

Peles

1662 PRIMER NOTES

The number of alleles, product size and heterozygosity at each of the eight microsatellite loci are shown in Table 1. All of the examined loci showed a distinct allelic variation ranging from 2–8 alleles in the bears examined. Alleles at each locus differed by multiples of two in size. All the polymorphic loci conformed to Hardy–Weinberg expectations except for MSUT-3, which may have null alleles (Table 1). The eight loci showed relatively low allelic variations and low heterozygosities. This may be caused by a small sampling area for this species or by the population being isolated. To elucidate the cause, the genetic diversity of the present population has to be compared with that of other larger populations.

To the best of our knowledge, no original microsatellite DNA loci for this species have yet been described, although several sets of microsatellite primers have been developed for other bear species (Paetkau & Strobeck 1994; Paetkau *et al.* 1995; Taberlet *et al.* 1997), some of which may be applicable to this species. The present microsatellite loci will become a potent DNA marker to investigate genetic variations in the Asiatic black bear.

Acknowledgements

We wish to thank T. Kawahara and N. Ohnishi for their kind assistance for genetic analyses. We are also indebted to T. Shimada, Y. Segawa, and M. Yabuta for specimen management.

References

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Burke T, Rainey WE, White TJ (1992) Molecular variation and ecological problems. In: *Genes in Ecology* (eds Berry RJ, Crawford TJ, Hewitt GM), pp. 229–254. Blackwell Scientific Publications, Oxford.

Hashimoto Y, Yasutake A (1999) Seasonal changes in body weight of female Asiatic black bears under captivity. *Mammal Study*, 24, 1–6.

Horino S, Miura S (2000) Population viability analysis of a Japanese black bear population. *Population Ecology*, 42, 37–44.

Isagi T, Kanazashi T, Suzuki W, Tanaka H, Abe T (1999) Polymorphic microsatellite DNA markers for *Magnolia obovata* Thunb. and their utility in related species. *Molecular Ecology*, 8, 698–700.

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, 4, 347–354.

Paetkau D, Strobeck C (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, 3, 489–495.

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248–249.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Servheen C (1990) The Status and Conservation of the Bears of the World. *International Conference on Bear Research and Management Monograph Series*, 2, 32pp.

Taberlet P, Camarra J-J, Griffin S *et al.* (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, 6, 869–876.

The Mammalogical Society of Japan, ed. (1997) *Red List of Japanese Mammals*. (in Japanese with a list in English) Bun-ichi Sogo Shuppan, Tokyo.

Microsatellites in the hermaphroditic snail, *Lymnaea truncatula*, intermediate host of the liver fluke, *Fasciola hepatica*

S. TROUVÉ,\*† L. DEGEN,\* C. MEUNIER,‡ C. TIRARD,§ S. HURTREZ-BOUSSÈS,‡ P. DURAND,‡ J. F. GUÉGAN,‡ J. GOUDET\* and F. RENAUD‡

\*Institut Zoologie et Ecologie Animale, Université, 1015 Lausanne, Switzerland, †Laboratoire Ecologie-Evolution, UMR CNRS 5561 Biogéosciences, 6 Bd Gabriel, Université Bourgogne, 21000 Dijon, France, ‡Centre d'Etude sur le Polymorphisme des Micro-Organismes, Centre IRD Montpellier, 911 av. Agropolis, 34032 Montpellier Cedex 1, France, §Laboratoire Fonctionnement et Evolution des Systèmes Ecologiques, Université Pierre et Marie Curie, 7 quai St. Bernard, 75252 Paris Cedex 05, France

Keywords: DNA markers, genetic variability, heterozygosity, Lymnaeidae, mating system, mollusc

Received 20 February 2000; revision accepted 11 May 2000

Correspondence: S. Trouvé. Laboratoire Ecologie-Evolution, UMR CNRS 5561 Biogéosciences, 6 Bd Gabriel, Université Bourgogne, 21000 Dijon, France. Fax: 33 3 80 39 62 31; E-mail: sandrine.trouve@u-bourgogne.fr

Host-parasite interactions are strongly affected by differential gene flow in host and parasite populations. In this context, genetic markers are particularly useful to estimate population structure and heterozygosity level associated with infection. The freshwater snail, *Lymnaea truncatula* (Gastropod, Mollusc), is the main species acting as intermediate host in the life cycle of the liver fluke, *Fasciola hepatica* (Trematoda, Platyhelminth), which is responsible for important human health and veterinary problems worldwide. The mollusc is hermaphroditic and usually inhabits small temporary ponds and streams.

While isoenzymatic markers have already been developed in *L. truncatula*, a total monomorphism was encountered at 18 enzymatic loci in each of 19 populations originating from France, Portugal, Morocco and Bolivia (Jabbour-Zahab *et al.* 1997). Extinction colonization events due to the temporality of the habitat as well as a reproduction through self-fertilization could explain the low level of variability observed.

To investigate the role of mating systems and population dynamics in the genetic variability of *L. truncatula* as well as to analyse population genetic of host–parasite interactions, we developed polymorphic microsatellite markers.

A genomic library of 2174 clones was constructed and screened for (CA)<sub>10</sub> and (GA)<sub>10</sub> repeats using standard hybridization techniques (Estoup *et al.* 1993). A total of 18 positive clones were sequenced. We selected clones for which appropriate flanking sequence could be defined (i.e. nine loci: Table 1). For amplification of microsatellite loci, primers were designed using Primer 0.5 program (Lincoln & Daly 1991).

Each polymerase chain reaction (PCR) consisted of a 10.5-µL mixture containing 0.076 mM each of dCTP, dTTP,



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**Table 1** Microsatellite core sequences, primer sequences, size of cloned allele and genetic variability of microsatellite loci of the freshwater snail, *Lymnaea truncatula*.  $H_O$  and  $H_E$  are observed and expected heterozygosity, respectively

Locus*	Repeat motif	Primer sequence (5'-3')	Size (bp)	Individuals scored	No of alleles	$H_O$	$H_E$
16	(AAT) <sub>8</sub>	ATCGTGTAAAAATCGGG ATTCGGTATCTAAGATGGCAG	224	26	7	0.072	0.517
20	(CA) <sub>5</sub> (A) <sub>2</sub> (CA) <sub>3</sub>	CATCTTGACTAGCCCAG TTTGTGTGTGTGTGTGTG	121	7	4	0	0.667
21	(TA) <sub>4</sub> (TG) <sub>11</sub> TA(TG) <sub>4</sub> (T) <sub>3</sub> (GT) <sub>3</sub>	GAGATGCCCTTACACTTTTTC TGCACTAACCCCTAACTAC	112	22	5	0.033	0.443
24	(C) <sub>14</sub> (AC) <sub>7</sub>	AAACGTCACCTTTCGCATC ACTACTTGGACACTAGAG	223	24	6	0.1	0.541
29	(CT) <sub>9</sub> GCTCTTT(CT) <sub>9</sub> GT(CT) <sub>4</sub>	AATGTCCTGTCAAAGCTGG GTGTGTGATGAAAGGAGG	152	24	3	0.052	0.346
32	(CA) <sub>3</sub> TACATA(CA) <sub>7</sub>	CGTACTTTTATATACAGCCC CTCTAACCCAAACTACTC	149	7	2	0	0.333
36	(GT) <sub>7</sub> (G) <sub>11</sub>	AAGATTGGTCTTGTGAGG GGATTAAAGGAGATATACGG	190	8	3	0	0.242
37	(TC) <sub>11</sub> (T) <sub>2</sub> (TC) <sub>3</sub>	GTCCAGTCTTTGTATGTC GTTAAGTACCCAACCTCTCTC	120	7	4	0	0.667
43	(AG) <sub>8</sub>	GAGGGGGATGCAAAACAAG TGGGTGGCAATGACGTAG	105	7	2	0	0.667

\*GenBank Accession nos AF226978 to AF226986.

dGTP; 0.008 mM dATP; 0.3  $\mu$ Ci [<sup>32</sup>P]-ATP; 1.2 mM MgCl<sub>2</sub>; 1 $\times$  PCR buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl); 0.2 mg/mL BSA; 5 pmol of each primer; 0.5 U *Taq* polymerase (GIBCO BRL); = 5 ng of genomic DNA. Amplifications were performed in a PTC-100 thermocycler (MJ Research) using: an initial 3 min denaturation step at 95 °C, followed by 30 cycles of 1 min at 94 °C, 30 s at the annealing temperature of 52 °C, and 45 s at 72 °C, and then a final elongation step for 10 min at 72 °C. PCR products were denatured and separated on 6% polyacrylamide and 8 M urea sequencing gels. Clones served as size controls.

To characterize each locus we genotyped *L. truncatula* snails originating from seven populations in Switzerland. The number of individuals studied varied from 7–26 because of snail availability. The samples were studied at nine loci (Table 1). For locus 20, which presents a short microsatellite in the primer sequence, we particularly checked for repeatability following several PCR processes performed with different thermocyclers. The pattern obtained was always unambiguous and the size of the alleles was repeatable.

All the loci studied are polymorphic and the number of alleles ranges from 2–7 across the sampling area. Although this indicates a low to moderate polymorphism, this result appears interesting as the level of polymorphism obtained with microsatellite markers is much higher than the one observed with allozymes.

The mean observed heterozygosity is low ( $H_O = 0.029$ ) compared to the mean expected heterozygosity ( $H_E = 0.492$ ). This indicates a clear deficit of heterozygotes within populations. Although the population dynamic (bottlenecks) due to the temporality of the habitat might explain the low level of genetic variability (estimated by the number of alleles and heterozygosity) found in *L. truncatula*, compared with other

temperate freshwater snails (Jarne & Städler 1995 for review; Monsutti & Perrin 1999), a reproduction predominantly through selfing certainly constitute the main cause.

Cross-species amplification was tested on another *Lymnaea* from Switzerland, *L. ovata*. Among the seven primer pairs used (16, 20, 21, 24, 29, 36, 37), only two (20 and 21) successfully amplified the PCR products. Although only six individuals were screened, two alleles appeared for locus 21.

The diversity of these loci make them valuable tools for population genetic differentiation, mating system investigations as well as for host genetic analyses in relation to parasitism.

#### Acknowledgements

This work is dedicated to the memory of José Oviedo. Special thanks to J.C. Casanova, G. Dreyfuss, S. Mas-Coma, J.P. Pointier and D. Rondelaud for having kindly provided different *Lymnaea* samples. This work received the financial support from the CNRS and from the French Embassy in Switzerland. ST has benefited from a fellowship from the 'Fondation Singer-Polignac'.

#### References

- Estoup A, Solignac M, Harry M, Cornuet JM (1993) Characterisation of (GT)<sub>n</sub> and (CT)<sub>n</sub> microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. *Nucleic Acids Research*, **21**, 1427–1431.
- Jabbour-Zahab R, Pointier JP, Jourdan J *et al.* (1997) Phylogeography and genetic divergence of some lymnaeid snails, intermediate hosts of human and animal fascioliasis with special reference to lymnaeids from the Bolivian Altiplano. *Acta Tropica*, **64**, 191–203.
- Jarne P, Städler T (1995) Population genetic structure and mating system evolution in freshwater pulmonates. *Experientia*, **51**, 482–497.

Lincoln S, Daly M (1991) *Primer, Version 0.5*. Whitehead Institute for Biomedical Research, Cambridge, MA.

Monsutti A, Perrin N (1999) Dinucleotide microsatellite loci reveal a high selfing rate in the freshwater snail *Physa acuta*. *Molecular Ecology*, 8, 1075–1092.

## Characterization and isolation of DNA microsatellite primers in wood mice (*Apodemus sylvaticus*, Rodentia)

BETTINA HARR,\* KERSTIN MUSOLF† and GABRIELE GERLACH†

\*Institut für Tierzucht und Genetik, Veterinärmedizinische Universität Wien, 1210 Wien, Austria, †Universität Konstanz, Fakultät für Biologie, Postfach 5560, 78457 Konstanz, Germany

Keywords: *Apodemus flavicollis*, *Apodemus sylvaticus*, microsatellite loci, Rodentia

Received 29 April 2000; revision accepted 15 May 2000

Correspondence: Dr Gabriele Gerlach. Fax: + 49 7531 88–3059; E-mail: gabi.gerlach@uni-konstanz.de

The wood mouse (*Apodemus sylvaticus*) widely distributed in European forests and bush land is an interesting model animal to study the evolution of social behaviour and mate choice. In this context we were interested in the dynamics of the genetic structure of a population over several years that might indicate clustering of kin groups and recruitment of subadults. Mitochondrial DNA (mtDNA) studies revealed little variation in this species (G. Gerlach, unpublished results) and stressed the need for the development of microsatellite markers to accurately assess and monitor genetic variation and changes. In this paper we describe the identification of highly variable microsatellite markers which will provide a powerful tool for the investigation of the genetic structure of wood mice populations. DNA from muscle tissue from an individual of *A. sylvaticus* was extracted as described in

Sambrook *et al.* (1989) using the conventional proteinase K/chloroform method. Genomic DNA was digested with the restriction enzyme *Sau3A I* and electrophoresed in a 0.8% agarose gel (Rassmann *et al.* 1991). DNA fragments were excised and purified from a range of 400–900 bp. A size-selected library was constructed by ligating the DNA fragments into the vector pUC18 (Amersham Pharmacia Biotech) following the manufacturer's recommendations. Plasmids were transformed into electrocompetent *Escherichia coli* XL-1 Blue cells by electroporation (*E. coli* pulser, Bio-Rad) and grown on agar plates. Approximately 20 000 recombinant plaques were blotted on Hybond N + nylon membranes (Schleicher and Schüll) and were screened using digoxigenin labelled dinucleotide (GT)<sub>7</sub> and (GA)<sub>7</sub> using standard hybridization techniques (Schlötterer 1993). Forty-eight positive clones which showed a signal on the X-ray film after chemiluminescence detection (CSPD) were sequenced using the ABI PRISM dye terminator cycles sequencing-ready reaction kit (following the recommendations of the manufacturer) and analysed with an ABI PRISM 377 automated sequencer. Eight dinucleotide repeats (Table 1) could be revealed and primer sets were designed with the help of the program OLIGO (National Biosciences Inc., version 4.0). They amplified repeatedly and were polymorphic within a population of 30 individuals. These animals were live-trapped at different localities near Konstanz, Southern Germany. Before releasing the animals, pieces of tissue were collected from the ear, fixed with 100% ethanol and preserved until DNA extraction.

Allelic variability and heterozygosity of the loci was determined with DNA extracted from tissue samples of wood mice according to standard protocols (Sambrook *et al.* 1989) with proteinase K treatment. Polymerase chain reaction (PCR) amplification (Saiki *et al.* 1988) was carried out in a DNA thermal cycler (Biozym) in 10 µL of reaction mixture including about 10–100 ng of template DNA, 0.2 mM dNTP, 0.5 µM of each primer, ddH<sub>2</sub>O, 1 × PCR buffer (Amersham Pharmacia Biotech, 50 mM KCl, 1.1 mM MgCl<sub>2</sub>, 10 mM Tris-HCl), and 0.25 units of *Red-taq* polymerase (Amersham Pharmacia Biotech). Twenty-five PCR cycles were performed: denaturation at 94 °C for

**Table 1** Microsatellite loci in wood mice, *Apodemus sylvaticus*. Size range refers to the observed PCR product sizes. Number of alleles were determined from 30 individuals. Animals were caught at different places in the wild and therefore were assumed to be unrelated. (*n*, number of alleles; *H<sub>O</sub>*, observed heterozygosity; *H<sub>E</sub>*, unbiased expected heterozygosity; *T<sub>a</sub>*, annealing temperature). The sequences were submitted to GenBank (acc: accession nos)

Locus acc	Size	Repeat in clone	<i>n</i>	<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	<i>T<sub>a</sub></i> (°C)	Primer sequence (5'–3')
As-7 AF246520	114	(GT) <sub>19</sub>	10	0.74	0.81	47.8	F: CAGGTCCTATTCTTCCAGTTA R: ACAATTGATTAATAATGGAACC
As-11 AF246521	248	(GT) <sub>23</sub>	15	0.97	0.90	50.5	F: GGAAGTTTAGGGTCTGGTG R: GATCAGGATTTCTAGAAAGAA
As-12 AF246522	249	(TG) <sub>22</sub> (GA) <sub>24</sub>	14	0.73	0.88	53.6	F: TGTCAGGTCCTAACAGTAGG R: CTGTTTGGAGTTGTGTTCTG
As-20 AF246523	144	(GT) <sub>25</sub>	11	0.84	0.86	55.0	F: CAGTGAACACCCCTCCATAA R: AGCCACAGACCAATPAAGAAG
As-27 AF246525	138	(AG) <sub>19</sub>	7	0.85	0.86	55.0	F: TGATTTGACCCATGAGCAG R: CCCACACCATGGCCATACAC
As-34 AF246526	150	(AC) <sub>18</sub>	12	0.84	0.84	47.1	F: CCAGAAGTATGCTGTGGTTTT R: TTAAGAATGACTAAGGATCAG

VOLUME 9  
NUMBER 10  
OCTOBER  
2000

# MOLECULAR ECOLOGY



Published by  
Blackwell Science

PM 330  
11 OCT. 2000  
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