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RESEARCH ARTICLE

Isolation and characterization of microsatellite marker loci in the Wagner's mustached bat *Pteronotus psilotis* (Chiroptera: Mormoopidae) and cross-amplification in other related species

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Abstract. *Pteronotus psilotis*, a mormoopid bat, is an insectivorous, gregarious and strict cave-dwelling species that is found areas between the sea level and an elevation of about 1000 masl. This species is present in diverse habitats ranging from rain forest to dry deciduous forest. Nine microsatellite loci were developed for Wagner's mustached bat, *Pteronotus psilotis* using the next-generation sequencing approach, and their utility for population genetics studies was assessed. All loci were polymorphic (7–15 alleles) and characterized in 30 individuals from three *P. psilotis* populations, with the levels of observed and expected heterozygosity ranging from 0.280 to 0.867 and 0.584 to 0.842, respectively. One locus showed significant departures from Hardy–Weinberg expectations after Bonferroni correction. Cross-amplification in 11 other bat species was tested, for which eight microsatellites were successfully amplified, and of these seven were polymorphic. The development of these new microsatellite loci will contribute to investigations of genetic population structure, genetic diversity and gene flow in *P. psilotis* populations, as well as in other closely related bat species.

Keywords. microsatellites; cross-amplification; polymorphism; Mormoopidae; *Pteronotus psilotis*.

Introduction

Mormoopidae is one of the six bat families exclusive to the New World (Findley 1993). The family comprises of two genera: *Mormoops* (ghost-faced bats) and *Pteronotus* (mustached and naked-backed bats) whose taxonomic diversity has been traditionally accepted as 10 species, two of which are known only from fossils (Smith 1972; Simmons and Conway 2001). *Pteronotus personatus* (Wagner 1843), Wagner's mustached bat is an insectivorous, gregarious and strict cave-dwelling species, found in areas between Mexico and South America, inhabiting the lowlands of tropical semi-deciduous forests (De la Torre and Medellín 2010). A recent work, however, has indicated the need for taxonomic reorganization within *P. personatus*. Smith (1972) recognized morphologically two subspecies within *P. personatus* (*P. p. psilotis* and *P. p. personatus*), but Dávalos (2006) suggested, in a molecular revision, that the two subspecies should be considered as different species. Pavan and Marroig (2016, 2017) and Zárate-Martínez *et al.* (2018) have also recommended the elevation of *P. personatus* and *P. psilotis* to the species rank. In this investigation, we follow these recent taxonomic recommendations and, therefore, as the study specimens were collected in Mexico they correspond to the species *P. psilotis*.

P. psilotis is considered as 'least concern' according to the International Union for Conservation of Nature (IUCN) conservation categories (Dávalos *et al.* 2008).

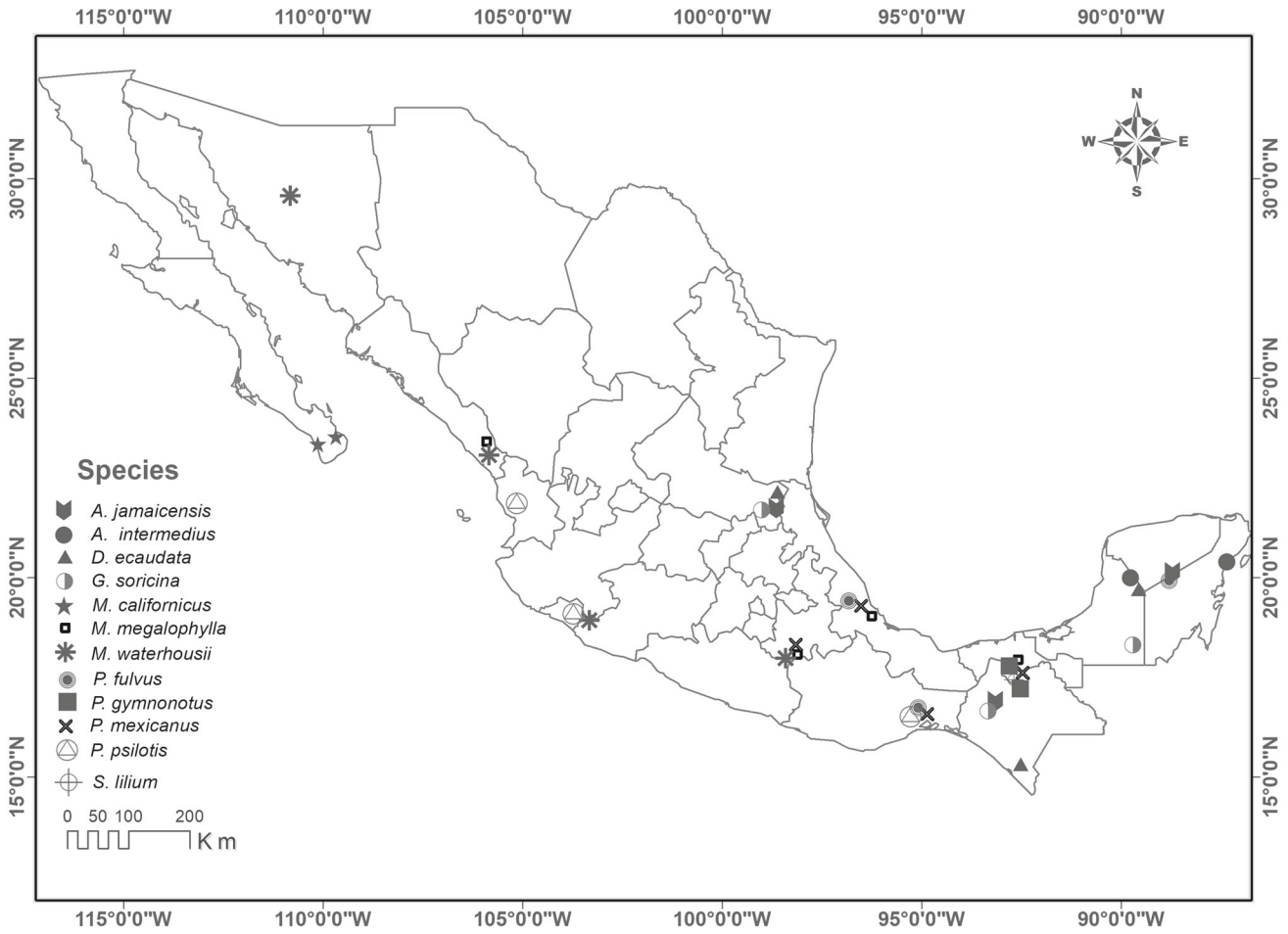

Figure 1. Sampling sites of *P. psilotis* and of 11 bat species used for cross-amplification.

Despite this assessment, land use is changing quite rapidly and the associated habitat fragmentation may cause threats in the near future, and thus a basic understanding of the genetic patterns in this species is needed before this trend continues. The generation of a library of microsatellite markers using a next-generation sequencing approach provides tool markers that will be useful in analyses to estimate genetic diversity or gene flow of the proposed lineages. In addition, cross-species transferability of these loci was assessed in 11 bats, four from the family Mormoopidae and seven from the family Phyllostomidae. These are the first microsatellite loci developed for mormoopid bats.

Materials and methods

In the present study, the required ethical guidelines were followed (Sikes 2016), and all specimens were collected under Mexican Government permits (SGPA/DGVS nos. 09131/14, 05853/13 and CC 08450/92). For library construction, genomic DNA was extracted from the muscular tissue of one *P. psilotis* individual (Santiago Ixcuintla, Nayarit, Mexico; voucher UAM-I RLW140304Ppe74) using a salt-extraction protocol (Aljanabi and Martinez 1997). The sample was sent to the Georgia Genomics Facility (University of Georgia, Athens, USA). DNA was sheared using Covaris S2. Fragmented DNA was ligated to Illumina universal TruSeq adapters containing 10 custom nucleotide indexes (Fisher *et al.* 2011; Faircloth and Glenn 2012). These fragments were enriched through polymerase chain reaction (PCR), purified and normalized, and sequenced using an Illumina HiSeq 2000 to produce paired-end 100-bp nucleotide reads. The resulting FASTQ files were demultiplexed and the readings were filtered and executed through the PALfinder pipeline to identify adequate microsatellites and for the design of primers (Castoe *et al.* 2012). More than 11 million 100-bp readings were obtained, and we registered 8321 loci with primer of which 12 were selected by applying the parameters indicated by Méndez-Rodríguez *et al.* (2015). All selected primers were perfect microsatellite dinucleotide motifs and the minimum number of repeats was 20.

A total of 30 wing membrane biopsies from *P. psilotis* individuals was collected from three natural populations: 10 biopsies from bats captured in each of the localities of La Venta, Oaxaca, Mexico (16°35.197′N 94°52.424′O),

Table 1. Characterization of nine polymorphic microsatellite markers for *P. psilotis*.

Locus	Primer sequence (5′ to 3′)	T_a (°C)	SSR motif	Allelic size range (bp)	GenBank accession number
Pps1	F: 5′-NED-GCAACCTCCTAACCTGGTGG-3′ R: 5′-GCCTGTAAACTCCTGCTGTGC-3′	62	$(TC)_{38}$	209–245	MH186029
Pps2	F: 5′-6-FAM-CTGGGTTGTTGAAAGTCCGC-3′ R: 5′-TCTAAGGATAGTGTGCGTGCG-3′	59	$(AC)_{28}$	120–132	MH186030
Pps3	F: 5′-VIC-AAGTCCTTGCTGCCTTGACC-3′ R: 5′-GGTGTTATTTGTGCCAGACCC-3′	60	$(AC)_{24}$	102–128	MH186031
Pps4	F: 5′-PET-GCCTTGGATTATGCTGTAGGG-3′ R: 5′-AACTGGGCAGGTTTGAGTGC-3′	60	$(AC)_{30}$	192–212	MH186032
Pps5	F: 5′-6-FAM- AACTTGTGTGGGACAGCACG 3′ R: 5′-CGCTCTTCCTGCCATTTCC 3′	60	$(AC)_{22}$	133–147	MH186033
Pps6	F: 5′-VIC-ATCATCCTGTGGGACTGGG-3′ R: 5′-TGAGGTTATCCTTCTTCACCCC-3′	60	$(TC)_{22}$	298–316	MH186034
Pps7	F: 5′-6-FAM-GGAGATGACCCTGCCATAGG-3′ R: 5′-GTTGCTGTGTATTTTCTGTCTTGC-3′	61	$(AC)_{28}$	258–280	MH186035
Pps8	F: 5′-VIC-TTAGAAGAGCGGTTCCCTGC-3′ R: 5′-GAGTTTTGGTTTCCCTCCACC-3′	61	$(AC)_{22}$	234–260	MH186036
Pps9	F: 5′-PET-GCACTTCCCAAACGAGTTCC-3′ R: 5′-AATGCTGCTTCCTCTGTCCC-3′	61	$(AC)_{22}$	195–217	MH186037

T_a, annealing temperature; SSR motif, simple sequence repeat pattern; size (bp).

Table 2. Measures of genetic diversity for *P. psilotis*.

Locus	Mexico, $n = 30$			
	N_a	N_e	H_o	H_e
Pps1	15	6.338	0.867	0.842
Pps2	7	5.631	0.280	0.822*
Pps3	12	5.643	0.800	0.823
Pps4	9	6.000	0.726	0.833
Pps5	7	3.482	0.667	0.713
Pps6	10	4.380	0.767	0.772
Pps7	8	4.724	0.633	0.788
Pps8	13	5.960	0.800	0.832
Pps9	8	2.406	0.600	0.584

N_a, number of alleles per locus; N_e, number of effective alleles; H_o, observed heterozygosity; H_e, expected heterozygosity.
*Significant deviation from the HWE ($P \leq 0.05$).

Las Viejas, Nayarit, Mexico (21°51.40′N, 105°08.81′O) and Los Ortices, Colima, Mexico (19°04.81′N, 103°43.59′O). For cross-amplification, biopsies from *P. fulvus*, *P. gymnonotus*, *P. mexicanus*, *Mormoops megalophylla* (Mormoopidae), *Diphylla ecaudata*, *Artibeus intermedius*, *A. jamaicensis*, *Macrotus californicus*, *M. waterhousii* and *Glossophaga soricina* (Phyllostomidae) were used, all of these were obtained from the tissue collection at the Mammal Biology and Ecology Laboratory of the Universidad Autónoma Metropolitana Iztapalapa. The geographic origins of these specimens are presented in figure 1.

DNA was extracted using a salt-extraction method (Aljanabi and Martinez 1997). PCR amplification was performed using a T100 thermal cycler (Bio-Rad Laboratories, Hercules, USA). The amplification was carried out for each locus separately. The 25-μL reaction volume contained 20 ng template DNA, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 0.25 μM of each primer (the 5′ tail of the forward primer for each selected primer pair was fluorescently labelled with 6FAM, NED, VIC or PET, Applied Biosystems), 3.0 mM magnesium chloride ($MgCl_2$) and 0.25 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, USA). Cycling conditions were: 2 min denaturation at 94°C; 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s with temperatures varying by locus (table 1) and an extension at 68°C for 30 s; final extension of 5 min at 68°C, using a MultiGene thermocycler (Labnet International, USA). To ascertain locus amplification intensity, consistency and polymorphism fragment visualization was performed at Macrogen (Korea, ABI3730XL, Applied Biosystems) using a standard size GeneScan 500 LIZ.

Allele size was estimated using GeneMarker 2.4.2 software (SoftGenetics, LLC, USA). Observed (H_o) and expected heterozygosity (H_e) were calculated using GenAlEx 6.501 (Peakall and Smouse 2012). Deviations from the Hardy–Weinberg equilibrium (HWE) by locus and population were tested using the GenePop 4.0 program

Table 3. Cross-amplification in 11 bats from the Mormoopidae and Phyllostomidae families.

Species (*n*)	Pps1	Pps2	Pps3	Pps4	Pps5	Pps6	Pps7	Pps8	Pps9
P. fulvus (4)	P (5) 254–277	0	P (5) 106–122	P (2) 184–188	0	P (6) 326–340	P (2) 264, 266	0	0
P. gymnonotus (4)	P (5) 253–263	P (4) 124–146	P (4) 116–126	M 184	0	P (6) 328–350	P (2) 270, 272	0	M 199
P. mexicanus (4)	P (4) 237–245	0	P (6) 104–134	P (2) 208, 210	P (5) 115–149	P (4) 314–336	P (5) 266–278	0	0
M. megalophylla (4)	0	0	0	P (2) 196, 198	0	0	0	0	0
D. ecaudata (4)	0	0	P (2) 112, 114	0	0	0	0	0	0
A. intermedius (4)	0	0	P (4) 116–122	0	0	0	0	0	0
A. jamaicensis (4)	0	0	P (4) 108–136	0	0	0	0	0	0
S. lilium (3)	0	0	P (5) 108–116	0	0	0	0	0	0
M. californicus (4)	0	0	0	0	M 137	0	0	0	0
M. waterhousii (4)	0	0	P (6) 110–138	0	P (3) 135–139	0	0	0	0
G. soricina (3)	0	0	P (5) 118–140	0	P (2) 135, 137	P (3) 302–306	0	0	0

n, Sample size; P, polymorphic in all samples (no. of alleles) and below the size range of alleles amplified; M, monomorphic; 0, unsuccessful amplification.

(Raymond and Rousset 1995). Linkage disequilibrium (LD) was evaluated using Arlequin 3.0 (Excoffier *et al.* 2005). For HWE and LD, an exact test was applied with sequential Bonferroni correction. The significance level was set at $P \leq 0.05$ (Rice 1989). The presence and frequency of null alleles was evaluated using MicroChecker 2.2.3 (Van Oosterhout *et al.* 2004). For each population, the number of alleles (N_a) per locus and effective alleles (N_e) were calculated using GenAlEx 6.501 (Peakall and Smouse 2012).

Results and discussion

Of the 12 microsatellite markers tested in all samples of *P. psilotis*, three did not amplify in any sample and nine, which were polymorphic, were successfully amplified by PCR (table 1). The number of alleles per locus ranged from 7 to 15, similar to those found for other bats of the family Phyllostomidae (Ortega *et al.* 2002; Romero-Meza *et al.* 2012) and Vespertilionidae (Piaggio *et al.* 2009). The H_o ranged from 0.280 to 0.867 with an average of 0.687, while the H_e ranged from 0.584 to 0.842 with a mean of 0.779 (table 2). These results are similar to values reported previously for other New World bats (Bardeleben *et al.* 2007; Ramírez *et al.* 2011). One locus showed significant departures from HWE as a result of heterozygote deficiency (Pps2). No evidence was found for LD among the loci. The presence of null alleles was detected in two loci (Pps2 and Pps7) (table 2).

Cross-species amplification indicates that seven polymorphic loci (Pps1–Pps7), in different degrees, will provide new insights into the intraspecific variation of mormoopid bats (table 3). Further, some polymorphic loci could be applied to phyllostomid bats, such as Pps3 for *D. ecaudata*, *A. intermedius*, *A. jamaicensis* and *S. lilium*, Pps3 and Pps5 for *M. waterhousii* and Pps3, Pps5 and Pps6 for *G. soricina* (table 3).

The nine microsatellite loci reported here represent the first set of molecular markers developed for the genus *Pteronotus*, and they will be useful for population genetic analysis in *P. psilotis*, where they have been shown to be highly polymorphic and reliable across populations. These markers can also be used to provide information about changes in the historical distribution and connectivity of mormoopid populations.

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