R E PO R T

CHARACTERIZATION AND ASSESSMENT OF 12 AUTOSOMAL MICROSATELLITE LOCI SUITABLE FOR POPULATION AND EVOLUTIONARY STUDIES OF THE NEOTROPICAL OTTER (*Lontra longicaudis*)

Cristine S. TRINCA[1,2,#], Helen F. WALDEMARIN[3], Fernando C. W. ROSAS[4], Eduardo EIZIRIK[2,5]

[1] *Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Prédio 43323, CEP 91501-970, Porto Alegre, RS, Brazil.*
[2] *Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, Prédio 12C, CEP 90619-900, Porto Alegre, RS, Brazil.*
[3] *Associação Ecológica Ecomarapendi / Projeto Ecolontras, Rua Paissandu, 362, Laranjeiras, CEP 22210-080, Rio de Janeiro, RJ, Brazil.*
[4] *Laboratório de Mamíferos Aquáticos, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo,2936, Aleixo, CEP 69060-001, Manaus, AM, Brazil.*
[5] *Instituto Pró-Carnívoros, Av. Horácio Neto, 1030, casa 10, Parque Edmundo Zanoni, CEP 12940-970, Atibaia, SP, Brazil.*
[#] *Corresponding Author*

(received 23rd March 2011, accepted 10th April 2012)

Abstract: We tested 12 microsatellite markers previously isolated from two different otter species (*Lutra lutra* and *Lontra canadensis*) in the Neotropical otter aiming to help in the choice of markers that could be used even in noninvasive samples of this species. Thirty-one individuals from several localities of Brazil were genotyped. The mean expected heterozigosity values for the ten best loci ranged from 0.613 to 0.891 (mean H_e for all loci = 0.769). To investigate the reliability of individual identification with the selected markers, we calculated the maximum $P_{(ID)}$ using the ten loci, and also a subset of seven loci exhibiting the shortest product length (< 300 bp), which are more likely to be useful in noninvasive samples. The maximum estimated $P_{(ID)}$ was 1.68×10^{-11} and 3×10^{-8}, respectively, indicating a good potential of both marker sets for secure individual identification. The results obtained in this study revealed that ten of the 12 loci analyzed are suitable for application in ecological and evolutionary studies of the Neotropical otters, even on the basis of noninvasive sampling.

Keywords: *Lontra longicaudis*; microsatellite loci; noninvasive sampling; population dynamic; phylogeographic studies

INTRODUCTION

Strategies for species conservation must integrate genetic and ecological data to allow for adequate planning and implementation. For such conservation efforts to be successful, accurate estimates of genetic diversity, geographic structuring, density, and population abundance are essential. In spite of their importance, these data are often difficult to obtain for most carnivore species, due to their elusive behavior.

In this context, microsatellite DNA has been extensively used as an informative type of molecular marker in population and evolutionary studies of carnivores (Walker *et al.* 2001; Blundell *et al.* 2004; Pertoldi *et al.* 2006; Carmichael *et al.* 2007). Microsatellites are highly polymorphic regions of DNA widely scattered throughout eukaryotic genomes (Schlötterer 1998, 2004). This kind of molecular

marker is a useful genetic tool that, in association with noninvasive sampling, increases the potential for collecting genetic, ecological and demographic data for elusive species, such as the Neotropical otter.

Recently, a variety of microsatellite loci has been isolated from mustelids (*e.g.* Dallas and Piertney 1998; Carpenter *et al.* 2003; Beheler *et al.* 2004, 2005) and tested for cross-amplification in related species (*e.g.* Davis and Strobeck 1998; Beheler *et al.* 2004, 2005). However, until now there is no published study that has attempted their use in the Neotropical otter (*Lontra longicaudis*). The characterization of polymorphic molecular markers in the Neotropical otter will allow the development of ecological and genetic studies on this species, expanding our knowledge on its biology and providing a suitable basis for planning adequate management and conservation strategies.

Here, we report the characterization of a set of 12 polymorphic markers suitable for studies in *L. longicaudis*, derived from cross-species testing of previously developed microsatellite loci of *Lutra lutra* and *Lontra canadensis*.

MATERIAL AND METHODS

The aim of this work was to identify a set of loci that: (i) show good amplification efficiency and (ii) are polymorphic in *L. longicaudis*; and (iii) present short amplification products (≤ 300 bp), facilitating their use in DNA recovered from noninvasive samples (*e.g.* hairs and scats). We tested 12 primer sets chosen on the basis on the following features reported for the original species (from which the markers were isolated) and, in some cases, additional mustelids also typed for the same loci: (i) number of observed alleles; (ii) heterozygosity; and (iii) product length.

The primers were tested in genomic DNA from thirty-one individuals of the Neotropical otter sampled from wild populations and kept in captivity. The samples were obtained from several localities in Brazil, including the states of Rio Grande do Sul, Santa Catarina, Paraná, Mato Grosso do Sul, Rio de Janeiro, São Paulo, Goiás, Minas Gerais a Amazonas. Tissue and blood samples were obtained opportunistically by collaborators from road-killed and captive individuals, respectively. Tissue samples were stored in ethanol 96% and blood samples were preserved in a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS). Both tissue and blood samples were stored at -20°C DNA prior the DNA extraction. Genomic DNA was extracted using a standard phenol-chloroform-isoamyl alcohol protocol following digestion of the tissue with Proteinase K (Sambrook *et al.* 1989).

The markers selected for this study were developed by Dallas & Piertney (1998) for *Lutra lutra* and by Beheler *et al.* (2004, 2005) for *Lontra canadensis*. We initially tested all 12 loci for 14 individuals, and selected the ten best markers (based on amplification performance, product size, and level of polymorphism) for genotyping in the remaining individuals.

Every forward primer was 5'-tailed with an M13 sequence (Boutin-Ganache *et al.* 2001), and used in combination with an M13 primer that had the same sequence but was dye-labeled on its 5' end. The PCR reactions were performed in 10 µl reactions containing 0.5 – 1.5 µl of empirically diluted DNA, 1x PCR Buffer (Invitrogen), 1.5 - 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of the reverse and M13-fluorescent primers, 0.013 µM of the M13-tailed forward primer, and 0.5 unit of Taq DNA Polymerase. The conditions were kept constant and general for all loci, to maximize standardization. The PCR cycles profile consisted of 10 cycles (touchdown) of 94 °C for 45s, 60-51 °C for 45s, 72 °C for 1.5 min; this was followed by 30 cycles

of 94 °C for 45s, 50 °C for 45s, 72 °C for 1.5 min and final extension of 72 °C for 30min. PCR reactions were carried out for each locus separately, and products from 1 to 3 loci were diluted and pooled together based on yield, size range and fluorescent dye, and then analyzed in a MegaBACE1000 automated sequencer (GE Healthcare). Allele sizes of PCR products were determined using the software GENETIC PROFILER 2.2 (GE Healthcare) and an internal size standard (ET Rox-400 or ET Rox-550, GE Healthcare).

We tested the selected loci for linkage disequilibrium and for Hardy-Weinberg disequilibrium using the software ARLEQUIN 3.01 (Excoffier *et al.* 2005) with the allowed percentage of missing values set to 0.1. We also estimated the maximum $P_{(ID)}$ using the panel of ten markers and also with a subset containing the shortest-product loci using the software CERVUS 3.0 (Marshall *et al.* 1998). Both marker sets were tested since the DNA recovered from noninvasive samples tends to be degraded, and therefore the reliable amplification and genotyping of large products may be compromised.

RESULTS AND DISCUSSION

The initial tests indicated that all loci were amplifiable in the Neotropical otter. However, two of them (LUT818 and RIO17) presented low amplification efficiency. The ten remaining loci presented the desirable features of good efficiency, short product and allelic diversity.

The number of observed alleles per locus ranged from six to 13 (mean number of alleles per locus = 8.6) and the mean expected heterozygosity values for the ten best loci ranged from 0.613 to 0.891 (mean H_e for all loci = 0.769) (Table 1).

Table 1. Characterization of polymorphic microsatellite loci in *Lontra longicaudis*. (N_I) number of analyzed individuals, (N_A) number of alelles, (H_e) expected and (H_o) observed heterozygosities, (HWE) Hardy-Weinberg equilibrium test P value and (PIC) polymorphic information content. Sample size = 31 individuals.

Locus name	Isolated from	N_I	N_A	Size range (bp)	H_e	H_o	HWE	PIC	Dye	Reference
Lut453	*Lutra lutra*	31	6	136-146	0.749	0.677	--	0.693	FAM	Dallas & Piertney 1998
Lut701	*Lutra lutra*	29	8	172-198	0.702	0.793	--	0.643	FAM	Dallas & Piertney 1998
Lut733	*Lutra lutra*	31	11	152-178	0.810	0.677	--	0.770	NED	Dallas & Piertney 1998
Lut782	*Lutra lutra*	31	6	178-198	0.613	0.516	--	0.552	NED	Dallas & Piertney 1998
RIO06	*Lontra canadensis*	30	7	261-293	0.718	0.600	--	0.660	FAM	Beheler *et al.* 2004
RIO07	*Lontra canadensis*	31	7	176-192	0.788	0.290	*	0.743	FAM	Beheler *et al.* 2004
RIO11	*Lontra canadensis*	31	10	168-186	0.891	0.903	--	0.864	NED	Beheler *et al.* 2005
RIO18	*Lontra canadensis*	30	13	142-174	0.834	0.500	--	0.801	NED	Beheler *et al.* 2005
RIO19	*Lontra canadensis*	31	9	286-306	0.744	0.548	*	0.718	NED	Beheler *et al.* 2005
RIO20	*Lontra canadensis*	30	9	257-273	0.844	0.333	*	0.809	HEX	Beheler *et al.* 2005

* significant after Bonferroni correction ($\alpha = 0.005$)

Furthermore, no locus was in linkage disequilibrium (LD) after Bonferroni correction ($\alpha = 0.001$) (Rice 1989). On the other hand, three loci (RIO07, RIO18 and RIO20) were out of Hardy-Weinberg equilibrium (HWE) even after Bonferroni correction, but this result can be biased by the sampling design, since we employed samples collected from a large geographic area whose genetic structuring is presently unknown. To further investigate this possibility, we repeated this analysis with samples collected from a more restricted area (Rio Grande do Sul state in southern Brazil; n = 11), and only one locus remained out of HWE (RIO20), supporting the hypothesis that population subdivision may account for the departure from equilibrium observed in the broader sample.

The maximum $P_{(ID)}$ obtained for the ten loci were 1.68×10^{-11}, and for the seven shortest-length microsatellites it was 3×10^{-8}, indicating a high probability of accurate

individual identification using either ten or seven loci. This result indicates that this set of markers should be effective in individual-based studies of *L. longicaudis* employing noninvasive samples.

The set of markers tested here demonstrated to have a great potential for application in population-genetic, ecological and evolutionary studies of the Neotropical otter, allowing their use even in studies based on noninvasive sampling. The characterization of these microsatellite loci in *L. longicaudis* opens a wide range of opportunities for studies addressing population structure and dynamics, kinship and mating system, as well as phylogeographic and historical investigations focusing on this so-far-ignored species. The data obtained from these studies will likely be of significant utility for the development of adequate conservation strategies for *L. longicaudis*, not only in Brazil but throughout its geographic distribution.

Acknowledgements *- We would like to thank all people and institutions that helped with the collection of biological samples used in this study. This work was supported by Centro Nacional de Pesquisas para a Conservação de Predadores Naturais – CENAP/IBAMA, Instituto Pró-Carnívoros and CNPq.*

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RÉSUMÉ

CARACTERISATION ET EVALUATION DE 12 LOCI MICROSATELLITAIRES POUR L'ETUDE DE L'EVOLUTION DES POPULATIONS DE LOUTRE A LONGUE QUEUE (*Lontra Longcaudis*)

Nous avons testé sur la Loutre à longue queue, 12 marqueurs microsatellitaires précédemment isolés chez deux espèces de loutres (*Lutra lutra* et *Lontra canadensis*) ceci afin de choisir les marqueurs qui peuvent être utilisés dans le cadre d'un échantillonnage non invasif de cette espèce. Trente et un individus ont été génotypés à partir de plusieurs localités du Brésil. Les valeurs moyennes attendues d'hétérozygotie pour les dix meilleurs loci variaient de 0,613 à 0,891 (Moyenne H_e=0,769 pour tous les loci). Pour évaluer la fiabilité de l'identification individuelle à partir des marqueurs sélectionnés, nous avons calculé le $P_{(ID)}$ maximum (polymorphisme) en utilisant les dix loci mais aussi un sous-ensemble de sept loci présentant les plus courtes séquences (<200 pb). Les $P_{(ID)}$ maximum estimés étaient respectivement de $1{,}68 \times 10^{-11}$ et 3×10^{-8}, indiquant un bon potentiel des complexes de marqueurs pour une identification individuelle fiable. Les résultats obtenus dans cette étude ont révélé que dix des 12 loci analysés sont appropriés pour une application dans les études écologiques et évolutives des loutres à longue queue, même sur base d'un échantillonnage non invasif.

RESUMEN

CARACTERIZACIÓN Y EVALUACIÓN DE 12 LOCI DE MICROSATÉLITES AUTOSÓMICOS APROPIADOS PARA ESTUDIOS POBLACIONALES Y EVOLUTIVOS DE LA NUTRIA NEOTROPICAL (*Lontra longicaudis*)

Probamos 12 marcadores de microsatélites previamente aislados de dos diferentes especies de nutria (*Lutra lutra* y *Lontra canadensis*) en la nutria neotropical con el objetivo de ayudar en la elección de los marcadores que podrían ser utilizados incluso en muestras no invasivas de esta especie. Treinta y un individuos de varias localidades de Brasil fueron genotipados. La media de los valores esperados de heterocigosidad para los diez mejores loci variaron desde 0.613 hasta 0.891 (la media *He* para todos los loci = 0.769). Para investigar la fiabilidad de la identificación individual con los marcadores seleccionados, calculamos el $P_{(ID)}$ utilizando los diez loci, y también un subconjunto de siete locis exhibiendo la longitud más corta del producto (<200 pb), que probablemente sean de más utilidad en muestras no invasivas. La máxima estimada $P_{(ID)}$ fue de 1.68×10^{-11} y 3×10^{-8}, respectivamente, indicando un buen potencial de ambos juegos de marcadores para asegurar la identificación individual. Los resultados obtenidos en este estudio revelaron que diez de los 12 loci analizados son apropiados para la aplicación en los estudios ecológicos y evolutivos de las nutrias neotropicales, incluso sobre la base del muestreo no invasivo.