freshwater gastropod (Caenogastropoda, Cerithioidea, Thiaridae) which recently invaded the whole intertropical belt (see Introduction and Fig. 1). This invasion is generally known from anecdotal records, but it has been thoroughly studied in the French West Indies (Pointier *et al*. 1993). *Melanoides tuberculata* is a cosmopolitan, tropical species able to colonize both permanent ponds and rivers. It is also very resistant to pollution and can occupy canals and sewers in urban environments (Dudgeon 1989). Cytological studies have indicated that *M. tuberculata* reproduces through obligate apomixis and lines exhibit various levels of ploidy (Jacob 1957, 1958). However, males have been detected sporadically in natural populations (Livshits *et al*. 1984; Heller & Farstay 1990; Samadi *et al*. 1999) and meiosis has been cytologically observed in their germ cells (Jacob 1958). Studies based on allozymic (Livshits *et al*. 1984) and microsatellite (Samadi *et al*. 1999) markers have suggested that sexual reproduction occurs sporadically. *Melanoides amabilis*, which has been described as a distinct species of *Melanoides* originating from Indonesia (Reeve 1860), recently invaded the Martinique island in the West Indies (Pointier 2001). It was included in the current analysis to clarify its systematic status relative to *M. tuberculata*. *Tarebia granifera*, another Asian thiarid snail introduced in the New World (Chaniotis *et al*. 1980; Pointier *et al*. 1998), served as outgroup in our analyses. Sampling was performed at the scale of the current distribution area of *M. tuberculata*, including the area of origin and invaded areas (Fig. 1; Table 1). We also sampled individuals from both *M. amabilis* and *T. granifera* (Table 1).

Morphs studied and morphological characterization

As mentioned previously, populations of *M. tuberculata* are composed of parthenogenetic lines that can be identified morphologically (morphs). Individuals of the same morph have the same microsatellite genotype (Samadi *et al*. 1999) and they were expected to exhibit similar haplotypes. We therefore analysed at least one individual per morph and population, which should be representative of the available

Table 1 Origin of samples of the three species studied

N	Species	Region	Locality	Morph	N_{si}	Year	Collector
1	*Melanoides tuberculata*	Caribbean	Martinique, FWI	PAP*	4	2000	JPP
2	*Melanoides tuberculata*	Caribbean	Martinique, FWI	MAD*	2	2000	JPP
3	*Melanoides tuberculata*	Caribbean	Martinique, FWI	FAL*	3	1999	JPP
4	*Melanoides tuberculata*	Caribbean	Martinique, FWI	PDC*	3	1999	JPP
5	*Melanoides tuberculata*	Caribbean	Martinique, FWI	CPF*	4	1999	JPP
6	*Melanoides tuberculata*	Caribbean	Martinique, FWI	FDF*	3	1999	JPP
7	*Melanoides amabilis*	Caribbean	Martinique, FWI	—	2	2000	JPP
8	*Tarebia granifera*	Caribbean	Martinique, FWI	—	2	2000	JPP
9	*Melanoides tuberculata*	Caribbean	Guadeloupe, FWI	PAP*	1	1998	JPP
10	*Melanoides tuberculata*	Caribbean	Montserrat, WI	MAD*	1	2001	MS
11	*Melanoides tuberculata*	Caribbean	Batabano, Cuba	MAD*	1	2002	JPP
12	*Tarebia granifera*	Caribbean	Isla de la Juventud, Cuba	—	1	2002	JPP
13	*Melanoides tuberculata*	America	Florida, USA	BCI	1	2000	SGJ
14	*Melanoides tuberculata*	America	Florida, USA	BCI	1	2000	SGJ
15	*Melanoides tuberculata*	America	Florida, USA	BCI	1	2000	SGJ
16	*Melanoides tuberculata*	America	Temascal, Mexico	VEL*	2	1993	DA
17	*Melanoides tuberculata*	America	Nachirelo, Colombia	MAD*	1	1999	LEV
18	*Melanoides tuberculata*	America	San Jeronimo, Colombia	COL	1	1999	LEV
19	*Melanoides tuberculata*	America	Tumbes, Peru	TUM	1	1994	JA
20	*Melanoides tuberculata*	America	Choroni, Venezuela	CHO	1	2001	PD
21	*Melanoides tuberculata*	America	Sumidouro, Brazil	SUM	2	1993	CC
22	*Melanoides tuberculata*	Europe	Aquarium shop in Douai, France	FRA	1	1999	PD
23	*Melanoides tuberculata*	Africa	Figuig, Morocco	MOF*	2	1993	HL
24	*Melanoides tuberculata*	Africa	Bouaké, Ivory Coast	BOU	1	1995	JPP
25	*Melanoides tuberculata*	Africa	Ede, Nigeria	ND	1	2000	MG
26	*Melanoides tuberculata*	Africa	Kinshasa, Zaire	ZAK*	2	1994	LAT
27	*Melanoides tuberculata*	Africa	Kisumu, Kenya	KIS	1	2000	MG
28	*Melanoides tuberculata*	Africa	Seychelles	ND	1	2000	MG
29	*Melanoides tuberculata*	Africa	Irondro, Madagascar	IRO	1	1999	NC
30	*Melanoides tuberculata*	Middle-East	Ilan, Israel	ISR*	1	1993	JH
31	*Melanoides tuberculata*	Middle-East	Afilayia, Oman	OMW*	2	2000	HM
32	*Melanoides tuberculata*	Asia	Chiang-Mai, Thailand	CHM	1	1999	BD
33	*Melanoides tuberculata*	Asia	Bangkok, Thailand	BAN	2	1998	BD
34	*Melanoides tuberculata*	Asia	Hoi Han, Vietnam	HOI	1	2001	JPP
35	*Melanoides tuberculata*	Asia	Seria, Brunei	BCI	1	2000	MH
36	*Melanoides tuberculata*	Asia	Bogor, Indonesia	BCI	2	1999	BD
37	*Melanoides tuberculata*	Asia	Lombok, Indonesia	ND	1	2000	MG
38	*Melanoides tuberculata*	Asia	Brumas-Tawan, Indonesia	BTA	1	1999	BD
39	*Tarebia granifera*	Polynesia	Tahiti, French Polynesia	—	1	1998	RG
40	*Melanoides tuberculata*	Polynesia	Moorea, French Polynesia	MOO	1	1999	RG
41	*Melanoides tuberculata*	Polynesia	Huahine, French Polynesia	MOO	1	1998	RG

The table gives the population number used in Fig. 1, the region and locality of sampling (FWI — French West Indies, WI — West Indies), the morph [*indicates that they were characterized by Pointier (1989) and Samadi *et al*. (1999) and ND that they were not determined], the number of individuals sequenced (N_{si}), the year of sampling and the name of collectors. Codes for collectors: BD, B. Delay; CC, C. Coelho; DA, D. Amaya-Huerta; HL, H. Laamrani; HM, H. Moné; JA, J. Arenas; JH, J. Heller; JPP, J.-P. Pointier; LAT, L.A. Tchuem-Tchuenté; LEV, L.E. Velásquez; MG, M. Glaubrecht; MH, M. Hossaert; MS, M. Stevens; NC, N. Charbonnel; PD, P. David; RG, R. Galzin; SGJ, S.G. Johnson. The symbol — corresponds to species presenting no distinct morphs (*M. amabilis* and *T. granifera*).

genetic diversity per region. Pointier (1989) provided the first description of morphological variation in *M. tuberculata* studying populations from the French West Indies. Individuals invariably belonged to one of four very distinct morphs with no intermediate forms, and offspring exhibited the same morph as their mother. All populations we have sampled since then have shown the same pattern. Morph identification is based on shape, sculptures and, most importantly, colour ornaments of the shell. Pointier (1989) and Samadi *et al*. (1999) have described 16 morphs from both the invaded and the assumed original areas. Each has been given a three-letter name, from the initials of the locality where they were first found. Eleven of these 16 morphs are studied here. Additional populations were

sampled adding 15 uncharacterized morphs to the previous list (Table 1). Only one of the morphs studied (ZAK) has been found in both the invaded area and in the Old World. Genetic analysis using microsatellite markers confirmed that individuals from both areas are identical (Samadi *et al*. 1999). Some of the other morphs have been found in several distant localities either within the invaded area, or within the area of origin. CPF and FDF constitute special cases. They were detected in Martinique for the first time in 1993 and 1997, respectively. Genetic analysis suggested that these morphs are locally produced hybrids between pre-existing morphs (CPF = PAP × FAL and FDF = PDC × FAL; Samadi *et al*. 1999). Hybrids then reproduce asexually and form new parthenogenetic lines. Samadi *et al*. (1999) have suggested that, as for many parthenogenetic animals (Turgeon & Hebert 1995), these hybridizations increase the ploidy level because unreduced gametes are involved. In *M. tuberculata*, hybrid genotypes display all the bands typical of the FAL genotype (for the two hybrids) and a subset of the PAP bands (for CPF), or of the PDC bands (for FDF). Therefore fertilization probably occurred between FAL unreduced gametes and PAP or PDC reduced gametes.

Our morphological analysis was modified from the method established by Samadi *et al*. (1999), and the morphological parameters used in this study (14 traits) are given in the Appendix. This morphological analysis was conducted on all individuals used in the genetic analysis except for three samples in which shells were too degraded for proper morph characterization (Table 1). We quantified differences among morphs to evaluate whether mitochondrial clades could be recognized morphologically. Shells were cleaned using bleach before analysis and scoring was performed independently by two observers. A morph was defined as a set of individuals (or populations) that were identical for the 14 morphological traits used. For each trait, a pairwise distance was calculated between morphs (0 when traits were identical, 1 otherwise), and the overall distance was the sum over the 14 traits studied (range 1–14). The correlation between pairwise morphological and genetic distances was evaluated using the Mantel permutation procedure (Mantel 1967). Pairs with identical morphology and sequences were excluded. We used the Tamura & Nei (1993) genetic distance measure.

DNA extraction and molecular characterization

The samples were manually collected, and individuals were killed by immersion (45 s) in 70 °C water. This ensured that individuals did not retract too deeply into their shell, which would prevent further penetration of tissues by alcohol. They were then stored in 96% ethanol. Total genomic DNA was extracted from foot muscle using a 'phenol–chloroform' protocol adapted for molluscs (Jarne *et al*. 1990). The polymerase chain reaction (PCR) was used to amplify two fragments of the mitochondrial DNA [mtDNA; 297 base pairs (bp) of the 16S rRNA and 262 bp of the 12S rRNA]. Conserved primers (Palumbi 1996) were used to obtain amplification products for some individuals, and these products were sequenced. This allowed us to define two pairs of primers that were subsequently used for all individuals. These primers were 16SF (5′-TAGCATGAATGGTCTGACGAAAGC-3′) and 16SR (5′-AAGGAGATTATGCTGTTATCCC-3′) for the 16S rRNA fragment, and 12SF (5′-AACTCAAAGGACTTGGCGGTGC-3′) and 12SR (5′-GTTTTTTTACTTTCAAGTCCTCC-3′) for the 12S rRNA fragment.

PCR amplification was performed in a total volume of 50 μL containing 6 μL of DNA extract, 60 pmol of each primer, 5 μL of 10× reaction buffer, 1.2 mM $MgCl_2$, 480 μM of dNTP, and 1 U of *Taq* polymerase. The reaction profile was 40 cycles of denaturation at 92 °C for 20 s, primer annealing at 48 °C for 40 s and extension at 68 °C for 1 min. Amplifications were run on a PTC-100 thermocycler (MJ Research). The PCR products were purified using the Wizard PCR DNA Purification System (Promega). Sequencing was performed on both DNA strands using the big-dye terminator cycle polymerase sequencing system (Applied Biosystems Inc.), and products were run on an automated sequencer (ABI-310, Perkin Elmer). Fifty-eight *M. tuberculata*, two *M. amabilis* and three *T. granifera* individuals were sequenced. Sequences were deposited in GenBank (Accession numbers AY290769–AY290786, AY283067–AY283084).

Sequence analysis

Sequence alignment was performed using CLUSTALW version 1.8 (Thompson *et al*. 1994) and optimized by visual inspection. For the following analyses the two data sets were concatenated to increase the power of phylogenetic reconstruction. After removing the gaps present in more than 25% of sequences, the total length of the analysed matrix was 534 bp. Several classical indices of diversity were estimated (N_{mito}, the number of haplotypes, and π, the nucleotide diversity; Nei 1987) using PROSEQ version 2.9 (http://helios.bto.ed.ac.uk/evolgen/filatov/proseq.html). We also estimated *K*, the number of segregating sites. These parameters were estimated within the whole sample as well as in subsamples corresponding to large geographical regions: (i) 'Africa' included samples from Africa, the Middle-East, Madagascar and the Seychelles, (ii) 'Asia' included samples from Thailand, Indonesia, Vietnam and Brunei, (iii) 'Old world' included African (as previously defined) and Asian samples, and (iv) 'America' included samples from America, the West Indies and Cuba.

Phylogenetic reconstructions were performed using genetic distance and the neighbour-joining (NJ) method, maximum parsimony (MP) and maximum likelihood (ML)

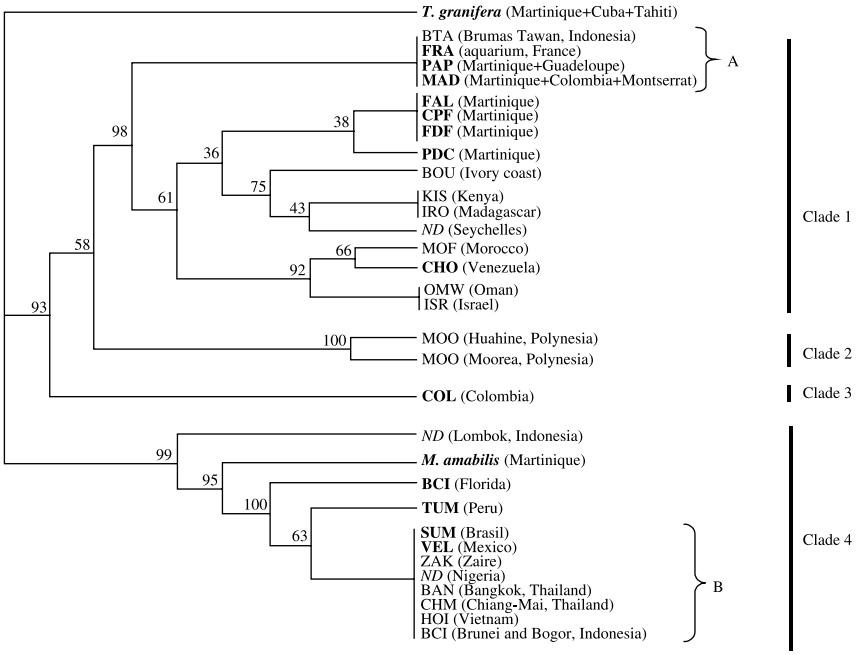

Fig. 2 One of the most parsimonious trees based on a 534-bp mitochondrial fragment (12S and 16S) using maximum parsimony to reconstruct the phylogenetic relationships among morphs of *Melanoides tuberculata*. The sampling site is indicated for each morph. *Tarebia granifera* was used as an outgroup. Haplotype A and haplotype B were represented in several morphs and localities. Samples from the invaded area are in bold type. Bootstrap scores are based on 1000 replicates. The number of individuals sampled for each morph and locality is given in Table 1.

methods implemented in PAUP* (Swofford 2000). The model of sequence evolution was evaluated using MODELTEST 3.0 (Posada & Crandall 1998). The HKY85 (Hasegawa–Kishino–Yano) + Γ_8 model provided the best fit to the data, and it was used for both NJ and ML reconstructions. The following ML model parameters were determined: base frequencies (A = 0.3494, C = 0.1528, G = 0.1845), TRatio (4.7646), and shape parameter of gamma distribution (0.1334). The ML topology was identified after an ML heuristic search conducted using the best NJ tree as starting tree, and Tree Bisection–Reconnection branch swapping. The stability of nodes was estimated by bootstrap (Felsenstein 1985), with 500 replicates of heuristic searches (NJ starting trees, ML parameters identically set to their optimal value for each replicate, and nearest neighbour interchange (NNI) branch swapping). The best MP tree was obtained by PAUP* after 100 replications of the heuristic search, and the corresponding bootstrap values were obtained with 1000 replicates of heuristic searches. Bootstrap values of the NJ tree were also obtained from 1000 replicates.

The best ML phylogeny was compared to a phylogeny forcing the monophyly of invaders with PAUP*, using the nonparametric KH test (Kishino & Hasegawa 1989), with correction for comparison of topologies defined *a posteriori* (KH–SH test; Shimodaira & Hasegawa 1999).

Results

Table 2 shows a summary of molecular diversity. One hundred and eighteen segregating sites were found (22%), and 82 were informative. Eighteen different haplotypes were identified among all samples. Individuals belonging to the same morph (Table 1) displayed exactly the same sequence, as expected for recently derived parthenogenetic lines, with two exceptions. (i) BCI was sampled in Florida and two localities in Indonesia (Java and Brunei), and the Floridian individuals differed by two substitutions from Indonesian individuals. (ii) A difference of four substitutions was found between the Moorea and Huahine sequences of the Polynesian morph MOO. Overall, this confirms that morphologically identical individuals generally have very recent common ancestors. However, the reverse was not necessarily true, because two sequences (haplotypes A and B) were found in several morphs (Figs 2 and 3) sampled in distant places around the world. As expected,

Table 2 Genetic and morphological divergence within *Melanoides* samples in different regions defined in text

Origin	N_{is}	N_{morph}	N_{mito}	π	K
Africa	12	7–9	6	0.0519 (0.0256)	61
Asia	9	5–6	3	0.0355 (0.0250)	52
Old World	21	12–15	8	0.0607 (0.0224)	69
America	35	13	9	0.0611 (0.0253)	83
Total	56	25–28	17	0.0658 (0.0298)	118

N_{is} is the number of individuals sequenced, N_{morph} is the number of morphs, N_{mito} is the number of haplotypes, π is the nucleotide diversity and K is the number of segregating sites. *Melanoides amabilis* was considered as a distinct morph of *M. tuberculata* (see Results) and the Polynesian samples were ignored because of their uncertain status (invaders or natives, see Glaubrecht 2000). A range of N_{morph} is given because of individuals whose morph could not be determined.

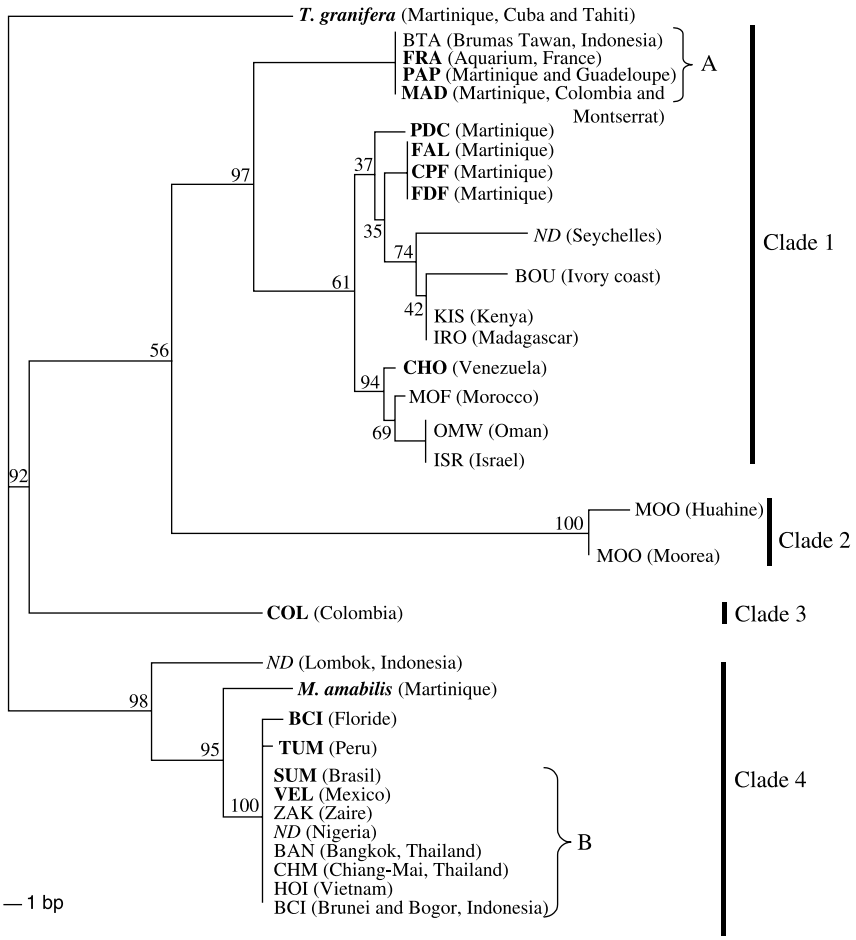

Fig. 3 Neighbour-joining tree constructed using a 534-bp mitochondrial fragment (12S and 16S). The legend is as in Fig. 2.

the hybrid morphs CPF and FDF displayed the same DNA sequence as FAL, their putative mother. The two *M. amabilis* individuals on the one hand and the three *T. granifera* individuals on the other had the same sequence.

A substantial amount of both genetical (Table 2) and morphological (Fig. 4 and Appendix) variation was found. The nucleotide diversity per site was high ($\pi = 0.0658$). Pairwise morphological distances between morphs ranged from 2 to 14 (over 14 morphological traits, with a mean of 8.7 (SD = 2.3). This mean morphological distance was positively (although weakly) correlated with the pairwise genetic distance (Mantel test, $r = 0.124$, $P = 0.03$). However, closely related morphs, or even morphs with completely identical sequences, could display strikingly different shell features (compare for instance VEL and HOI with identical DNA sequences in Fig. 4).

The MP and NJ trees are given in Figs 2 and 3, respectively. They show minor differences, mainly for terminal clades with low bootstrap values. The ML tree generated using the HKY model was not significantly different from the MP and NJ topologies ($\Delta \mathrm{Ln}L = 3.37476$, $P = 0.257$ and $\Delta \mathrm{Ln}L = 5.16758$, $P = 0.145$). Four major clades were supported by high bootstrap values in all trees. Clade 1 included seven invading morphs from the Caribbean region (West Indies and Venezuela), as well as seven morphs from Africa distributed in one subclade from Central Africa, Madagascar and the Indian Ocean (four morphs) and one subclade from Maghreb and the Middle East (three morphs). One Indonesian morph and one individual from an aquarium shop in France are also found within clade 1, together with Caribbean invasive lines. Clade 2 included two haplotypes from a single Polynesian morph. The Colombian morph (clade 3) was unrelated to any sequence, whether from the invaded area or from the area of origin. Clade 4 was a large clade including five of the six morphs from southeastern Asia, together with four invading morphs from continental America (from Florida to Brazil), *M. amabilis* from Martinique, and two African morphs. The relationships among the four clades were better considered as unresolved because all nodes were quite deep. However, a high bootstrap value (93) suggested the monophyly of clades 1, 2 and 3. *M. tuberculata* constituted a paraphyletic taxon, because *M. amabilis* was solidly rooted into clade 4 (95% bootstrap value).

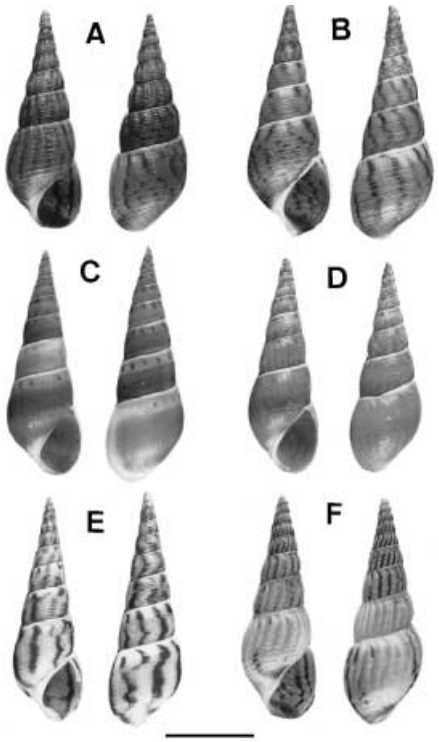

Fig. 4 Shells of *Melanoides amabilis* (C) and of several morphs of *M. tuberculata*: MAD (A), PAP (B), MOO (D), VEL (E) and HOI (F). Note that PAP and MAD on one side, VEL and HOI on the other side had the same haplotype.

The molecular diversity (Table 2) of the invaded area (New World, $\pi = 0.0611$) was high and similar to that of the assumed area of origin (Old World, $\pi = 0.0607$). The KH test strongly rejected the monophyly of New World samples of *Melanoides* spp. ($\Delta \mathrm{Ln}L = 118.8$, $P < 0.001$). The minimum number of independent invasions of the New World was estimated from well-supported groups of sequences using the MP tree (bootstrap values > 90; Fig. 2). When such groups contained populations from both the area of origin and the invaded area, this accounted for one invasion. For example, the subclade OMW, ISR, MOF and CHO of clade 1 is well-supported (bootstrap value 92% in Fig. 2), and was made of New World and Old World samples. That was considered as one invasion. Similar reasoning holds for clade 3, and one invasion event was also counted. From the MP tree, a minimum of seven invasion events were detected using this method, including (from top to bottom of Fig. 2) (A) PAP and MAD (West Indies and Colombia), (B) FAL, CPF, FDF and PDC (Martinique), (C) CHO (Venezuela), (D) COL (Colombia), (E) *M. amabilis* (Martinique), (F) BCI (Florida), and (G) TUM (Peru), VEL (Mexico), and SUM (Brazil). When we used the NJ tree (Fig. 3), we detected a minimum of six invasion events. Indeed, BCI would not necessarily constitute a separate New World invader. Six is a minimum number because some of these groups could account for several invasions (for example, PAP and MAD from group A could have been introduced independently). These invaders were not associated with a particular region of the Old World. Instead, they were distributed over the entire trees with the exception of the Polynesian clade (clade 2), and their closest relatives in the assumed area of origin were distributed in various regions of Africa (B, C) or Asia (all others except D), or were indeterminate (D).

Discussion

The phylogeography of Melanoides tuberculata *in its area of origin*

Our data support the view that *M. tuberculata* is not a homogeneous taxon, but contains several quite divergent clades, three of which seem to be typical of different parts of the world (Africa and the Middle East for clade 1, Pacific for clade 2 and southeastern Asia for clade 4) while one is an 'orphan' invading line (Colombia). The multiplicity of invasions (see below) adds to the complexity of the picture. Not only does it make it difficult to attribute each invading line to a particular region of origin without more intensive sampling (as in the case of the Colombian line), but it also introduces uncertainty as to the native status of the snails collected in the area of origin (Old World). Indeed, there seems to be little reason for invasions to occur only in America (where several distinct lines currently coexist in some places such as Martinique), and not in regions already occupied before 1950 (the so-called area of origin). Invasions in the Old World would certainly often remain cryptic because the presence of *M. tuberculata*, not of any particular morph or line, is usually reported and / or checked for. The occurrence of a single Indonesian morph (BTA) in the predominantly African and Mid-oriental clade 1, as well as that of two morphs from Zaire (ZAK) and Nigeria (ND) in the predominantly southeastern Asian clade 4, might be attributed to such cryptic invasions, all the more that they are identical in sequence to definitely invasive lines found in America (respectively, PAP and MAD from the Caribbean, and VEL and SUM from Mexico and Brazil). The case of the ZAK morph, found both in Africa (Zaire) and America (Brasil, see Samadi *et al*. 1999), is especially interesting. Its sequence identity with several southeast-Asian lines suggests an Asian origin, followed by introduction to both Africa and the New World.

However complex the recent invasion history of *Melanoides* might be, the different haplotypes observed in the invaded area emerged well before invasions began. The estimated level of molecular divergence in *M. tuberculata* is among the highest for molluscs at the within-species level (see Thomaz *et al*. 1996; Angers *et al*. 2003; for comparison). Using the upper range of the divergence rate for 16S

sequences in invertebrates (1.6–2.2% per million years (Myr), DeJong *et al*. 2001) and given the approximately 10% average divergence among clade 1 and clade 4, a minimal estimate of 4.5–6.2 Myr can be calculated for the age of their last common ancestor. These estimates should be taken with caution since the data set seems to reject a molecular clock model ($P < 0.005$).

The invasion history: how many independent introductions?

Our phylogenetic reconstructions provide several interesting results with regard to the number of independent introductions of *Melanoides* in the New World. First, invasive morphs are not monophyletic. Second, they are distributed throughout the phylogeny of *Melanoides* and exhibit molecular divergence as large as that of morphs from the area of origin. We can notice here that another freshwater mollusc, *Biomphalaria glabrata*, is known to have great variability in the New World (Campbell *et al*. 2000). However, in this case, in contrast to *Melanoides*, the great variability in the New World is attributable to the South American origin of *B. glabrata*.

Concerning *Melanoides*, the results showed that at least six independent founding events occurred in the New World. Even at a small geographical scale (e.g. the Martinique Island), several morphs, whose divergence predates the invasion of the New World, currently coexist. This is also the case in Guadeloupe, Venezuela and Colombia (Pointier 1989; this study). Unfortunately our limited samples, and the fact that the literature does not usually report the morphs, prevent us from inferring the situation in the other countries studied here (Brazil, Mexico, or Peru). The distributions of the different morphs are largely unknown, though some of them (such as MAD, observed in Cuba, most of the lesser West Indies, Venezuela and Colombia; J.P. Pointier, personal observation) already have quite large distributions. Moreover, the local morph composition can change in time, as in Martinique, where successive invasions by new morphs have been observed at a rate of one every 2 or 3 years (Pointier *et al*. 1993).

Our study documents multiple invasion events of *Melanoides* in the New World. How can such a pattern be generated? The number of introductions has been so large that the invasion of *Melanoides* has not been associated with reduced molecular and morphological variation, as often found in invasive species (Amsellem *et al*. 2000; Meunier *et al*. 2001; Meusnier *et al*. 2001; Downie 2002). A first possibility is the development of invasion corridors. This occurs when humans create a continuous connection between the invaded area and a particular region in the area of origin, providing the opportunity for distinct taxa of this region to invade the same place independently. The most well-known case is the invasion of the North American Great Lakes by Ponto-Caspian invertebrate species (Ricciardi & MacIsaac 2000), travelling in the ballast water of transoceanic ships. However, *Melanoides* invaders originate from most of the area of origin in the Old World, and corridors cannot be inferred based on the current data set. Alternatively, if human-mediated migration does not create localized corridors, a high probability of successful invasion may rely on a taxon-specific ability to invade, rather than on its presence in a particular geographical region. *Melanoides* exhibits at least three characteristics often mentioned to increase invasion ability (Lodge 1993): (i) parthenogenetic reproduction, which allows single individuals to found new populations, (ii) viviparity, which increases juvenile survival, and (iii) good adaptation to anthropomorphic habitats. The development of thiarid invasions since the 1950s is related to the worldwide increase in the trade of aquarium plants (Madsen & Frandsen 1989; see also our sample from an aquarium store, Table 1).

How many invaders?

An important aspect when evaluating the consequences of invasions is the number of invasive species at a given place (e.g. Williamson 1996; Sax *et al*. 2002). This number is relevant in sexually reproducing taxa, because each species ends up as a single invading entity whatever the number of introductions. Indeed, several independently introduced populations or genotypes of the same species can merge and form a single entity with coherent dynamics of spread and population growth. As a consequence, the spread of a sexual invasive species can be represented on a single map. This is not necessarily true in obligately or facultatively asexual taxa, which pose two specific problems. (i) Are we able to count the number of invasive species (a taxonomy problem)? (ii) Can we use information on mating systems to delimit coherent invasive entities?

Taxonomical problems arise because an invading species is not necessarily known under the same name in different parts of its distribution. For example, the American species *Physa heterostropha* is known under different names in recently invaded areas: *Physa acuta* in Europe and *Physa cubensis* in the West Indies (Dillon *et al*. 2002; L. Bousset & P. Jarne, unpublished data). In addition, an invasive species might in fact include several morphologically similar species. Both problems are found in *M. tuberculata*. Since its first description (Müller 1774), many new species names have been given, as a consequence of substantial morphological variability, and subsequently synonymized with *M. tuberculata* (Starmühlner 1976). The distinctive morphology of *M. amabilis* preserved it from being pooled with *M. tuberculata*. Our results indicate that morphology is a

relatively poor indicator of genetic divergence, as illustrated by the weak correlation between morphological and genetic distances (see Results). Also, *M. amabilis* is firmly rooted into clade 4, which is otherwise made of different *M. tuberculata* morphs. *Melanoides tuberculata* is therefore currently a paraphyletic taxon and an invalid species, at least in the phylogenetic sense (i.e. a taxon with at least one autapomorphy, Mishler & Donoghue 1982). From a taxonomic point of view, we are left with two solutions: (i) *M. amabilis* is not a valid species, and should be considered a clone of *M. tuberculata*; (ii) *M. tuberculata* should be considered as several species. In any case, counting the number of invasive species in the genus *Melanoides* is not an easy task.

We also think that, even if species could be unambiguously counted, the species level might not be appropriate to count invasive entities in parthenogenetic taxa. In an obligate parthenogen, each independently introduced clone could be considered as a distinct invasive entity, especially when clones have distinctive morphologies and life-history traits. This is indeed the case of *M. tuberculata* morphs (Pointier *et al*. 1992). However, to complicate the pattern, truly obligate asexuals are rare (Little & Hebert 1996). Documenting the frequency of biparental reproduction is crucial in this case. Frequent or regular episodes of sexual reproduction (e.g. in aphids) can lead to a situation similar to that of sexual species. In contrast, in some taxa, such episodes can be so rare that the parthenogenetic lines have largely independent population dynamics in the invaded area. In *M. tuberculata* for example, some clones occasionally crossbreed, producing hybrids that become established as new lines. Microsatellite data indeed suggested that CPF and FDF are hybrids between FAL and PAP, and FAL and PDC, respectively (Samadi *et al*. 1999). In agreement, identical haplotypes were identified in FAL, CPF and FDF, showing that FAL served as the maternal parent. A further argument is that males have regularly been observed in PAP and PDC, though never in FAL (Samadi *et al*. 1999). These hybridization events were easily detected, because hybrid morphs were found in the same sites as their parents during regular surveys conducted in Martinique since the early 1990s. However, a similar scenario cannot in principle be excluded for morphs sharing the same mtDNA sequence (e.g. within haplotype A or B). Counting invasive entities should therefore take into account potential hybridization events. However, hybridization is so rare that *Melanoides* parthenogenetic lines have essentially independent population dynamics. For example, Pointier *et al*. (1993) showed that the population dynamics of three morphs of *M. tuberculata* at several sites on Martinique (invaded area) during 6 years were not affected by biparental reproduction. Hybridization does not create substantial mixture among lines from a population dynamics point-of-view, rather it creates new lines that can themselves invade.

In conclusion, invasions of *Melanoides* should be counted on a morph-by-morph basis: no less than 14 invading morphs, including *M. amabilis*, are represented in our study (Fig. 2).

Consequences of repeated invasions

A unique feature of recent *Melanoides* invaders is their phylogenetic heterogeneity, which consists of a mixture of divergent lines coming from very different (apparently random) origins. Furthermore, new genetic diversity can be created *in situ* as a consequence of rare events of sexual reproduction (hybrid morphs). For example, in Martinique, seven invading lines of *Melanoides* currently coexist, of which five have been introduced during the last 30 years (PAP, FAL, MAD, PDC and *M. amabilis*, Pointier 1999) and two have been produced *in situ* through hybridization (FDF and CPF). However Pointier *et al*. (1993) provided evidence of competitive replacement between successively introduced morphs of *M. tuberculata*. The present diversity of invaders could be only transitory if some morphs are in the process of being excluded.

In the long term, the multiplicity of invasions and high diversity of introduced taxa could pose serious threats, because they increase the probability that one of the invaders has a large impact on the local fauna. At present, in Martinique, only one freshwater mollusc species has disappeared following the invasion of Thiarids, the planorbid *B. glabrata* (Pointier & Jourdane 2000). This invasion can be seen actually beneficial to humans because *B. glabrata* is the intermediate host of a human parasite (*Schistosoma mansoni*) which was an important problem of public health in Martinique. Long-term surveys (J.-P. Pointier, P. David, P. Jarne, unpublished data) do not indicate that other local species are currently declining. However, the native freshwater malacofauna in Martinique was limited to 13 species (Pointier 2001) and was devoid of Thiarids. We have little idea of the consequences of the introduction of Thiarids on the more diverse guilds in continental America. Finally, in the case of Thiarids, the taxa most threatened by invasions could paradoxically be the invaders themselves. Thiarids have been introduced several times in the New World from a large number of Old World sources, quite probably as a consequence of intense economic activity in the aquarium trade. This should warn us about loss of biodiversity of this taxon in its area of origin. As we have seen, this biodiversity perhaps dates back 10 Myr, and could be seriously endangered if exotic morphs can travel throughout the Old World as quickly and efficiently as they do in the New World. Repeated introductions could threaten Thiarid biodiversity in two ways: (i) through competitive elimination of local morphs by introduced ones, and (ii) through genetic homogenization by hybridization between local and introduced morphs.

Freshwater molluscs might well face homogenization, as do other freshwater guilds (Lodge *et al*. 1998; McKinney & Lockwood 1999; Rahel 2000).

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B. Facon's PhD focuses on the biology of invasions from an interdisciplinary perspective, using *Melanoides* snails as model organisms. J.-P. Pointier is regularly visiting the French Antillas, among other exotic destinations, mostly to follow invasion waves of freshwater snails. M. Glaubrecht is a systematician working on gastropods. C. Poux has substantial experience in phylogeny reconstruction. P. Jarne and P. David are malacologists, and sometimes population biologists.

Appendix

Qualitative description of morphs based on 14 shell traits. They refer to the background colour (three traits), ornaments (four traits), columellar band (two traits), general shape (two traits) and sculptures (three traits)

	Background colour			Ornaments				Columellar band		General shape		Sculptures		
Morph	IN	TI	HE	DO	SP	SO	HO	SH	SC	CO	RO	GR	RI	RD
MAD	4	1	0	1	2	2	2	3	3	2	2	2	2	3
CPF	1	1	0	2	2	2	2	1	*	2	2	3	3	2
PDC	3	2	0	1	3	1	3	3	2	2	2	1	0	*
MA	2	2	1	0	*	*	3	2	2	2	1	0	0	*
FDF	1	1	0	2	2	1	2	1	*	2	2	2	3	2
FAL	1	4	0	1	3	1	1	1	*	1	3	3	3	2
PAP	3	1	0	2	2	2	2	2	2	2	2	3	0	*
ISR	2	1	0	1	3	1	1	2	1	1	3	3	3	3
BCI	3	3	0	2	1	3	1	3	3	2	2	3	0	*
BOU	3	3	0	2	1	1	1	2	2	2	1	1	0	*
HOI	1	1	0	1	2	2	2	2	2	3	3	2	2	3
COL	2	1	0	1	2	2	1	2	2	3	2	2	3	1
BAN	2	1	1	1	3	1	3	2	3	2	3	2	0	*
TUM	1	4	0	1	1	3	1	1	*	1	2	2	0	*
OMW	2	3	0	2	2	2	1	2	1	1	3	3	0	*
MOO	3	1	0	0	*	*	3	1	*	2	3	1	0	*
VEL	2	3	0	2	0	3	2	3	2	1	2	3	0	*
SUM	1	4	0	1	1	2	1	1	*	2	2	2	3	1
MOF	1	1	0	2	1	2	1	3	1	1	2	2	3	1
ZAK	2	1	0	2	0	3	1	3	1	1	3	1	0	*
FRA	4	3	0	2	1	2	1	2	1	2	2	3	0	*
KIS	4	3	0	2	3	1	1	2	3	2	3	3	3	2
IRO	1	1	0	1	1	2	1	3	1	3	3	3	3	1
CHO	2	1	0	2	2	2	2	2	1	2	2	3	0	*
CHM	4	3	0	1	0	3	2	3	3	2	3	1	0	*
BTA	4	3	0	1	2	1	2	2	2	1	2	1	0	*

IN = intensity of the shell background colour: (1) very pale, (2) pale, (3) medium, (4) dark.
TI = background tint of the shell: (1) yellow to brown, (2) greenish, (3) orange to reddish, (4) white.
HE = heterogeneity of the background colour among different parts of a shell whorl: (0) homogeneous, (1) a distinctly darker band below the suture.
DO = overall density of colour ornaments on the whole shell, except the zone just below sutures: (0) no ornaments, (1) medium, (2) dense.
SP = type of ornaments, expressed as the proportion of spots vs. flames: (0) only flames, (1) more flames than spots, (2) more spots than flames, (3) only spots.
SO = size of the ornaments: (1) small spots or narrow flames, (2) medium, (3) large spots or wide flames.
HO = heterogeneity of ornamentation among different parts of the whorl: (1) homogeneous, (2) slightly different ornaments below suture, (3) ornaments below suture very different from the rest of the shell.
SH = presence and sharpness of a dark band on the axial edge of the aperture (columellar band): (1) absent, (2) diffuse, (3) sharp.
SC = size of the columellar band, when present: (1) narrow, (2) medium, (3) wide.
CO = conicity of the shell: (1) acute, (2) medium, (3) blunted cone.
RO = roundness of body whorls: (1) flat, (2) slightly rounded, (3) well-rounded.
GR = spiral grooves: (0) absent, (1) shallow grooves, (2) intermediate, (3) very deep grooves.
RI = density and width of axial ribs: (0) none, (1) a few narrow ribs, (2) a few large ribs, (3) many narrow ribs.
RD = depth of axial ribs when present: (1) shallow, (2) medium, (3) deep.
*indicates when the trait is absent on the studied shell.