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METHODS AND RESOURCES ARTICLE

Validation of non-invasive genetic tagging in two large macaw species (*Ara macao* and *A. chloropterus*) of the Peruvian Amazon

George Olah[1,2] · Robert G. Heinsohn[1] · Donald J. Brightsmith[3] · Jose R. Espinoza[4] · Rod Peakall[2]

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Abstract Genetic tagging is the unique identification of individuals by their DNA profile. This technique is well established in mammals, but it has not yet been widely adopted for birds. Extraction methods for minute amounts of DNA even enable the use of genetic tagging from non-invasive samples, like hair, scat, or feather. In this study, we evaluate the potential for non-invasive genetic tagging by using molted feathers of two sympatric macaw species in the Peruvian Amazon. Correct species identification is critical when relying on feathers for genetic analysis, so we describe multilocus methods for species identification. We evaluate the quality of naturally shed macaw feathers in tropical environmental conditions and present new primers for molecular sexing on the feather samples. We successfully validated 11 microsatellite markers for use in genetic tagging studies on large macaws and confirmed that DNA from blood and feather samples yields equivalent population genetic patterns. The techniques described here can be implemented for other birds with higher conservation concern.

Keywords Parrots · Macaws · Feather · Genetic tagging · Microsatellite · Molecular sexing

Introduction

Genetic tagging, the technique of unique identification of individuals by their DNA profile, is now well-established (Andreou et al. 2012; Palsboll 1999). Genetic tagging became feasible with the development of methods allowing access to highly variable genetic markers such as codominant microsatellites, which are still the international standard for forensic analysis despite major advances in next generation sequencing methods (Bruford and Wayne 1993; Guichoux et al. 2011; Paetkau et al. 1995; Peakall et al. 2006; Phillips et al. 2014). Genetic tagging was first used with invasively collected samples like skin biopsies of whales, fin clips of fishes, or ear tissues of small mammals (Andreou et al. 2012; Palsboll et al. 1997; Peakall et al. 2006). For non-invasive genetic tagging studies of mammals, DNA has been obtained from hair and scat samples (Arrendal et al. 2007; Coster et al. 2011; Ruibal et al. 2009, 2010; Taberlet and Luikart 1999). Genetic tagging can also provide data of value beyond individual identification. For example, it is standard practice to include a sex typing marker, since information about the sexes of individuals is needed for population demography or studies of sex-biased dispersal (Beck et al. 2008; Blackmore et al. 2011; Wright et al. 2005).

Despite its wide use in mammals, genetic tagging has not yet been widely adopted for birds (Horváth et al. 2005;

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✉ George Olah
george.olah@anu.edu.au

[1] Fenner School of Environment and Society, The Australian National University, Frank Fenner Building No 141, Canberra 2601, Australia

[2] Research School of Biology, The Australian National University, Canberra 2601, Australia

[3] Department of Veterinary Pathobiology, Schubot Exotic Bird Health Center at Texas A&M University, College Station, TX, USA

[4] Unidad de Biotecnología Molecular, Laboratorios de Investigación y Desarrollo (LID), Universidad Peruana Cayetano Heredia, Lima, Peru

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Segelbacher 2002; Taberlet and Luikart 1999). Furthermore, even studies assessing the reliability of molted feathers as a DNA source are scarce (Gebhardt et al. 2009; Segelbacher 2002), and the conclusions sometimes contradictory. Some studies have recommended avoiding the use of plucked or cut feathers due to low DNA quality of such samples (McDonald and Griffith 2011; McDonald and Griffith 2012), while others advocate their use (Katzner et al. 2012). Due to their degraded DNA content, feathers in museum samples are likely to be particularly problematic (Sefc et al. 2003). Despite the limitation of low quality and quantity of DNA, naturally molted feathers can still provide an important source for genetic tagging when no other samples are easily available (Gebhardt and Waits 2008b; Heinsohn et al. 2007; Monge et al. 2015). However, damaged feather samples can still present a challenge for reliable sex typing (Gebhardt and Waits 2008b), and for genetic tagging more generally in birds.

One-third of the extant parrot species are classified as threatened on the IUCN Red List (IUCN 2014; Olah et al. 2016). Capturing parrots for genetic samples often requires a large effort due to such attributes as their high mobility, preference for the forest canopy and their often remote habitats (Heinsohn et al. 2007; Masello et al. 2002; Murphy et al. 2007; Olah et al. 2015). Despite these challenges there are some population genetic studies on parrots (Brock and White 1992; Chan et al. 2008; Heinsohn et al. 2007; Masello et al. 2011, 2015; Melo and O'Ryan 2007; Monge et al. 2015; Wenner et al. 2012; Wright and Wilkinson 2001).

Here we focus on two sympatric macaw species (scarlet macaw, *Ara macao* and red-and-green macaw, *Ara chloropterus*) from the lowland Peruvian Amazon, where they frequently visit 'clay licks' to supplement their dietary sodium by eating clay (Brightsmith and Villalobos 2011; Lee et al. 2010; Powell et al. 2009). Both species are considered as globally of Least Concern (IUCN 2014), and the availability of their shed feathers at clay licks make them a suitable test species for developing, validating, and applying genetic tagging for the first time on a large sample of wild parrots. We build on previous studies that applied genetic tagging to other species (Palsboll 1999; Peakall et al. 2006), tested non-invasive molecular sexing in parrots (Gebhardt and Waits 2008b; Presti et al. 2013), demonstrated species identification in macaws (Abe et al. 2012), and showed reliability of feather genotyping compared to blood samples (Maurer et al. 2010; Segelbacher 2002).

The goal of our study was to assess the potential for non-invasive genetic tagging with 11 microsatellite loci by using molted feathers of macaws sampled in the wild. Here we (1) evaluate the DNA quality for genetic analyses of naturally shed feathers left on the ground by macaws in tropical environmental conditions; (2) describe multilocus methods for species identification using DNA from feathers; (3) present new primers for molecular sexing on damaged feather samples; (4) validate eleven microsatellite markers for use in genetic tagging studies on large macaws; and (5) confirm that DNA from blood and feather samples yields equivalent population genetic patterns.

Methods

Target species and study site

The study was conducted in the lowland rainforest of Peru in the regions of Madre de Dios and Puno. The tropical moist and subtropical wet forest extends from 250 to 800 m elevation and receives on average 3200 mm of rain per year (Brightsmith 2004; Tosi 1960). Our systematic collection of samples focused on two coexisting macaw species, the scarlet macaw (hereafter SCMA) and red-and-green macaw (hereafter RGMA). Both species have similar ecology (Brightsmith 2005a) and nest in emergent canopy trees during the rainy season (November–April) in Peru (Brightsmith 2005b).

A total of 1263 naturally shed feathers were collected during the rainy season in the years 2009–2012. Collections were made across 10 main clay licks spread over 1000 km of the Piedras, Heath, Tambopata, Candamo Rivers and their tributaries (Brightsmith and Aramburú Muñoz-Najar 2004; Brightsmith and Villalobos 2011). DNA was extracted from 886 samples (70 % of the total), and 500 (40 %) of these were used in the analyses of this study after the quality screening. Although the majority (84 %) of these samples were collected from clay licks, some feathers were also collected in the forest, below nesting trees, and in nest hollows. Upon collection, samples were photographed with a measuring scale and stored individually in paper envelops in airtight boxes with silica gel to avoid further degradation.

To compare population genetic results between blood and feather samples in this study, we used 33 blood samples (28 SCMA and 5 RGMA) collected from captured adults and nestlings around the Tambopata Research Center (TRC; 13°8.070′S, 69°36.640′W) from both species as described in Olah et al. (2015).

DNA extraction and genotyping

DNA extraction was performed using the Qiagen DNeasy Blood and Tissue kit (QIAGEN, California) following the manufacturer's instructions with some modifications to improve yield. These included longer incubation times,

higher temperatures, and double elution on the spin columns in the last step, following Gebhardt et al. (2009). For feathers >20 mm in size DNA was extracted from the blood clot from the superior umbilicus (Horváth et al. 2005). The entire shaft was used as the DNA source for small feathers after cleaning the surface with 70 % ethanol.

In a pilot study of 40 molted feather samples intentionally consisting of DNA of varying quality, we screened 30 previously described microsatellite markers specifically designed for SCMA and also known to amplify in RGMA (Olah et al. 2015). From this pilot set of 30 loci, the 11 loci that yielded the highest amplification success across the trial DNA samples of lower quality were selected for this study. These 11 loci mainly amplified smaller fragment sizes (overall means of 122–284 bp). The locus SCMA 32 was found to only amplify samples of higher quality DNA. Thus amplification success at this locus was highly correlated with amplification success at the other loci. Therefore, we used this locus to pre-select samples for the full analysis.

M13 PCR tags were attached to all forward primers (Schuelke 2000) and we amplified all loci individually. PCR products of 4 loci were multiplexed in the same lane using different fluorescent tags (Table S1) and genotyped on an ABI 3130XL sequencer (Applied Biosystem) with the size standard GS500 (-250) LIZ. We used a negative control for contamination check and a positive control to ensure consistent size scoring across all genotyping runs. Results were scored with Geneious version R6 (http://www.geneious.com, Kearse et al. 2012) and full genotypes were constructed. Most of the samples were genotyped once, with genotyping errors estimated from randomly selected samples (7–55 per locus) that yielded full genotype data for all 11 loci during the first scoring. This represents about 10 % of the PCR reactions. Samples with 5 or more missing loci were excluded from the final analysis.

The following 11 microsatellite markers were used to construct the genotype data: SCMA 02, SCMA 09, SCMA 14, SCMA 22, SCMA 26, SCMA 27, SCMA 30, SCMA 31, SCMA 32, SCMA 33, and SCMA 34 (Olah et al. 2015). Given all our loci were already pre-screened for the presence of null alleles in Olah et al. (2015) from genotyping of high quality DNA from blood, we used heterozygote deficit (homozygote excess) as an indicator of DNA quality in this study. Therefore, we tested deviations from Hardy–Weinberg equilibrium in GenePop 3.4 (Raymond and Rousset 1995) by exact probability test (Markov chain parameters were set to 100 batches with 1000 iterations per batch), and we assessed the degree of heterozygote deficit (if any). We also included blood samples that were previously genotyped during the microsatellite development for some relevant analyses (Olah et al. 2015).

Statistical analysis of feather quality

Feathers were provisionally assigned to the target species in the field based on their shape, size, and color pattern. The size of each feather sample was derived from the photographs using ImageJ (http://imagej.nih.gov/ij/index.html). Each sample was also visually categorized by quality (good, medium, damaged) and whether it was covered by clay (yes/no). We also calculated the number of days between the collection and the DNA extraction dates for each feather. Finally, all samples were assigned a binary response variable of 1 (amplification of a fragment greater than 100 fluorescent units in the expected size range of SCMA 32) or 0 (failure to amplify). A linear logistic regression model was used to test the likely determinants of PCR amplification success at the SCMA 32 locus. Akaike information criteria (AIC) and Bayesian information criteria (BIC) were used to determine the best model containing all significant terms. Model was selected with the lowest AIC values and simultaneously having the lowest BIC values. Statistical models were computed using GenStat 13.7 (Payne 2009).

Species identification

A total of 14 parrot/parakeet (*Amazona*, *Pionus*, *Pionites*, *Pyrilia*, *Aratinga*, *Pyrrhura*, *Brotogeris*, *Touit*, *Forpus*) and 6 macaw species (*Ara*, *Orthopsittaca*, *Primolius*) are found in the study area, some of them with similar plumage patterns to our two target species, thus genetic species identification was crucial. Each feather was given a unique number and provisionally identified in the field. We used three independent genetics approaches for species filtering. First, we used the AgGT17 locus that was expected to provide allelic differences between our two study species (Abe et al. 2012; Gebhardt and Waits 2008a). However, these earlier studies were based on less than 30 samples. In this study, we uncovered additional species specific alleles by using a larger sample size.

In the next step we compared the identifications based on the AgGT17 locus with assignment tests based on allele frequencies of 11 other loci (Paetkau et al. 1995, 2004). We also applied a Bayesian approach with the program STRUCTURE version 2.3.4 to assign individual feather samples to species, based on their multilocus genotype (Pritchard et al. 2000). STRUCTURE implements the Bayesian Markov chain Monte Carlo (MCMC) method to assign individuals to k clusters. In order to separate clusters as species we used the no-admixture model, with independent allele frequencies among clusters. Burn-in was set to 50,000 iterations, followed by 50,000 MCMC iterations and replicated ten times for each value of k, from one to five. To avoid any bias in the species allocation, the

AgGT17 locus was excluded from the assignment tests and STRUCTURE analysis.

By sequencing the COI gene on the mtDNA with primer pair of BirdF1/BirdR1 (Hebert et al. 2004), we barcoded five RGMA and ten SCMA samples further confirming the validity of our nuclear DNA methods for species identification. We used the software Geneious R6 (Kearse et al. 2012) to generate sequence alignments.

Molecular sexing

The most widely employed method for molecular sexing of birds is based on the conserved CHD gene in the avian sex chromosomes (Ellegren 1996). In this test the primers produce one amplified fragment for males and two different size fragments for females due to retroposon insertions in the females' Z chromosome (Suh et al. 2011). In our pilot study we tested the widely used P2/P8 primers that amplify DNA fragments between 300 and 400 bp (Griffiths et al. 1998) and the 2550F/2718R primers that show much better agarose gel resolution (ranging between 400 and 1000 bp) and higher confidence in sex determination on agarose assay over a wide range of bird taxa (Ong and Vellayan 2008). Both primer combinations showed very low amplification success on our molted feather DNA in the pilot study, probably because of our more degraded DNA samples.

In order to achieve robust molecular sexing from degraded DNA, we therefore designed new primers for our target species that would yield results for small fragment size differences with capillary electrophoresis. Our assay targeted a 189 bp fragment of CHD-Z and a 215 bp fragment of the CHD-W yielding a difference of 26 bp (Fig S1). The primer design was based on an alignment of CHD gene sequences of SCMA from GenBank (accession numbers: KF425691, KF412778; http://www.ncbi.nlm.nih.gov/genbank). Geneious version R6 was used to obtain the alignment and optimize primer design. The sequences of the new primers (5′ to 3′) are:

P8_SCMA_F: TGCAAAACAGGTRTCTCT
P2_SCMA_R: GAWTAAGTAGTTCAAAGCTA

We compared the new primers for macaw samples of known sex, and on blood samples previously sexed using the 2550F/2718R primers.

Population genetic analyses

GenAlEx 6.5 (Peakall and Smouse 2006, 2012) was used to compute all population genetic analyses, unless otherwise stated. These calculations included allele frequencies, observed and expected heterozygosities, probability of identity (*PI*), and probability of identity for siblings (PI_{sibs}).

The *PI* value across loci provides an estimate, under the assumptions of Hardy–Weinberg equilibrium, of the average probability that two independent samples will have the same identical genotype (Waits et al. 2001). It thus provides an estimate of how many loci are needed to discriminate among individuals. The theoretical estimate of the *PI* is usually lower than the observed value, hence the calculation for PI_{sibs} was introduced in forensic science (Evett and Weir 1998), to estimate the probability when full siblings occur in the dataset that share very similar alleles. To empirically confirm how many loci were needed for recovering all genotypes, we computed the genotype recovery rates by adding increasing number of loci, in order of their effective number of alleles. Lastly, we pinpointed complete genotype matches for conspecific samples in the genetic tagging analysis. We manually checked each near match for samples that only differed at 1–3 loci and resolved any scoring errors.

In order to compare between previously genotyped blood samples in TRC (Olah et al. 2015) and feathers collected in a 3 km radius around the same location, the genetic differentiation (F_{ST}) between these two groups was estimated by an analysis of molecular variance (AMOVA). Allele frequencies, observed and expected heterozygosities were calculated for blood and feather samples separately. Pairwise estimate of Shannon's Mutual Information Index was also performed (Peakall and Smouse 2012; Smouse et al. 2015), providing an alternative allele frequency based estimate of genetic differentiation. In order to validate that samples from clay licks ($N_{SCMA} = 96$ feathers) give similar population genetic results to samples from nests ($N_{SCMA} = 40$ blood samples and 38 feathers), we also performed an AMOVA between these two types of sampling sites for SCMA.

Results

Feather quality, microsatellite amplification, and population statistics

The size of feathers and the number of days between collection and DNA extraction did not significantly affect the PCR amplification success ($GLM_{Feather\ size}$: $\chi^2_1 = 1.47$, $P = 0.225$; $GLM_{Days\ since\ collection}$: $\chi^2_1 = 3.10$, $P = 0.078$). However amplification success was significantly lower for poor quality feathers ($GLM_{Feather\ quality}$: $\chi^2_2 = 108.87$, $P < 0.001$; Fig. 1a) and when clay was present on feathers at collection ($GLM_{Clay\ on\ feather}$: $\chi^2_1 = 14.14$, $P < 0.001$; Fig. 1b).

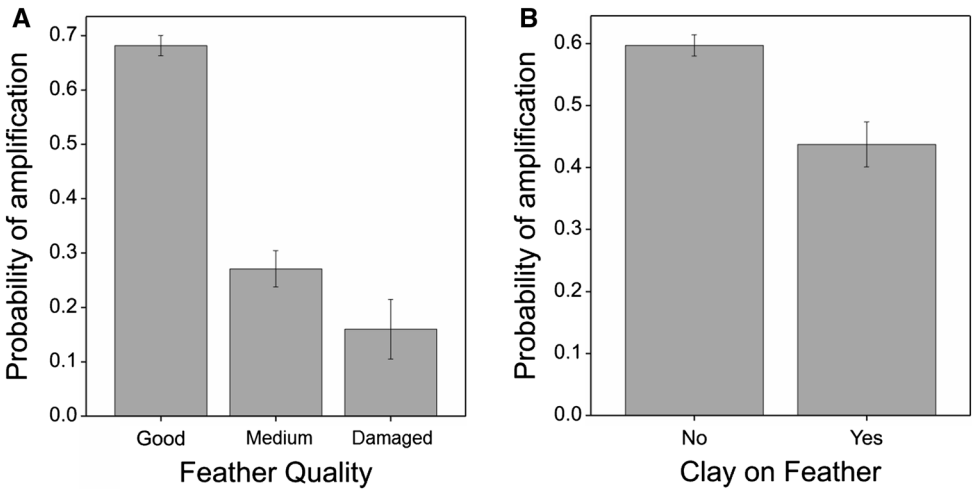

Fig. 1 Predicted effect of significant variables from a linear logistic regression model on the probability of PCR amplification of SCMA32 locus: **a** feather quality and **b** clay on feather

In total 500 feather samples were genotyped across the 11 loci with only 27 samples discarded from subsequent analyses because they had 5 or more missing loci. Allele frequency and heterozygosity estimates by locus are shown in Table 1 for both target species. Across all loci the allele number (*N*a) ranged from 12 to 20 per locus for SCMA and from 9 to 18 for RGMA. The mean expected heterozygosity (H_E) was 0.892 for SCMA and 0.772 for RGMA. The observed heterozygosity (H_O) values across loci ranged from 0.733 to 0.908 for SCMA and from 0.553 to 0.892 for RGMA.

The average amplification success over the 11 markers was 94 % for SCMA and 95 % for RGMA. The lowest overall amplification success (Table S2) across both species (N = 473) occurred at SCMA 31 (18.6 %), SCMA 02 (14.2 %), and SCMA 27 (6.3 %). Scoring errors at genotype level were calculated from about 50 randomly selected samples that had no missing loci during the first scoring before the repeats. Genotyping errors (Table S2) occurred mainly due to allelic dropouts at the larger allele (at loci SCMA 02, SCMA 14, SCMA 26, SCMA 30, and SCMA 32), but in some cases at the smaller allele (AgGT17 and SCMA 27), and sometimes due to false alleles (SCMA 02, SCMA 14, SCMA 27, SCMA 30, and SCMA 32). The total numbers of genotyping errors were as follows: SCMA 02 (2/7 replicated samples), SCMA 27 (4/15), SCMA 14 (5/48), SCMA 32 (3/44), SCMA 30 (2/42), SCMA 26 (1/48), and AgGT17 (1/55). We found no genotyping errors at SCMA 09, SCMA 22, SCMA 31, SCMA 33, SCMA 34, and P2/P8_SCMA.

Subsequently three overlapping dataset were analyzed: set (1) the 6 loci with no error and low amplification failure, set (2) combination of set (1) and 3 additional loci with some error or higher amplification failure, and set (3) combination of set (2) including two loci with both scoring error and higher amplification failure (Table 1). Using all 11 loci only eight repeated samples showed mismatched genotypes where 8-10 loci were adequate (see below), suggesting that genotyping errors did not affect the genetic tagging analysis. Two of these samples were confirmed siblings from the same nest.

Species identification

The AgGT17 locus for nuclear DNA based molecular identification of the target species amplified in all but two samples, potentially providing a technique to separate these two species. Based on 11 loci (excluding AgGT17), the STRUCTURE analysis (Fig S2a) and assignment tests (Fig S2b) independently allocated 18 samples into a third group probably representing a different or several different species that were not the target of this study. Most of these 18 samples also showed unusual alleles at the AgGT17 marker. After sequencing a mtDNA region of ten SCMA and five RGMA samples identified by our nuclear DNA methods, the alignments showed two different groups. After performing a nucleotide blast to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank), the two groups matched the nucleotide sequences of our two study species correctly.

In total 142 SCMA and 313 RGMA feathers were identified and confirmed independently by the AgGT17 genetic marker, the STRUCTURE analysis (Fig S2a), and the assignment test (Fig S2b). In the field 28 % of the feather samples were misidentified based on their color. Thus species ID was corrected in light of this genetic analysis.

Molecular sexing

The P8_SCMA_F/P2_SCMA_R primers produced two amplified fragments in females (CHD-Z and CHD-W) and

Table 1 Population statistics for microsatellite markers in non-invasive feather samples from scarlet macaw (*Ara macao*) and red-and-green macaw (*Ara chloropterus*)

# loci	Locus	Scarlet macaw (*Ara macao*)									
		N	Size range (bp)	MS (bp)	N_a	N_e	H_O	H_E	*F*	P_{HWE}	P_{HED}
6 loci	SCMA 22	131	114–160	134	19	12.1	0.908	0.918	0.01	0.221	0.412
	SCMA 32	128	175–211	192	16	10.9	0.828	0.908	0.088	0.037	0.003
	SCMA 34	132	151–189	173	17	8.5	0.803	0.882	0.09	0.018	0.005
	SCMA 33	134	174–212	193	20	11.2	0.881	0.91	0.033	0.696	0.156
	SCMA 26	130	210–240	225	14	9.4	0.808	0.894	0.096	0.002	0
	SCMA 09	132	112–136	123	12	5	0.75	0.802	0.065	0.836	0.103
9 loci	SCMA 14	131	220–252	238	14	8.8	0.733	0.886	0.173	0	0
	SCMA 30	124	206–246	229	17	9.8	0.871	0.898	0.03	0.908	0.029
	SCMA 31	108	137–169	152	16	8.7	0.861	0.885	0.027	0.235	0.197
11 loci	SCMA 02	111	268–300	284	17	12.9	0.793	0.922	0.141	0.003	0
	SCMA 27	120	209–245	226	18	11.3	0.858	0.912	0.059	0.243	0.026
	AgGT17	132	102–138	119	18	6.5	0.833	0.846	0.015	0.63	0.208
	Mean			191	16.4	9.9	0.827	0.892	0.069		
# loci	Locus	Red-and-green macaw (*Ara chloropterus*)									
		N	Size range (bp)	MS (bp)	N_a	N_e	H_O	H_E	*F*	P_{HWE}	P_{HED}
6 loci	SCMA 22	278	122–150	135	14	8.5	0.892	0.882	–0.012	0.478	0.287
	SCMA 32	279	173–199	184	11	3.1	0.631	0.677	0.069	0.005	0.001
	SCMA 34	279	157–181	169	13	5.4	0.799	0.816	0.02	0.002	0.019
	SCMA 33	280	166–190	179	10	2.4	0.575	0.586	0.019	0.571	0.269
	SCMA 26	277	222–240	231	10	5.1	0.715	0.803	0.11	0.097	0.001
	SCMA 09	280	112–132	122	11	4	0.739	0.751	0.015	0.889	0.382
9 loci	SCMA 14	273	212–238	228	9	2.4	0.553	0.59	0.063	0.124	0.103
	SCMA 30	270	206–248	230	17	4.9	0.715	0.796	0.102	0	0
	SCMA 31	220	135–165	153	12	7.5	0.836	0.867	0.036	0.89	0.197
11 loci	SCMA 02	238	260–300	283	18	5.9	0.668	0.831	0.196	0	0
	SCMA 27	267	211–245	227	17	9	0.76	0.889	0.145	0	0
	AgGT17	282	98–112	105	4	1	0.007	0.011	0.331	0.006	0.006
	Mean			187	12.9	5.3	0.717	0.772	0.091		

Presented are species, number of locus used in the analyses, locus name, number of samples (*N*), fragment size ranges, mean fragment size (MS), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E), probability of departure from HWE (P_{HWE}), and probability of heterozygote deficit (P_{HED})

one amplified fragment (CHD-Z) in males, which was easily visualized by capillary electrophoresis (Fig S1). The optimized primers yielded results matching 20 blood samples of known sex for both target species. When applied to the feather DNA samples, typing was achieved for sex in 85 % of SCMA samples (66 males, 55 females, 21 unknown) and 95 % of RGMA samples (183 males, 114 females, 16 unknown).

Probability of identity and genetic tagging

The probability of identity (among siblings) analysis was calculated using the best 6 loci (Table 1). For SCMA the five most variable loci ($PI_{sibs(5)} = 0.002$) and for RGMA the six most variable loci ($PI_{sibs(6)} = 0.003$) were predicted to recover all unique genotypes, given the sample sizes of this study ($N_{SCMA} = 142$, $N_{RGMA} = 313$). This prediction was supported empirically, for example we did not recover more unique genotypes in feathers after the two most variable loci of SCMA (Fig. 2a) and after the five most variable loci of RGMA (Fig. 2b) when including the best six loci (similar results for 9 or 11 loci). We therefore used only these six microsatellite markers for subsequent genetic tagging.

Among the 142 feather samples of SCMA we identified five complete genotype matches (total of 137 unique genotypes). When we added 86 previously genotyped SCMA blood samples we found eight additional genotype matches between blood and feather samples (Fig S3). Out of 313

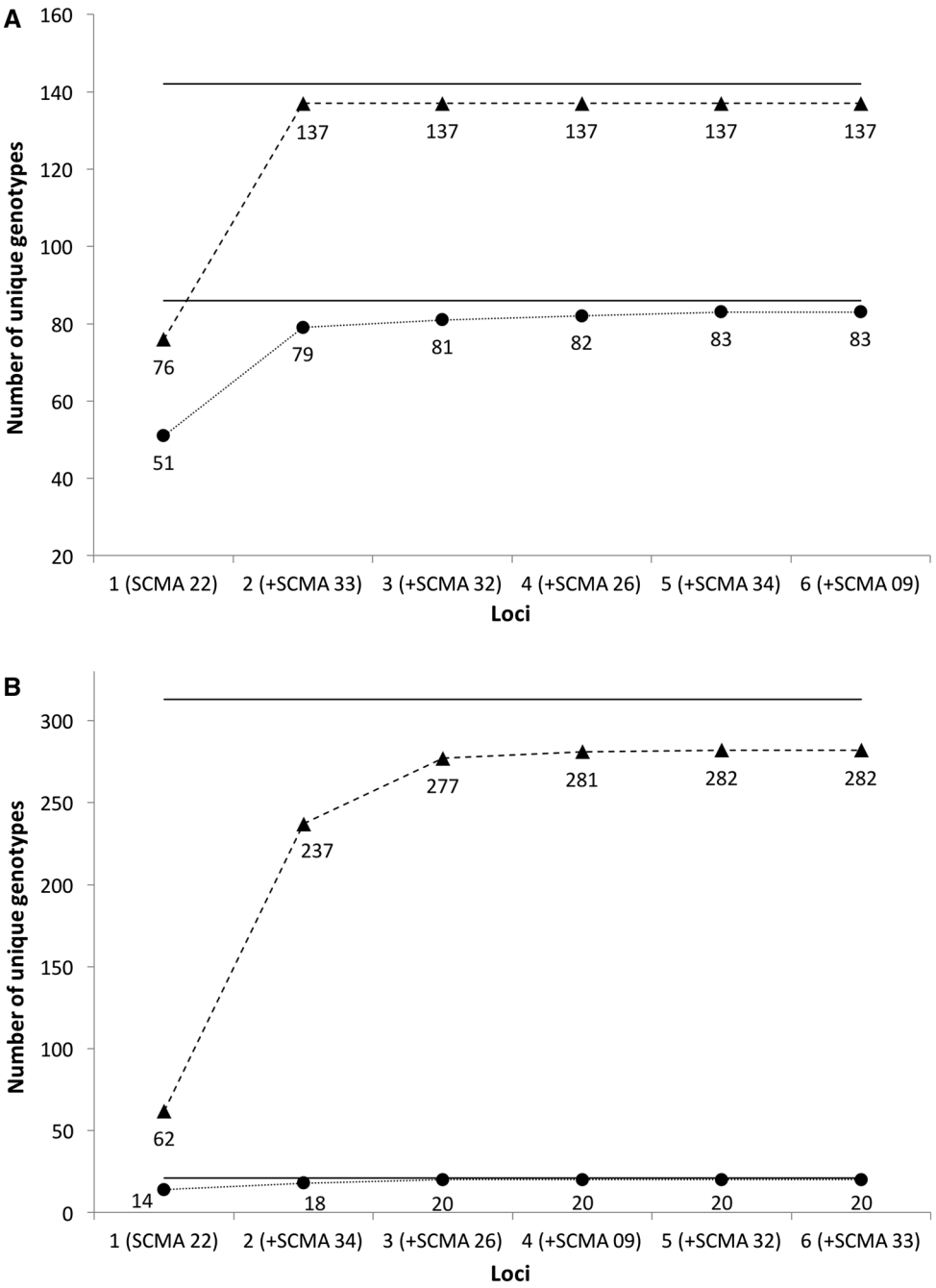

Fig. 2 Recovery of unique multilocus genotypes for increasing combinations of loci for **a** scarlet macaw (*Ara macao*) and **b** red-and-green macaw (*Ara chloropterus*). The order of loci was defined by their number of effective alleles (*from highest to lowest*) for the two species respectively. Triangles (*dashed line*) indicate genotype recovery using only feather samples; and circles (*dotted line*) show recovery when using blood samples from related individuals (including parent/offspring and full siblings)

RGMA feather samples there were 23 matches (total 282 unique genotypes). As expected, across both species the most frequent type of 'recapture' was in the same location from the same sampling event (15). Further matches occurred (a) among or within nests (6), (b) between nests and clay licks (6), and (c) among or within clay licks in different time (9).

Reliability of non-invasive feather samples

Within the 3 km vicinity of TRC we had a comparable number of blood and feather samples from both species to test whether the invasive and non-invasive samples yielded similar population genetic results. We found similar allele frequencies, observed and expected heterozygosities between blood and feather samples of SCMA (Table 2). The genetic distance based AMOVA with the 6 most reliable loci showed no significant differentiation between the two sample types for SCMA ($N = 73$, $F_{ST} < 0.001$, $P = 0.447$) or RGMA ($N = 23$, $F_{ST} < 0.001$, $P = 0.444$), and similar results were yielded with 9 and 11 loci (Table S3). The Shannon's allele frequency based analysis also failed to detect any significant genetic differentiation

Table 2 Population statistics for microsatellite markers on blood and feather samples from scarlet macaw (*Ara macao*) in TRC

# locus	Type	N	N_a	N_e	H_O	H_E	F
6 loci	Blood	27.8 ± 0.1	12.1 ± 1.0	7.70 ± 0.8	0.873 ± 0.030	0.863 ± 0.012	−0.010 ± 0.025
	Feather	44.3 ± 0.2	14.1 ± 0.9	9.01 ± 1.0	0.872 ± 0.015	0.880 ± 0.015	0.008 ± 0.020
9 loci	Blood	27.8 ± 0.1	11.8 ± 0.8	7.52 ± 0.5	0.892 ± 0.021	0.862 ± 0.007	−0.033 ± 0.020
	Feather	43.1 ± 1.1	13.7 ± 0.7	8.88 ± 0.6	0.850 ± 0.023	0.881 ± 0.010	0.034 ± 0.027
11 loci	Blood	27.9 ± 0.0	12.1 ± 0.6	7.86 ± 0.5	0.898 ± 0.018	0.868 ± 0.007	−0.034 ± 0.016
	Feather	42.5 ± 1.0	14.0 ± 0.6	9.13 ± 0.5	0.841 ± 0.021	0.885 ± 0.008	0.049 ± 0.025

Presented are number of loci used in the analysis, type of samples, number of samples (N), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (F). Numbers are mean values ± SE

(Table S3). Across all the samples we also found no significant genetic differences between samples from nests and clay licks for SCMA (AMOVA: N = 174, $F_{ST} < 0.001$, $F'_{ST} = 0.049$, $P = 0.326$).

Discussion

We have developed, validated, and applied a genetic tagging method for feather samples collected from wild populations of two sympatric macaw species in the southeastern Peruvian Amazon. Our results demonstrate that feathers are valuable sources of DNA for genetic tagging as tested using 11 highly variable microsatellite loci.

Feather sampling in a tropical environment

Feathers are a promising non-invasive source of DNA but there are some conflicting views on their utility (Katzner et al. 2012; McDonald and Griffith 2011; McDonald and Griffith 2012). In their experimental setup with feathers of domestic goose, *Anser anser domesticus*, Vili et al. (2013) showed that humidity, direct sunlight, and heat have the most degrading effect on feather DNA quality. As expected, not all feathers collected from our tropical study site yielded sufficient and high enough quality DNA for molecular sexing and genetic tagging. Gebhardt et al. (2009) found that molted macaw feathers at clay licks provide promising DNA samples, but they also reported a high error rate in molecular sexing of samples (Gebhardt and Waits 2008b). Here we found by logistic regression analysis that damaged feathers had significantly lower amplification success over intact feathers, consistent with other studies (Gebhardt et al. 2009; Hogan et al. 2008). Despite thoroughly washing the feathers with 70 % ethanol, samples with clay still had significantly lower amplification success. In addition, clay particles appear to inhibit PCR reactions as also observed by Yankson and Steck (2009). Unlike the studies on a large grouse, *Tetrao urogallus* (Segelbacher 2002), or on large macaws, *Ara* spp. (Gebhardt et al. 2009), we found that the size of the feathers did not significantly affect the quality DNA yields.

For future genetic studies using feather samples in tropical environments we recommend (a) collecting only good quality and intact feathers, (b) collecting mainly clean feathers free of clay, and (c) considering feathers in a wide range of size, in order to maximize quality and quantity of DNA. If feathers are stored appropriately (e.g. in dry box with desiccant), the time interval between sample collection and DNA extraction appears to be flexible, at least over a time window of 2-5 years.

Species and sex identifications by molted feathers

Correct species identification remains the critical first step when relying on feathers for population genetic analysis or genetic tagging (Rudnick et al. 2007). Identification of species by the morphology and color of feather samples can be challenging, and in our study we initially misidentified almost one-third of our feather samples in the field. DNA barcoding, mainly based on mtDNA COI gene, is the standard genetic technique for species identification (Abe et al. 2012; Tavares and Baker 2008). However, in this study we were able to distinguish species using nuclear DNA and the same multilocus genotyping methods employed for our population genetic analyses. This minimized the need for DNA sequencing, reducing the cost of the project.

Although molecular sex typing of birds initially required a blood sample (Fridolfsson and Ellegren 1999; Griffiths et al. 1998) primers are now available for freshly plucked or collected feathers (Bosnjak et al. 2013; Ong and Vellayan 2008; Presti et al. 2013). Typically the molecular sexing of birds targets sex specific DNA fragments that are visualized on agarose gel electrophoresis, providing a low cost and simple laboratory assay (Miyaki et al. 1998; Ong and Vellayan 2008). For this technique the DNA rich avian blood with nucleated erythrocytes is usually used (Fridolfsson and Ellegren 1999; Griffiths et al. 1998). However, other studies have successfully applied the method to

plucked feathers from captive birds (Bosnjak et al. 2013; Ong and Vellayan 2008). Gebhardt and Waits (2008b) even evaluated the performance of primer sets on molted feathers of SCMA and reported high overall error rates and high dropout rates. Presti et al. (2013) also found that most primers did not amplify well on molted macaw feathers and suggested the use of primers amplifying even shorter PCR fragments. With our new primers that target a shorter PCR fragment, we were able to confidently identify sexes in 84 % of SCMA and 94 % of RGMA molted feather samples.

Genetic tagging, tracking macaws without capture

The probability of identity values calculated for siblings in our dataset of SCMA and RGMA indicated that five or six of the most variable loci were enough to recover unique genotypes for the two species respectively, given our sample sizes. We confirmed this empirically by comparing the number of unique genotypes recovered for increasing combinations of loci, including previously genotyped SCMA blood samples with many related individuals, e.g. parent/offspring and full siblings (Fig. 2a).

Our study recovered a total of 36 genotype matches among samples, and according to the *PI* values and the genotype recovery rates we were confident that these were 'recaptures' of the same individuals. Recaptures found between blood and feather samples further demonstrate the feasibility of this technique. Adult SCMAs are often observed feeding their chicks with seeds mixed with clay, and crop samples of these chicks showed high content of clay (Brightsmith et al. 2010; Cornejo et al. 2011). We suspected that adult macaws visit the nearest clay licks to their nests for sodium supplementation but no evidence has been shown to confirm this (D.J. Brightsmith, pers. comm.). In the present study we found genetic evidence that juvenile SCMAs returned to their fledging site and used the nearest clay lick (e.g. feathers of fledglings from the nests Amor & Franz were recovered at the nearest clay lick to the nests in the next year; Fig S3).

Our ability in this study to recover individual genotypes with 5–6 strategically chosen informative markers demonstrates the potential for population and individual-based genetic studies in macaws, which can help better understand their movements in subsequent analyses. We have previously observed at least four banded breeding pairs of SCMA returning to their nesting site in subsequent breeding seasons in TRC, often re-using the same nest hollows (G. Olah, pers. obs.). In this study we were able to confirm the re-use of the same nests for breeding around TRC by the genetic tagging analysis. Berkunsky and Reboreda (2009) also showed high nest fidelity of blue-fronted parrots, *Amazona aestiva*, based on observation of banded females. This behavior of secondary cavity nesting parrots could reflect preferences for nests associated with better characteristics. SCMA has also been showed to prefer nesting in cavities (or artificial nests) with higher previous success (Olah et al. 2014).

We compared blood versus feather samples, and samples sourced from/around nests versus samples from clay licks, and found no genetic differentiation between these groups. These findings further show that feathers can indeed be considered as representative samples of the local population. The microsatellite markers of this study were originally designed from the full genome sequence of SCMA (Seabury et al. 2013), hence we were able to select highly variable di-nucleotide repeats for SCMA that also showed variability for the closely related RGMA (Olah et al. 2015). However, the mean numbers of alleles, effective alleles, observed and expected heterozygosity (Table 1) were lower in RGMA (cogenic species) than in the focal species (SCMA), possibly due to ascertainment bias (Ellegren et al. 1997; Peakall et al. 1998). With these possible limitations in mind, the genetic tagging technique developed for these macaws will be widely applicable to other related species of higher conservation concern. In addition, for many threatened parrots the non-invasive genetic sampling of molted feathers may be the only available DNA source and its use can also help to address the ethical concerns of catching wild individuals.

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