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Cross-genus amplification and characterisation of microsatellite loci in the large-eared free tailed bat, *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar

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Primers developed for the Brazilian free-tailed bat, *Tadarida brasiliensis*, were successfully used to cross-amplify microsatellite loci in two Afro-tropical *Otomops* species. Seventy one (71) bats from two species were genotyped for two dinucleotide and four tetranucleotide loci, yielding 1 to 15 alleles per locus. For the combined sample, the observed and expected heterozygosities ranged from 0.125 to 1.000 and 0.125 to 0.919, respectively. The polymorphism information content (PIC) values were 0.295 to 0.905 (mean 0.687) for *Otomops martiensseni* and 0.110 to 0.797 (mean 0.442) for *Otomops madagascariensis*. Five *O. martiensseni* loci deviated significantly from Hardy-Weinberg equilibrium. These six loci provide genetic markers that will be useful in investigating the population genetic structure of Afro-Arabian *O. martiensseni* and Malagasy *O. madagascariensis*, with potential application to Asian species of *Otomops* and possibly other genera within the Molossidae.

Key words: Bats, *Otomops*, Chiroptera, microsatellites, Molossidae, cross-genus amplification.

INTRODUCTION

Microsatellites or short tandem repeats are popular markers in population genetic studies as they show high levels of polymorphism and are useful for estimating parameters such as gene flow, inbreeding, migration rates, population size and kinship (Selkoe and Toonen, 2006; Barker, 2002; Zane et al., 2002. As development of new microsatellite markers is relatively expensive and time-consuming (Zane et al., 2002; Abdelkrim et al., 2009), cross-amplification of microsatellites using primers developed for another species is considered a cost-effective and viable option (Barbará et al., 2007). Cross-species amplification of microsatellites has been utilised in various taxa, including plants (Datta et al., 2010; Eliott et al., 2013), insects (Chen and Dorn, 2010), fish (Dubut et al., 2010) and mammals (Kaňuch et al., 2007; Kretschmer et al., 2009; Sanvito et al., 2013). This method does, however, have limitations since the primers work best for the species for which they were developed. Loci are less likely to amplify successfully as the genetic distance between the original and target species increases, and those which amplify usually exhibit lower levels of polymorphism than in the original species (Primmer et al., 2005). Projects based on cross-species amplification should therefore be preceded by a preliminary study which assesses the ability of candidate primers to amplify suitably variable microsatellites in the target species (Schlötterer, 2000; Scribner and Pearce, 2000).

The Molossidae are one of the less studied families

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Table 1. Primer sequences from Russell et al. (2005) used in the cross-amplification of microsatellites in *O. martiensseni* and *O. madagascariensis.*

Locus	Primer sequences (5' – 3')
TabrA10	F: AAG TGG TTG GGC GTT GTC
	R: GCG ATG CAC TGC CTT GAG A ϖ
TabrA30	F: AGT CGC GGG TTT GAT TCC AGT TA
	R: ACC CCT TCC CTT TGT TCC TTC AG ω
TabrD10	F: CCC CAC TCA TTT ATC CAT CCA CA ϖ
	R: ATC TCG CAG CTA TTG AAG TA
TabrD15	F: AGT CCT GGC TCC TAT TCT CAT TG
	R: CTA TCC GTC TAC CTG TCC GTC TAT ω
TabrH6	F: ATC TCT CCA GTC CTT ACC A ϖ
	R: TTT ACC CTC CAC AGT CTC A
TabrH12	F: CCA TGT GAG CCA ATT CCT A ω
	R: GTC AGG ACT CTC CAG AGA

F = forward primer; R = reverse primer. Primer labels are indicated: ϖ6-FAM, ωNED.

within the Chiroptera, and phylogenetic and population genetic studies on this family have been based primarily on mitochondrial and nuclear sequence data (for example, Lamb et al., 2011; Ammerman et al., 2012). However, Russell et al. (2005) used microsatellites to study the population genetics of the American species, *Tadarida brasiliensis*, and recently Naidoo et al. (2013) reported on the utility of the primers of Russell et al. (2005) to cross-amplify polymorphic microsatellites in the molossid species, *Chaerephon pumilus* sensu lato from south eastern Africa.

Afro-tropical members of the Old World genus *Otomops*, *Otomops martiensseni* from Africa (including the Arabian Peninsula) and *Otomops madagascariensis* from Madagascar, have a wide but somewhat sparse distribution throughout the region (Peterson et al., 1995; Simmons, 2005; Lamb et al., 2008). According to the 2008 IUCN (The World Conservation Union) Red List of Threatened Species, *O. martiensseni* has been classified globally as having a "Near Threatened" status (Mickleburgh et al., 2008). Although species-level phylogenetic and phylogeographic investigations of *Otomops* have been undertaken (Lamb et al., 2006, 2008), fine-scale genetic investigations within the genus have been limited, leaving many unanswered questions including the number of species and taxonomic status of Afro-tropical individuals.

Our aim was to test the ability of primers developed to amplify hypervariable nuclear microsatellites in the American genus *T. brasiliensis* (Molossidae) (Russell et al., 2005) to cross-amplify and reveal polymorphism in two Afro-Malagasy species of the molossid genus *Otomops,* namely *O. martiensseni* and *O. madagascariensis*. If successful, these primers may also be useful for population genetics studies on Asian species of *Otomops*, and possibly other genera within this pan-tropical bat family. Consequent studies on gene-tic variation, gene flow and kinship in *Otomops* may prove useful in the amendment of current legislations used for the protection and conservation of this genus.

MATERIALS AND METHODS

A total of 71 individuals from two *Otomops* species, *O. martiensseni* (n=63) and *O. madagascariensis* (n=8), were tested for successful genotyping of the loci TabrA10, TabrA30, TabrD10, TabrD15, TabrE9, TabrH2, TabrH3, TabrH6 and TabrH12 (Russell et al., 2005). Genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (QIAGEN Inc.). The optimised polymerase chain reaction (PCR) amplifications were performed in 25 µl reactions containing 30 to 60 ng template DNA, 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Fermentas), 0.2 µl 5 U/µl *Taq* polymerase (Super-Therm) and 4 µl of 6 µM primer dilution (forward and reverse) per reaction (primer sequences in Table 1). The thermal cycling parameters used were as follows: 95°C for 1 min; followed by 39 cycles of (95°C for 30 s, primer-specific annealing temperature for 30 s and 72°C for 2 min); followed by 72°C for 10 min. Annealing temperatures for each primer pair were optimised using gradient PCR (Table 2). Genotyping was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems) at the South African Sugar Research Institute, Mount Edgecombe, South Africa. Each reaction comprised 1 µl of pre-diluted PCR product, labelled with dyes 5' 6-FAM or 5' NED (Applied Biosystems), 0.5 µl of LIZ® 600 Size Standard (Applied Biosystems) and 8.5 µl of Hi-Di™ Formamide (Applied Biosystems).

Raw allelic data were analysed and called using STRand v.2.4.59 (Toonen and Hughes, 2001; Hughes, 2006) and 1000 randomizations were performed in Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004) to check the *O. martiensseni* and *O. madagascariensis* data separately for null alleles, stuttering and large allele dropout. Additionally, FreeNA software (Chapuis and Estoup, 2007) was used to determine whether null alleles detected in the data were introducing bias in the analyses, where pairwise F_{ST} values were calculated between *O. martiensseni* and *O. madagascariensis* with and without the excluding null alleles (ENA) method applied. The ENA method corrects for the presence of null alleles. GenAlEx 6.5b4 (Peakall and Smouse, 2006, 2012) was used to calculate the number of alleles and the observed (H_O) and expected (H_E) heterozygosities. Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010) was used to determine deviation from Hardy-Weinberg equilibrium (HWE) and Cervus v.3.0 (Kalinowski et al., 2007) was used to calculate polymorphism information content (PIC) values.

RESULTS AND DISCUSSION

Six of nine *T. brasiliensis* primer pairs successfully cross-amplified microsatellites in *O. martiensseni* and *O. madagascariensis*, namely TabrA10, TabrA30, TabrD10, TabrD15, TabrH6 and TabrH12. The remaining loci were not useable due to the presence of null alleles (TabrH3) or ambiguity in the peak data (TabrH2 and TabrE9) which rendered us unable to score these loci with confidence. Russell et al. (2005) also reported difficulty in amplifying TabrH2 across all Tadarida populations tested. Naidoo et al. (2013), who successfully cross-amplified six of the above loci in C. pumilus, were successful with TabrA10, TabrA30, TabrD10, TabrD15, TabrH6 and TabrE9. Repeat motifs of all loci were the same in Otomops and

Table 2. Characteristics of six microsatellite loci cross-amplified in *Otomops martiensseni*, *O. madagascariensis* and *Chaerephon pumilus* sensu lato using primers developed for *Tadarida brasiliensis* (Russell et al., 2005).

Locus	Specie	Genbank accession number	Repeat motif	T_a (°C)	Allele size range	Number of alleles	H_O	H_E	PIC
TabrA10	*O. martiensseni*	KC701453	GA	65	230 – 290	5	0.286	0.668*	0.610
	O. madagascariensis	KF112058	GA	65	270 – 280	2	0.500	0.400	0.305
	C. pumilus	KC896691	TAGA/TGGA	60	178 – 254	9	0.51	0.69	0.69
	T. brasiliensis	AY954900	GA	65	226 – 268	20	0.756	0.934*	-
TabrA30	*O. martiensseni*	KC701454	GA	65	282 – 314	9	0.143	0.303*	0.295
	O. madagascariensis	KF112059	GA	65	284 – 300	3	0.125	0.342	0.294
	C. pumilus	KC896690	GA	65	240 – 296	9	0.78	0.64	0.61
	T. brasiliensis	AY954901	GA	57	193 – 281	27	0.326	0.333	-
TabrD10	*O. martiensseni*	KC701455	GATA	60	330 – 374	13	0.545	0.866*	0.845
	O. madagascariensis	KF112060	GATA	60	340 – 348	2	0.125	0.125	0.110
	C. pumilus	KC896693	GATA	60	331 – 379	13	0.81	0.81	0.80
	T. brasiliensis	AY954902	GATA	50	308 – 376	15	0.773	0.818	-
TabrD15	*O. martiensseni*	KC701456	GATA	60	350 – 414	16	0.857	0.919	0.905
	O. madagascariensis	KF112061	GATA	60	350 – 382	7	1.000	0.875	0.797
	C. pumilus	KC896692	GATA	60	148 – 284	10	0.06	0.54	0.51
	T. brasiliensis	AY954903	GATA	58	235 – 395	29	0.872	0.945*	-
TabrH6	*O. martiensseni*	KC701457	TAGA	60	210 – 258	13	0.683	0.905*	0.889
	O. madagascariensis	KF112062	TAGA	60	256 – 284	6	0.750	0.783	0.702
	C. pumilus	KC896695	TAGA	60	139 – 318	14	0.46	0.64	0.61
	T. brasiliensis	AY954907	TAGA	55	187 – 357	47	0.936	0.952	-
TabrH12	*O. martiensseni*	KC701458	TAGA	60	330 – 374	6	0.492	0.640*	0.577
	O. madagascariensis	KF112063	TAGA	60	260 – 260	1	-	-	-
	T. brasiliensis	AY954908	TAGA	57	126 – 366	54	0.846	0.959	-
TabrE9	*C. pumilus*	KC896694	GA	60	329 – 365	15	0.84	0.80	0.79
	T. brasiliensis	AY954904	GA	52	349 – 461	52	0.814	0.952*	-

T_a, Optimised annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content. Significant deviations from Hardy-Weinberg equilibrium for each locus are indicated (*, $P < 0.05$ after sequential Bonferroni correction). Data for *Chaerephon pumilus* sensu lato (Naidoo et al., 2013) and the Argentinean population of *Tadarida brasiliensis* (Russell et al., 2005) are included for comparative purposes.

T. brasiliensis (Russell et al., 2005), whereas *C. pumilus* showed a different repeat motif for marker TabrA10 (Naidoo et al., 2013) (Table 2). Analysis of *O. martiensseni* data in micro-checker detected possible scoring error due to stuttering in 2 loci (TabrA10 and TabrH12) and the presence null alleles in 5 of the 6 loci tested (TabrA10, TabrA30, TabrD10, TabrH6 and TabrH12). *O. madagascariensis* data showed no null alleles or scoring error due to stuttering. None of the loci from either species showed any large allele dropout. To determine whether any substantial bias was introduced through the presence of the null alleles, pairwise F_{ST} values were calculated between *O*. *martiensseni* and *O*. *madagascariensis* with (0.248) and without (0.249) the ENA algorithm. As the difference between the corrected and uncorrected estimates of genetic differentiation was not substantial, we report analyses performed on uncorrected data only. Null alleles, stuttering and large allele dropout were not reported for microsatellites cross-amplified in *C. pumilus* (Naidoo et al., 2013).

All of the *O. martiensseni* loci were polymorphic, with 5 to 16 (mean 10.33) alleles per locus. *O. madagascariensis*, however, showed lower levels of polymorphism; five of six loci were polymorphic, with polymorphism levels ranging from 1 to 7 (mean 3.5) alleles per locus. The lower level of polymorphism in *O. madagascariensis* is likely a reflection of the smaller sample size used for this species. Polymorphism levels in cross-amplified *C. pumilus* sensu lato microsatellites [9 to 15 (mean 11.7) alleles per locus] (Naidoo et al., 2013) were slightly higher than, but comparable to those of *O. martiensseni*. This is somewhat unexpected, as the higher divergence (RAG2 genetic distance) between *T. brasiliensis* and *C. pumilus* s.l. (4.6%) than between *T. brasiliensis* and *O. martiensseni* (3.2%) (Lamb et al., 2011) leads to an expectation of lower polymorphism in *C. pumilus* s.l. (Primmer et al., 2005).

Polymorphism levels in all cross-amplified microsatellites were considerably lower than those in *T*. *brasiliensis*, the species for which the primers were developed [15 to 54 (mean 36.67) alleles per locus]. This is to be expected as number of amplified loci and the level of polymorphism tends to decrease with increasing genetic distance between the original and cross-amplified taxa (Primmer et al., 2005), and we are dealing here with cross-genus rather than cross-species amplification. The lower levels of polymorphism in *Otomops* and *Chaerephon* species are likely to reflect divergence which has occurred since *Tadarida* and *Otomops* (24.7 MYA) and *Tadarida* and *Chaerephon* (26.1 MYA) last shared common ancestors (Ammerman et al., 2012).

There was considerable variability in observed (H_O) and expected (H_E) heterozygosities across *Otomops* samples (Table 2). Consistent with expectation, the expected fractions of polymorphic offspring, as indicted by PIC values, are a little lower than the expected heterozygosities. The PIC of the *O. martiensseni* microsatellites ranged from 0.295 to 0.905 (mean 0.687), comparable to that of the similarly-sized sample of cross-amplified *C. pumilus* s.l. microsatellites, 0.51 to 0.80 (mean 0.67) (Naidoo et al., 2013). The PIC of the *O. madagascariensis* samples was generally lower (0.110 to 0.797 (mean 0.442), possibly due to the smaller sample size. PIC values showed some markers to be more informative than others, for example, TabrD15 was the most informative, with values of 0.905 and 0.797 in *O*. *martiensseni* and *O*. *madagascariensis*, respectively.

Markers with PIC values > 0.4 are considered moderately informative and those with values > 0.7 are considered highly informative (Hildebrand et al., 1992; Xu, 2010). Thus most of the loci tested in *O*. *martiensseni* can be deemed informative for linkage analysis, as three markers had PIC values >0.7 and two had PIC values >0.4 (Table 2). Only two markers in *O*. *madagascariensis* can be considered highly informative, that is, TabrD15 and TabrH6, but this may be due to the low sample number used. By comparison, all markers tested in *C*. *pumilus* appear to be informative, with TabrD10 having the highest PIC value (0.80) (Naidoo et al., 2013).

Analyses revealed the existence of significant linkage disequilibrium among 7 pairs of loci in *O*. *martiensseni* (TabrA10 and TabrA30, TabrD10 TabrD15 and TabrH6; TabrD10 and TabrA30 and TabrH12; and TabrD15 and TabrH12) and 1 pair in *O*. *madagascariensis* (TabrA30 and TabrD15) after standard Bonferroni correction ($P < 0.001$). All *O*. *martiensseni* loci except TabrD15 showed a significant deviation from HWE; the possible presence of population stratification, migration, mutation, natural selection or assortative mating within this species (Wigginton et al., 2005) needs further investigation. Three *T*. *brasiliensis* loci (TabrA10, TabrD15 and TabrH12) exhibited deviation from HWE (Table 2); in contrast, no *O*. *madagascariensis* loci and one *C*. *pumilus* locus (Naidoo et al., 2013) deviated significantly from HWE.

In summary, six of nine microsatellite markers reported for *T*. *brasiliensis* (Russell et al., 2005) have been successfully cross-amplified in two species of the molossid genus *Otomops*. These nuclear markers do not have as high a level of polymorphism as in the originally-studied species, *T*. *brasiliensis*, but PIC values indicate that they are sufficiently polymorphic for use in population-, colony- and individual-level genetic studies. This will allow for future work on intra- and inter-colony relationships in *Otomops*. Additionally, these markers may also be useful in population genetic studies on the other *Otomops* species, such as *O*. *wroughtonii* from southern India and *O*. *formosus* from Java. Comparison of marker statistics in cross-amplified *Otomops* and *Chaerephon* microsatellites (Naidoo et al., 2013) revealed some similarities, for example, relatively lower allele numbers and PIC values, which may be attributed to mutations which have occurred in these lineages in the 24.7 to 26.1 million years since they last shared a common ancestor with *T. brasiliensis.* The markers developed by Russell et al. (2005) have been successfully cross-amplified in two other molossid genera, *Otomops* and *Chaerephon*, and may therefore have the

potential to be used for population genetic studies of not only *Otomops*, but also other poorly-studied molossid genera in the future.

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