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TECHNICAL NOTE

Y chromosome microsatellite isolation from BAC clones in the greater white-toothed shrew (*Crocidura russula*)

LORI J. LAWSON HANDLEY* and NICOLAS PERRIN
Département d'Ecologie et d'Evolution, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland

Abstract

We constructed a microsatellite library from four *Crocidura russula* Y chromosome-specific bacterial artificial chromosome (BAC) clones. Only one of eight microsatellites was male-specific, despite genome walking to obtain more flanking sequence and testing of 93 primer combinations. Potential reasons for this low success are discussed. The male-specific locus, *CRY3*, was genotyped in 90 males, including *C. russula* from across the species range and two related species. The large difference in *CRY3* allele size between eastern and western lineages supports earlier reports of high divergence between them. Despite polymorphism of *CRY3* in Morocco, only one allele was found throughout the whole of Europe, consistent with previous studies that suggest recent colonization of Europe from a small number of Moroccan founders.

Keywords: bac library, chromosome, microsatellite library, retro-elements, shrews

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There is considerable interest in using the Y chromosome in molecular ecology due to its paternal inheritance (e.g. Petit *et al*. 2002). Although Y-linked markers are routinely used in human population genetics (Jobling & Tyler-Smith 2003), their use in nonhuman populations has been limited due to difficulties in marker isolation (Petit *et al*. 2002) and low levels of nucleotide diversity (e.g. Hellborg & Ellegren 2004). A substantial sequencing effort is required to uncover informative single nucleotide polymorphisms (SNPs). In contrast, variability of Y-linked microsatellites is thought to be similar to that of autosomal ones (e.g. Kayser *et al*. 2004) and they are therefore likely to be much more informative within species.

Recent analyses of mitochondrial DNA (mtDNA) and Y and X chromosome sequences in the greater white-toothed shrew, *Crocidura russula*, indicate large divergence between western (Morocco and mainland Europe) and eastern (Tunisia and Sardinia) lineages (Brändli *et al*. 2005; Cosson *et al*. 2005). Colonization of the Iberian Peninsula from Morocco ≥ 38 thousand years ago, and rapid European population expansion, has been demonstrated using mtDNA (Brändli *et al*. 2005), but investigations using the Y chromosome were limited by low nucleotide diversity. We aimed to overcome this limitation by characterizing rapidly evolving Y-linked microsatellites.

Correspondence: Lori J. Lawson Handley, *Present address: Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK; Fax: +44 (0) 1223 333 992; Email: ljl27@cam.ac.uk

We developed a Y chromosome-specific enriched microsatellite library from a *C. russula* bacterial artificial chromosome (BAC) library. This strategy has rarely been used to obtain Y-linked microsatellites (but see Ward *et al*. 2001 and Wallner *et al*. 2004). A non-arrayed BAC library with 5× genomic coverage was constructed from *C. russula* male DNA by BIO S&T (Montreal, Canada) using pIndigoBAC vector with a *Hin*dIII cloning site (EPICENTRE). Approximately 96 000 clones containing an insert ≥ 150 kb were screened to identify Y-linked target sequences using 'pooled PCR (polymerase chain reaction)' (Liu *et al*. 2000). Briefly, a sublibrary of twelve 96-well plates was constructed, with each well containing several hundred colonies. Sublibrary plates were pooled into one 'screening plate', which was screened by PCR using two *C. russula* Y-specific primer combinations (*UTY11*, 1 kb, Hellborg & Ellegren 2003 and *sry* HMG-box, 159 bp, Matsubara *et al*. 2001; see Brändli *et al*. 2005). Positive wells were identified in the screening plate after agarose gel electrophoresis, and corresponding wells in the sublibrary were PCR-screened again. Candidate wells in the sublibrary were then plated out and screened by colony hybridization. Four positive clones, with insert size 150–200 kb were identified. BAC DNA was purified

Table 1 Locus details

Locus	Total length sequenced (bp)	Repeat	GenBank Accession nos	Homology	Homology—nonretroelements Accessions	% identity (length)	LINE/SINE
CRY1	666	$(GT)_7A(TG)_2TT(TG)_{10}$	DQ096796	UTY intron	AY918450–60	100 (69 bp)	L1MA5A
CRY2	1653	$(GT)_7A(TG)_2TT(TG)_2$	DQ096797	UTX exon	AL138744	94 (121 bp)	SOR-1 (× 2)
CRY3	523	$(GT)_7A(TG)_2TT(TG)_{10}$	DQ096798	None > 25 bp			None
CRY4	1224	Complex (CCT)	DQ096799	None > 25 bp			None
CRY5	1045	$(TG)_{14}$	DQ096800	DBY intron	AY918427–38	88 (45 bp)	SOR-1
CRY6	576	$(T)_{13}(GT)_9$	DQ096801	DBY intron	AY918427–38	89 (112 bp)	SOR-1
CRY7	816	$(TG)_9$	DQ096802	DBY intron	AY918427–38	88 (126 bp)	SOR-1
CRY8	1451	$(TG)_{19}$	DQ096803	DBY intron	AY918427–38	86 (126 bp)	SOR-1 (× 2)

Only *CRY3* is male-specific. Homologous regions were identified by searching the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/). LINE (L1MA5A) and SINE (SOR-1) repeats were identified using REPEAT MASKER (http://repeatmasker.genome.washington.edu/). See Accessions for more details.

using the Large Construct Kit (QIAGEN), and used as template DNA to construct a microsatellite library following the universal linker method of Hamilton *et al*. (1999). Hybridizations were carried out with five biotinylated probes ($(TC)_{10}$, $(TG)_{10}$, $(CTG)_8$, $(CAC)_8$ and $(CCT)_8$), and enriched products were ligated into pBluescript II SK(+) and transformed into XL-2 Blue ultracompetent cells (Stratagene) according to the manufacturer's protocols. Five hundred thirty positive colonies were PCR-screened using standard M13 forward and reverse primers. Fifty-nine colonies with insert size 300–600 bp were sequenced using BigDye Terminator chemistry on an ABI PRISM 3100 sequencer (Applied Biosystems) with M13 primers.

Eight different microsatellites were found (Table 1). One locus was a (CCT)-rich repeat, while all others were (TG) or interrupted (TG) repeats. All loci except one (*CRY3*) amplified in both males and females. For the seven nonmale-specific loci, genome walking was carried out to increase the opportunity for specific primer design. Genome walking was performed in both forward and reverse directions with the APAgene™ Genome Walking Kit (BIO S&T) using reagent concentrations and thermal profiles specified by the manufacturer, including a streptavidin-binding step to remove nonspecific amlplicons. PCR products ≥ 500 bp were gel-purified using the Geneclean® SPIN Kit (Qbiogene) and subcloned using the Zero Blunt® TOPO® PCR cloning kit with One Shot® TOP10 competent cells (Invitrogen). Colonies were PCR-screened with M13 primers, and four positive clones from each ligation reaction sequenced as before. After genome walking, 576–1653 bp were available for each locus, and primers were designed along the length of each sequence. Ninety-three different primer combinations were tested under a range of annealing temperatures and $MgCl_2$ concentrations in two male and two female samples from *C. russula russula*, *C. russula yebalensis*, *C. russula ichnusae*, *C. russula agilis*, *C. leucodon* and *C. canariensis*. Single bands of expected size were obtained for each of the seven loci; however, none of the primer combinations were male-specific and no difference in size was obtained between males and females. This analysis was not taken further since these non male-specific loci are unlikely to be suitable for standard population genetic analyses (if for instance the loci are X- and Y-linked, analysis will be complicated by copy number, mutation rate and recombination rate differences between the sex chromosomes). Note that autosomal microsatellites have previously been characterized in *C. russula* (Favre & Balloux 1997; Duarte *et al*. 2003).

The male-specific locus, *CRY3*, was amplified in 81 males from across the species range of *C. russula*, six males of *C. leucodon*, and three males of *C. canariensis* (Table 2). No PCR products were obtained with females of these species. PCR conditions were: 0.25 μM of each primer (CRY3-F2 5′–3′ AGCAGAAAGTTGCAATGG, and CRY3-R2 5′–3′ TGGGCGTGGGGTTTGGGTG), 0.2 mM dNTPs, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (QIAGEN) and *c*. 20 ng DNA in a 20 μL volume, with a thermal profile of 95 °C for 10 min, 30 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, and 72 °C for 10 min. Genotyping was performed on an ABI 377 (Applied Bioystems). Note that PCR products of similar size to those from *Crocidura* were obtained in *Sorex* shrews (*S. coronatus*, *S. granarius*, *S. antinorii* and *S. araneus*) using an annealing temperature of 55 °C, but *CRY3* was not male-specific in these species.

We found 10 alleles at *CRY3* in *Crocidura*, ranging in size from 160 to 208 bp (Table 2). First, we found a large difference in allele size (24 bp) between eastern and western lineages of *C. russula* compared to the two outgroup species (Table 2). Our results therefore support the large divergence between the two lineages, and we note that *CRY3* would be a useful diagnostic tool to investigate male gene flow across the contact zone, which is believed to exist in Algeria (Sarà & Vogel 1996). Second, we found only one

Table 2 Distribution of *CRY3* alleles in 90 males of *Crocidura russula*, *C. canarienis* and *C. leucodon*

				Alleles (bp)									
Species	Country	No. of localities	*N*	160	164	170	172	174	176	178	180	204	208
C. r. yebalensis	Morroco	6	18			1	3	1	7	5	1		
C. r. russula	Spain	2	7				7						
	Portugal	1	5				5						
	France	3	11				11						
	Switzerland	17	35				35						
	Germany	1	1				1						
C. r. agilis	Tunisia	1	3									2	1
C. r. ichnusae	Sardinia	1	1									1	
C. russula total			81			1	62	1	7	5	1	3	1
C. canariensis	Canary Islands	2	3	3									
C. leucodon	Switzerland	4	6		6								
Total			90	3	6	1	62	1	7	5	1	3	1

C. russula yebalensis and *C. russula russula* constitute the western lineage of *C. russula*, while *C. russula ichnusae* and *C. russula agilis* constitute the eastern lineage.

CRY3 allele in Europe compared to six in Morocco, even though the number of individuals and the geographic distribution of samples were much larger in the former (Table 2). This supports the recent colonization of Europe by a small number of Moroccan founders and/or selection on the Y chromosome (Brändli *et al*. 2005).

Potential reasons for our finding of only one out of eight male-specific loci are the following. (i) Our BAC clone templates were not Y-specific, although this seems unlikely since the primers used for screening were strictly male-specific, and PCR/sequencing of the BAC clones confirmed their presence. (ii) BAC clones were from the Y chromosome but included a stretch of the pseudo-autosomal region. (iii) Our BACs contain sequences with homology to other genomic regions, incorporated via retrotransposition or gene conversion. This is plausible for two reasons. First, six of the seven nonspecific loci contained retro-elements (Table 1). A LINE-1 repeat (L1MA5A) was found adjacent to *CRY1*, whereas all others were close to one or more SOR-1 (Soricidae family-specific, Borodulina & Kramerov 2001) type SINEs (Table 1). SOR-1 elements (unlike many other SINEs) are not implicitly linked to microsatellites (Borodulina & Kramerov 2001); hence, this cannot explain their association with the microsatellites found here. Second, we observed short stretches of homology with other Y-linked regions and an X-linked gametolog (Table 1) in six out of the eight microsatellite-containing clones. Note that recombinant PCR products is a possible but unlikely explanation for this result since size of PCR products from genomic DNA corresponded to those predicted from cloned sequences.

Despite our low success in obtaining male-specific sequences, we feel that this strategy still has considerable promise due to public availability of BAC libraries and the publication of conserved Y-specific primer pairs for screening (e.g. Hellborg & Ellegren 2003). The most difficult and expensive aspect of BAC library construction is arraying the clones, which is unnecessary for the procedure described here. A limiting factor might be the length of Y-specific BAC insert used as template to construct the microsatellite library. We therefore recommend screening using several genes, distantly located if possible to avoid overlap, obtain maximum amount of Y-specific template and increase the prospects of finding male-specific markers.

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