Conserv Genet (2017) 18:1037–1046
DOI 10.1007/s10592-017-0954-6

RESEARCH ARTICLE

The application of non-invasive genetic tagging reveals new insights into the clay lick use by macaws in the Peruvian Amazon

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

Received: 30 October 2016 / Accepted: 9 March 2017 / Published online: 26 April 2017

Abstract Genetic tagging, the unique identification of individuals by their DNA profile, has proven to be an effective method for research on several animal species. In this study we apply non-invasive genetic tagging from feather samples to reveal the genetic structure and estimate local population size of red-and-green macaws (*Ara chloropterus*) without the need to capture these animals. The study was centered in the Tambopata region of the Peruvian Amazon. Here macaws frequently visit clay licks and their naturally molted feathers provide a unique source of non-invasively sampled DNA. We analyzed 249 feathers using nine microsatellite loci and identified 221 unique genotypes. The remainder revealed 21 individuals which were 'recaptured' one or more times. Using a capture-mark-recapture model the average number of different individuals visiting clay licks within one breeding season was estimated to fall between 84 and 316 individuals per clay lick. Analysis of population genetic structure revealed only small genetic differences among regions and clay licks, suggesting a single red-and-green macaw genetic population. Our study confirms the utility of non-invasive genetic tagging in harsh tropical environment to obtain crucial population parameters about an abundant parrot species that is very difficult to capture in the wild.

Keywords Parrot · *Ara chloropterus* · Clay lick · Feather · Genetic tagging · Microsatellite · CMR

Scientific video abstract featuring authors and main results of the study can be found in the link (https://youtu.be/knjkWi-Ftww).

Electronic supplementary material The online version of this article (doi:10.1007/s10592-017-0954-6) contains supplementary material, which is available to authorized users.

✉ George Olah
george.olah@anu.edu.au

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

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

[3] Department of Veterinary Pathobiology, Schubot Exotic Bird Health Center, Texas A&M University, College Station, TX 77845-4467, USA

Introduction

A high proportion of the 398 extant species of parrots (28%) are classified as threatened (IUCN 2014). The major threats faced by these birds include habitat destruction and fragmentation, poaching, and invasive alien species (Olah et al. 2016a; Owens and Bennett 2000; Pires 2012). Most parrots are forest dependent secondary cavity nesters, hence forest destruction decreases the availability of nesting sites and therefore reproductive success, and it can also lead to the loss of key food resources (Brightsmith 2005; Forshaw 2011). Stochastic factors in small and fragmented parrot populations can also cause extreme population decline, for example a hurricane caused significant reduction of the critically endangered Puerto Rican parrot, *Amazona vittata* (Wunderle 1999). Small populations may also suffer loss of genetic diversity and inbreeding depression. The reduced viability of such small populations in the face of environmental changes could consequently drive them towards extinction (Frankham et al. 2004). These processes were identified in the last population of Spix's macaw, *Cyanopsitta spixii*, which is now extinct in the wild (Caparroz et al. 2001).

The first step towards the conservation of any species in the wild is the acquisition of information about its biology.

For example, knowledge of a species' home range, population size, and dispersal patterns can be crucial for management. Tracking individual birds by satellite telemetry tags is often used to obtain information about their home range, but this technique is still limited by the number of birds that can be tagged affordably and remains most feasible for larger species (Groom et al. 2015; Limiñana et al. 2015; Webster et al. 2002; but see Bridge et al. 2011). Traditional methods for population size estimates require individual 'tagging' of the animals for capture-mark-recapture (CMR) methods. In populations of birds this is normally achieved by capturing individuals and tagging them with leg bands, but capture/recapture of the required number of individuals for a robust population size estimate is far from straight forward for many avian, amphibian, and fish monitoring studies (Pollock et al. 2002; White and Burnham 1999). Dispersal patterns hold important information about the study species but they often remain undetected without genetic tools, for example as calculated by isolation-by-distance and related methods to infer the extent of dispersal (Prugnolle and de Meeus 2002; Smith et al. 2016; Wright et al. 2005).

Genetic tagging, the unique identification of individuals by their DNA profile, has proven to be a highly effective method in molecular ecology, offering a powerful tool for 'tagging' whales (Palsboll et al. 1997), fishes (Andreou et al. 2012; Sekino et al. 2005), amphibians (Ringler et al. 2015), seals (Hoffman et al. 2006), bears (Woods et al. 1999), and small mammals (Peakall et al. 2006; Ruibal et al. 2010). DNA can now be sourced non-invasively even from footprints, as it was shown for an arctic fox (Dalén et al. 2007). In populations of birds, non-invasive sampling of feathers is a potential source of avian DNA (Bush et al. 2011; Horváth et al. 2005). Naturally shed feathers of eagles have been used to identify and monitor adult birds individually (Bulut et al. 2016; Rudnick et al. 2005). Other studies using feathers of a large grouse species, *Tetrao urogallus* have demonstrated the value of genetic tagging for estimating local population size (Jacob et al. 2009; Mollet et al. 2015; Moran-Luis et al. 2014; Rudnick et al. 2008; Segelbacher 2002). Taking advantage of the new high throughput sequencing techniques, feathers and blood samples have been used from Wilson's warbler, *Cardellina pusilla* to monitor its migration patterns by SNP data (Ruegg et al. 2014). However, similar techniques have not been used to estimate population size or other demographic parameters in parrots.

Here we use non-invasive genetic tagging in combination with mark-recapture modeling to infer demographic information for the red-and-green macaw, *Ara chloropterus*. In the Peruvian Amazon, where our study was based, these birds are abundant and visit so called clay licks (or colpas) to consume soils rich in sodium (Brightsmith 2004; Powell et al. 2009). However, it is not known if these clay licks act as 'magnets', in effect drawing in birds from vast areas, or whether they simply facilitate feeding aggregations of local populations. Hundreds of naturally dropped feathers are left behind at clay licks, offering excellent opportunities to obtain non-invasive DNA samples (Gebhardt et al. 2009). In turn, genetic tagging from these samples potentially allows one to obtain species-specific information on clay lick use by individuals and populations that are not easily captured in large numbers.

In this paper we use more than 200 genetic samples collected from feathers at clay licks to (1) test for genetic differentiation and possible isolation-by-distance among birds using different clay licks; (2) compare the proportions of males and females using the clay licks over time and possible relatedness among groups; (3) use non-invasive genetic tagging to gain information about the local movements of the macaws; and (4) estimate the number of birds visiting these clay licks.

Methods

Target species and study sites

The red-and-green macaw has a large range over South America from southern Panama to southeastern Paraguay. They mainly reside in subtropical and tropical moist lowland and montane rainforest from sea level to 1000 m (Forshaw 2011) and nest in hollows of emergent trees (Brightsmith 2005; Renton and Brightsmith 2009).

Our study area was situated in lowland rainforest of the southeastern Peruvian Amazon that receives an average annual rainfall of 3200 mm (Brightsmith 2004). Our study area was distributed in both protected (Tambopata National Reserve, Bahuaja-Sonene National Park, Los Amigos Conservation Concession) and unprotected areas (Rio Madre de Dios, Rio Las Piedras; Fig. 1). The areas with highest human populations in the region include Puerto Maldonado and other settlements along the Inter-oceanic highway (Baraloto et al. 2015; Conover 2003).

Sample collection

Our study area falls within the region of South America with the highest known number of clay licks that are regularly used by animals (Lee et al. 2010), offering an opportunity to sample molted feathers non-invasively and repeatedly. Furthermore, many aspects of clay lick ecology have been already studied in Tambopata including the distribution of clay licks (Lee et al. 2010), parrot behavior at clay licks (Brightsmith and Villalobos 2011; Burger and Gochfeld 2003), clay lick preference (Powell et al. 2009), soil characteristics (Brightsmith and Aramburú Muñoz-Najar

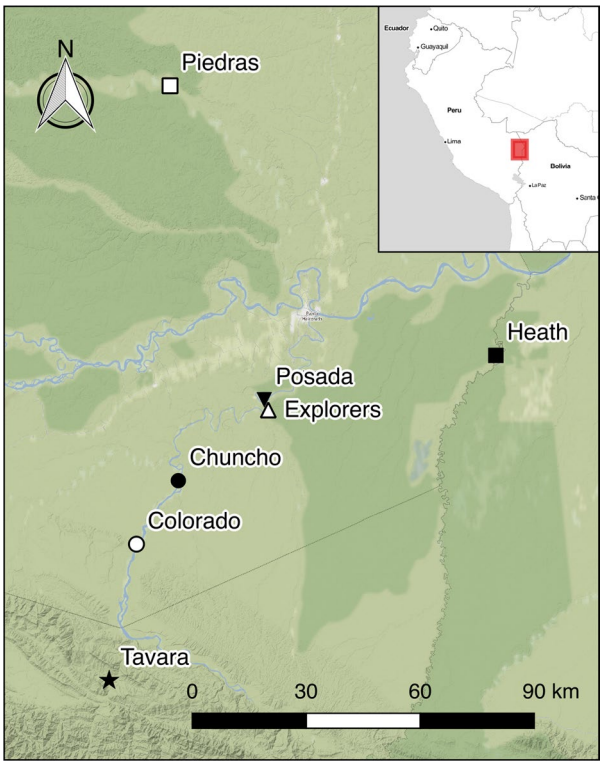

Fig. 1 Sampling locations of molted red-and-green macaw (*Ara chloropterus*) feathers at seven clay licks in southeastern Peru

2004; Brightsmith et al. 2008), and the effect of climate on geophagy (the intentional consumption of soil; Brightsmith 2004). However, we know very little about population sizes, sex ratio, and parrot density around clay licks.

In this study we collected a total of 249 molted contour and flight feathers from seven major clay licks (Fig. 1) that we visited systematically (every 2–4 weeks) during each breeding (rainy) season (November–April) between 2009 and 2012. Prior to the first collection of each season we cleared the feathers of unknown age from the clay licks in order to be more confident of the date range when the feathers were left behind during the collection season. Upon collection, we stored samples individually in paper envelopes in airtight boxes with silica gel to avoid further degradation (Olah et al. 2016b). As 20 parrot species are found in the study area, we provisionally identified the feathers from our target species in the field by shape and color, and later the species was confirmed by genetic techniques in the lab (for more details see Olah et al. 2016b).

Genotyping for individual identification

For DNA extraction from feathers we used the Qiagen DNeasy Blood and Tissue kit (QIAGEN, California) following manufacturer instructions with modifications (Gebhardt et al. 2009). Based on the full genome of scarlet macaw, *Ara macao* (Seabury et al. 2013) we previously developed 30 highly variable di-nucleotide microsatellite loci for the same species and also for red-and-green macaws (Olah et al. 2015). We selected nine microsatellite markers (SCMA 09, SCMA 14, SCMA 22, SCMA 26, SCMA 30, SCMA 31, SCMA 32, SCMA 33, and SCMA 34) *a priori* out of our set of 30 markers, based on how well they performed (usually shorter in bp size) on more degraded DNA from feather samples (Olah et al. 2016b), and then constructed full genotypes. M13 PCR tags were attached to all forward primers and we amplified all loci individually (for more details see Olah et al. 2016b). We previously tested the nine markers for Hardy–Weinberg Equilibrium (HWE), null alleles, and for amplification failures and genotyping error rates (Olah et al. 2016b, 2015). The selected loci showed no indication of null alleles, 95% amplification success and low error rates (2.7% on average across all loci).

We also demonstrated the power of the nine loci used, with the estimates of probability of identity. Only six of these loci were required to recover unique genotypes in the sampled red-and-green macaw population ($PI_{\text{sibs}(6)} = 0.003$), hence the probability of finding exact matches at nine loci among different individuals was extremely low. Furthermore, all of the genotype matches were manually checked locus by locus, as were all near genotype matches that differed at up to three loci. Finally, as a further check, we re-amplified all samples with full genotype matches.

Genetic differentiation among clay licks and isolation-by-distance

We used GenAlEx 6.502 (Peakall and Smouse 2006, 2012) to perform the population genetic analyses, unless otherwise stated. To test for genetic differentiation among macaws using clay licks, we used the analysis of molecular variance (AMOVA) framework, with all duplicated genotypes excluded from this analysis. We defined seven 'populations' of samples *a priori*, corresponding to the seven clay licks (Tavara, Colorado, Chuncho, Explorers, Posada, Heath, and Piedras; Fig. 1), and pooled all samples from a clay lick across the sampling intervals.

The AMOVA analysis provided estimates of overall and pairwise population genetic differentiations (F_{ST}) (Wright 1965; Excoffier, Smouse & Quattro 1992; Peakall, Smouse & Huff 1995), and their standardized [0,1] equivalents (Meirmans 2006; Meirmans and Hedrick 2011; Peakall and Smouse 2012). We performed tests for departure from the null hypothesis of no genetic differentiation by using 10,000 random permutations, and interpolated loci with missing data within each population (Peakall and Smouse 2006). We also tested isolation-by-distance across the study site by using a Mantel test (Mantel 1967) with

departure from the null hypothesis of no significant relationship between genetic and geographical distances tested by 10,000 random permutations at the individual level (Smouse and Long 1992; Smouse et al. 1986).

Sex ratios and relatedness of individuals at clay licks

Molecular sexing of samples was conducted using the P8_SCMA_F/P2_SCMA_R primers, as part of the genotyping runs (Olah et al. 2016b). A G-test was performed to compare the proportion of males and females across clay licks (Peakall and Smouse 2012).

It is possible that family groups may use the same clay licks. Therefore, to test for this possibility we calculated *r* pairwise relatedness estimates (Lynch and Ritland 1999) for each pair of individuals and also the mean pairwise relatedness for each clay lick with 95% confidence intervals around the mean pairwise *r* values estimated via bootstrapping (Beck et al. 2008). We also used random permutation of the data set to generate a distribution for the null hypothesis of no relatedness among individuals within groups and to provide a test for significance. All bootstrapping and permutation tests were performed 1,000 times. We presented the means for the pairwise relatedness estimates and included a control group of 20 individuals from seven known family groups (nests), which were previously genotyped (Olah et al. 2015).

Genetic recaptures and population size estimates at clay licks

In our dataset we treated each genotype as a 'capture' and when an identical genotype was found we considered it a 'recapture'. By definition, the minimum time between sampling events was 14 days. If two sampling events at the same clay lick occurred within this time frame we considered them as a single sampling event. We derived estimates of population sizes at clay licks (the number of individuals visiting the same clay lick) using two capture-mark-recapture (CMR) modelling approaches. As genetic recaptures occurred at various clay licks in different seasons, we used subsets of our genetic data for best fitting the assumptions of each model.

We used the program CAPWIRE (Miller et al. 2005) that allows the use of multiple detections of individuals within a single sampling session and accounts for heterogeneous feather molting patterns among individuals. This way CAPWIRE was able to incorporate recapture events that occurred in the same sampling session while also modeling capture heterogeneity. We summarized each dataset into the total number of observations made for each individual over the sampling period (e.g., also used the total number of feathers without recaptures) and these dataset were fitted using two model types. The (1) Two Innate Rates Models (TIRMs) view populations as a mixture of two types of individuals, the seldom caught type and the often caught type. This model type is used to estimate the probability that an individual belongs to a mixture and assigns individuals to a type. In the (2) Equal Capture Probability Model (ECM) all individuals are equally likely to be captured. A likelihood ratio test (LRT) was used to determine the most appropriate model and 95% confidence intervals about the mean were estimated with 1000 bootstrap replicates. We used CAPWIRE to estimate the number of birds using the Posada and Explorers clay licks (pooled together as Lower Tambopata complex because of their close proximity), and the Chuncho clay lick, using pooled data over the whole study period. These sites were selected to take advantage of their high recapture events even within single sessions over the whole study period. It has been shown by simulations that population size estimates based on single-session sampling (e.g., in CAPWIRE) are as reliable as other estimates (e.g., MARK) based on multisession sampling (Petit and Valiere 2006; Puechmaille and Petit 2007).

We also used conventional closed CMR models (null M_0, temporal M_t, behavioral M_b, and heterogeneity M_h) in the program MARK 8.0 (White and Burnham 1999). Closed population models were selected *a priori* because the fundamental assumptions of demographic (births and deaths) and geographic (migration in or out) closure were reasonable for this species. We only used this model to estimate the population size at the Lower Tambopata complex, as we found the highest number of recaptures here from different sessions but within a single closed period, providing sufficient data for this analysis. Here we used data from one breeding season (December 2010–April 2011) to avoid the violation of the assumptions of closure. Deaths are likely to be minimal during this interval in this long living species (>50 years; Brouwer et al. 2000). Migration was assumed to be negligible within the 5-month interval consistent with observations that individuals are using the same areas for nesting every year (D. J. Brightsmith, pers. obs.).

Results

Genetic differentiation among clay licks and isolation-by-distance

Across all clay licks we found that the allele number ranged from 9 to 17 per locus and the overall mean expected heterozygosity was 0.725. The observed heterozygosity values per clay lick ranged from 0.666 to 0.747 (Table 1). The AMOVA test attributed 1% of the variation among the seven clay licks ($F_{ST}=0.006$, $F'_{ST}=0.025$, $P=0.004$). This result indicates that there were small but statistically

Table 1 Genetic variation of red-and-green macaws (*Ara chloropterus*) at seven clay licks in southeastern Peru, based on nine polymorphic microsatellite loci

Clay lick	$N_{feather}$	N	N_{Female}	N_{Male}	N_u	Na	Ne	H_O	H_E	F	*CAPWIRE*	*MARK*
Tavara	23	22	8	10	4	7.44 ± 0.72	4.12 ± 0.47	0.711 ± 0.035* (1)	0.731 ± 0.031	0.02 ± 0.02	–	–
Colorado	13	10	2	8	–	5.88 ± 0.56	3.47 ± 0.61	0.688 ± 0.061* (1)	0.652 ± 0.044	−0.0 ± 0.06	–	–
Chuncho	120	114	41	69	4	10.1 ± 0.77	4.85 ± 0.74	0.73 ± 0.041* (1)	0.749 ± 0.04	0.02 ± 0.02	316 (221–486)	–
Explorers	30	18	6	9	3	6.55 ± 0.70	4.43 ± 0.63	0.666 ± 0.067* (1)	0.719 ± 0.052	0.06 ± 0.07		
Posada	15	12	6	6	–	6.44 ± 0.64	4.06 ± 0.38	0.747 ± 0.047	0.734 ± 0.027	−0.0 ± 0.05	89 (52–122)	84 (47–202)
Heath	23	23	7	14	2	7.55 ± 0.91	4.44 ± 0.48	0.685 ± 0.047	0.75 ± 0.029	0.08 ± 0.04	–	–
Piedras	25	22	7	13	2	8.22 ± 0.83	4.40 ± 0.57	0.716 ± 0.043* (1)	0.74 ± 0.035	0.03 ± 0.03	–	–
Total	249	221	77	129	15	7.46 ± 0.31	4.25 ± 0.21	0.706 ± 0.018	0.725 ± 0.014	0.02 ± 0.01		

Number of feathers sampled ($N_{feather}$), number of unique genotypes (N), number of females (N_{Female}), males (N_{Male}), and unknown sex (N_u), number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F), population size estimates (number of individuals and 95% CI) by CAPWIRE and MARK are given. Clay licks are arranged from south to north

*Significant ($P < 0.05$) departure from HWE, with the number of loci given in parentheses

significant genetic differences among the birds visiting these clay licks (Table 1). The pairwise comparisons of F_{ST} values of clay licks showed that the Tavara and Heath clay licks were the most significantly different from other clay licks, while the Colorado and Posada clay licks were not significantly different from any others (Table S1). At the individual genotype level, we found low but significant isolation-by-distance across the whole study area (Mantel test > 160 km, N = 221, $r = 0.089$, $P = 0.009$).

Sex ratios and relatedness of individuals at clay licks

We identified 129 males and 77 females (15 samples could not be sex typed) with unique genotypes (Table 1). In total we found an overall significant bias towards males when the samples from all clay licks were pooled ($N_{males} = 129$, $N_{females} = 77$, $G = 13.27$, $df = 1$, $P < 0.001$). However, we detected no significant differences in sex ratios when each clay lick was considered individually ($G = 2.83$, $df = 6$, $P = 0.83$). The mean pairwise relatedness estimates did not indicate higher than expected relatedness at any clay licks, except in the control group consisting of known related individuals (Fig. 2).

Genetic recaptures by non-invasive genetic tagging

Out of a total of 249 feather samples collected from clay licks we found 21 matching genotypes (Fig. 3). There were 16 cases with 2 matching feathers, 3 cases with 3 matching feathers, and a further 2 cases with 4 matching feathers, resulting in 221 unique genotypes in total. 12 of the 21 genotype matches occurred in the same location and the same sampling event within the 2–4-week window (8 males and 4 females). The other nine genotype matches (six males and three females) represented recaptures between different sampling events or different clay licks (Fig. 3).

All 21 samples were re-amplified to confirm repeatability with 19 of 21 samples giving a perfect match across all nine loci. One sample showed a one allele mismatch at two loci and another sample at one locus. The choice of nine loci therefore allowed the matching of genotypes, even allowing for some errors. Fig. S1 plots the frequency distribution of linear codominant genetic distances (Smouse and Peakall 1999) across all the samples, and shows a large gap between complete genotype matches and the average genetic distances, equivalent to genetic differences at more than four loci on average. Thus we can be confident about the reliability of our results.

Population size estimates at clay licks

The temporal model (M_t) gave the most parsimonious model in MARK as determined by the Akaike information

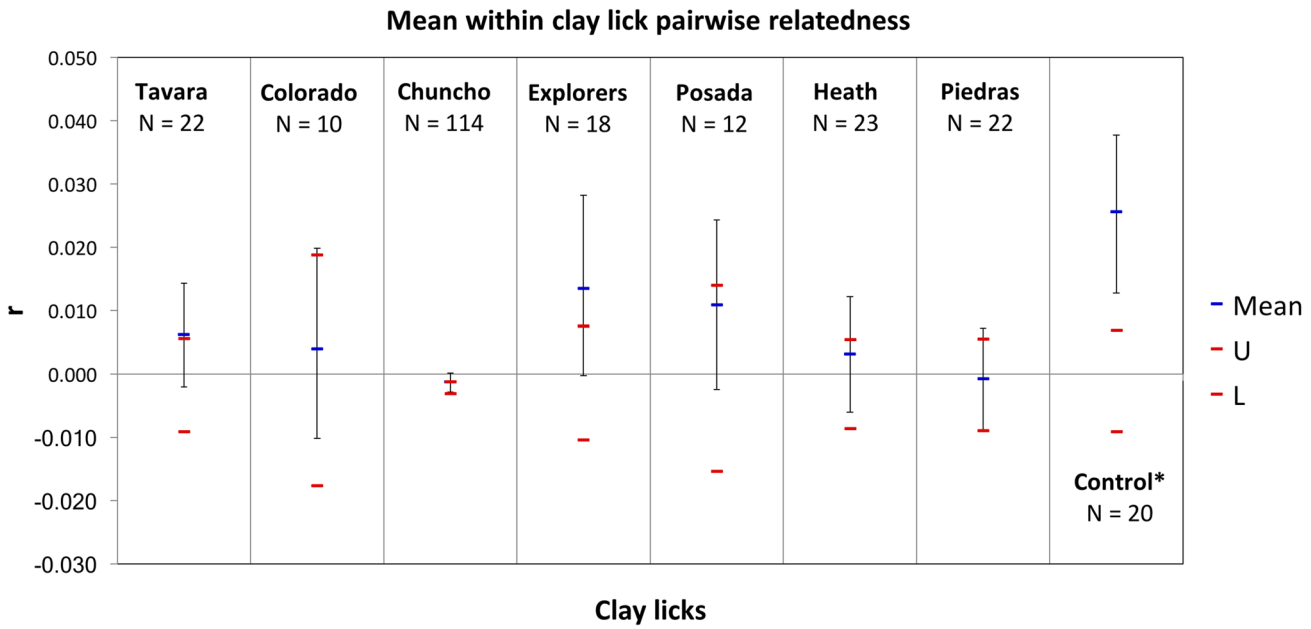

Fig. 2 Pairwise relatedness estimates of Lynch and Ritland (1999) among clay licks of red-and-green macaw (*Ara chloropterus*). *Red lines* represent permuted 95% confidence intervals—upper (U) and lower (L)—around the null hypothesis of zero relatedness and error bars represent bootstrapped confidence intervals around the mean.*Control group includes known related individuals

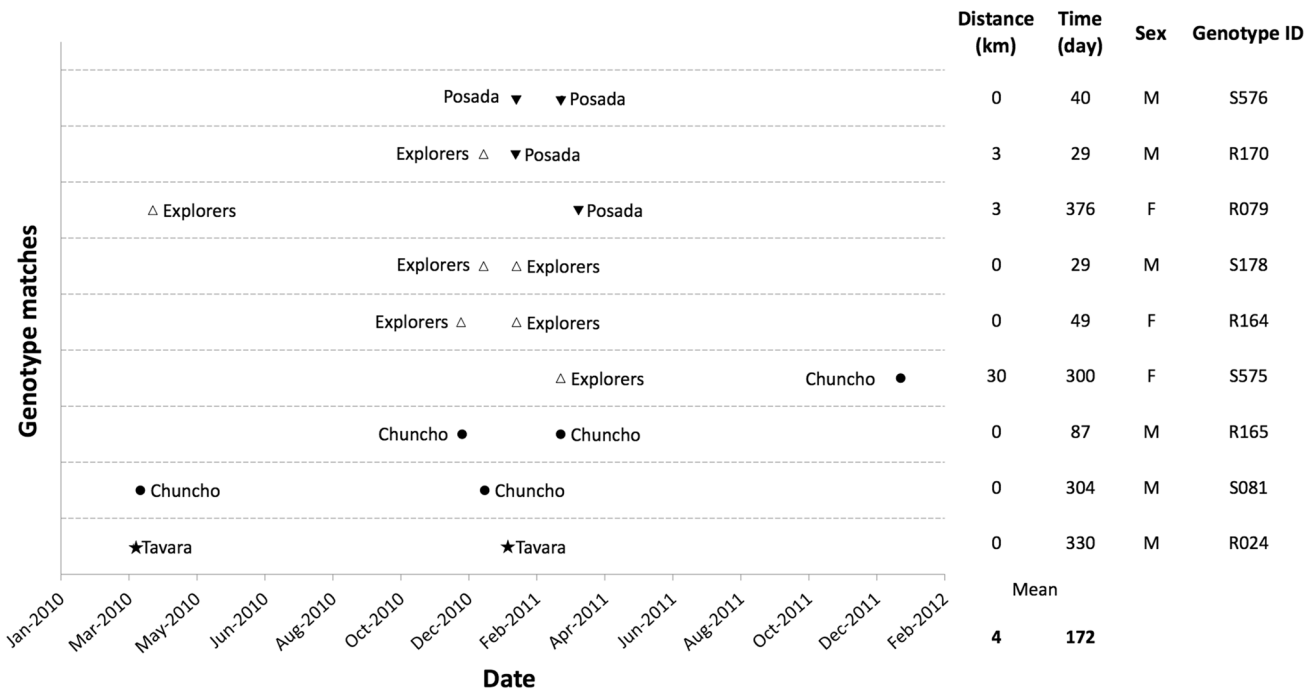

Fig. 3 Complete genotype matches of red-and-green macaws (*Ara chloropterus*) in Tambopata, Peru showing recoveries in different sampling events, the distance (km) and the time interval (day) between recaptures, the sex of the bird, and genotype ID. Only those nine genotype matches are indicated that were recovered in a different location or at a different time. Symbols of the clay licks correspond to Fig. 1

criterion (AIC). This model estimated the population size of the Lower Tambopata complex (Posada + Explorers) to be 84 individuals (95% CI: 47–202 birds; Table 1). For the same area CAPWIRE estimated 89 individuals (95% CI: 52–122 birds; Table 1). For the much larger Chuncho clay lick the CAPWIRE program provided an estimate of 316 individuals (95% CI: 221–486 birds; Table 1).

Discussion

In this study we used 249 red-and-green macaw feather genotypes collected over three years along more than 1000 km of riparian habitat in the Peruvian Amazon. Using nine hypervariable microsatellite genetic markers we were able to detect 21 duplicated genotypes in the landscape. These genetic data provided novel information about the clay lick use of the enigmatic red-and-green macaw. Below we evaluate our findings, compare the outcomes with the literature and conclude with consideration of the implications for conservation.

Genetic differentiation among clay licks and isolation-by-distance

We found small but significant genetic differences among the seven clay licks studied. This may indicate that red-and-green macaws are not drawn completely randomly from the population when supplementing their mineral intake at clay licks. However, the AMOVA revealed only low genetic differentiation (1%) over the whole study area. These findings are broadly consistent with two other genetic studies also based on microsatellite markers of macaws but with much lower sample sizes. Marques (2010) reported genetic diversity estimates of red-and-green macaw in Brazil based on six microsatellite loci and 84 samples (H_O ranging from 0.483 to 0.902), low genetic structure (AMOVA: N = 84, 1.6% among populations, $\Phi_{ST} = 0.016$, $P = 0.120$) with possibly high gene flow. Schmidt (2013) described 0.12% of genetic variation among core breeding sites of scarlet macaw, *Ara macao cyanoptera*, in Guatemala up to 35 km apart ($F_{ST} < 0.009$, $P > 0.05$). Our population genetic results also indicated individual genotype level isolation-by-distance across large geographical scales, which could indicate some restriction on dispersal over this large scale (>160 km), or high levels of philopatry for this species.

Sex ratios and relatedness of individuals at clay licks

Our results suggest an overall bias towards the detection of males. Feathers were collected explicitly in the breeding season of the macaws, when females incubate the eggs and care for hatchlings, while males search for food (Nycander et al. 1995). This might explain the overrepresentation of males at clay licks during this period. Further support for this is provided by the observation that at clay licks we found twice as many genotype recaptures of males than females (Fig. 3). We did not find higher than average relatedness among birds at clay licks (Fig. 2), which indicates that family groups might visit several different clay licks. This also supports the applicability of non-invasive genetic sampling at clay licks, as it gives a good representation of the whole population and not just groups of relatives.

Genetic recaptures by non-invasive genetic tagging

While the low number of recaptures might not be sufficient for precise population size estimates, some interesting results were nevertheless revealed from the data. The longest period between recaptures was 376 days, and the longest distance was 30 km, with a mean distance of 4 km. Interestingly, many of the matches occurred in the same sampling event. This result could be due to the simple process of multiple shedding of feathers at the same location and time. Alternatively, as macaws in the tropics seem to shed continuously over the year (D. J. Brightsmith, pers. obs.) it more likely indicates that the same birds make multiple visits to the clay lick during our 2–4-week sampling intervals. At a nearby clay lick in the Manu Biosphere Reserve, close-up photographs of red-and-green macaws were used to identify them by the lines of red facial feathers and beak shape (Munn 1992). Within a month period they found that 40–60% of the photos taken in different days showed same individuals.

Our genotype data confirmed and refined previously known or suspected aspects of red-and-green macaw natural history. For example, we confirmed that the macaws re-used the same clay licks (e.g. Posada, Explorers, Chuncho, Tavara), sometimes even over long periods (e.g., 330 days at Tavara clay lick; Fig. 3). Red-and-green macaws often reuse the same nests over many years (D. J. Brightsmith, pers. obs.), so it was assumed that they would re-use the nearby clay licks too. We repeatedly recovered feathers from 20 red-and-green macaw individuals between the same or nearby clay licks within a three km range (Posada & Explorers) and once between clay licks 30 km apart (Explores & Chuncho). The lack of longer-range recaptures suggests that the macaws at clay licks are drawn from local populations. Recaptures varied from within a month up to a year (Fig. 3).

Population size estimates at clay licks

Estimating population size of species in remote areas can be challenging. Capturing and later re-capturing or sighting uniquely identified macaws in a large tropical landscape

is a difficult task and has not been attempted for macaws before. In our study we used individual genotypes of feathers and samples with matching genotypes to test their efficacy for CMR based population size estimates. We acknowledge that these population estimates are for the numbers of macaws visiting clay licks, which may not equate to population size estimates. Furthermore, given the very low recapture rate in our study, we only used CMR models for two clay licks with the highest number of recaptures in our dataset.

The genetic CMR methods we used provide some of the first estimates of the number of macaws using clay licks over the breeding season. Our 1-month observational data from the Chuncho clay lick with a maximum number of 109 red-and-green macaws observed in one event seems to support the magnitude of our estimate. In general, our population estimates of the number of birds using a clay lick are similar to those in Manu near Tambopata. Here photographic IDs over a month interval yielded estimates of the total number of red-and-green macaws using that clay lick falling between 241 and 282 individuals (Munn 1992).

Future recommendations for non-invasive genetic tagging at clay licks

The amount of information gained from feather samples seems remarkable given the tropical conditions and high likelihood that DNA will degrade quickly. However, to maximize the efficacy of genetic tagging we recommend the following sampling strategy at clay licks: (a) a pilot study should be conducted to determine the most reliable locations for feathers; (b) the focus should be on only a few sampling locations with the highest number of available samples; (c) feathers should be collected on a regular basis (e.g., every week); (d) detailed location of feathers on clay licks should be recorded to be able to differentiate depositing events of feathers with matching genotypes; (e) collected feathers should be handled as recommended by Olah et al. (2016b); and (f) a concentrated sampling effort at the most frequently used clay licks is highly desirable in order to improve population size estimates of the area.

Conclusion

Prior to this study we had limited insights into how red-and-green macaws use the clay licks. Here we found no evidence that red-and-green macaw communities at different clay licks consisted of related individuals. The matching genotypes detected show that individual macaws can use multiple clay licks, and that macaw communities using various clay licks can overlap in space. However, the locations of the genotype recaptures despite sampling over a large study area were highly restricted, indicating that local movements might occur more frequently. Our estimates of the number of different individuals indicate that hundreds but not thousands of macaws locally use clay licks, leading us to conclude that these communities probably represent feeding aggregations drawn from local populations. Thus, with sufficient sampling this technique can be used for population size estimates, especially for species and locations where large number of non-invasive samples are easily available. The distribution of parrot clay licks in South America supports the hypothesis of geophagy due to sodium deficient natural diet (Lee et al. 2010). Parrot geophagy has also been reported in Central America (Valdéz-Peña et al. 2008), Africa (May 2001), and Papua New Guinea (Symes et al. 2006). These sites may offer similar sampling opportunities to our study, suggesting that the same genetic tagging techniques could be used to study clay lick use and population sizes of other species.

Acknowledgements This research was supported by funds from the Rufford Small Grants Foundation, Loro Parque Foundation, Idea Wild, and The Australian National University. Thanks to the Peruvian crew of this project for their enormous help with sample collection and field logistics: Braulio Poje Mishaja, Andres Veras, Jerico Solis, Crissel Vargas, Lizzie Ortiz, Gabriela Vigo, Nancy Carlos, Mabe Aguirre, Gustavo Martinez, the full staff and guides of Rainforest Expeditions, and the volunteers of the Tambopata Macaw Project. Thanks to George Powell and the WWF for providing some extra feather samples from Peru. Thanks for laboratory technical support to Christine Hayes and Cintia Garai. We thank for the laboratory space provided by the Unidad de Biotecnología Molecular, Laboratorio de Investigación y Desarrollo, Universidad Peruana Cayetano Heredia in Lima, Peru. Samples were collected under research permits from the Servicio Nacional de Areas Naturales Protegidas (SERNANP) in Peru. Genetic access to the samples was granted by the Servicio Nacional Forestal y de Fauna Silvestre (SERFOR) in Peru. CITES permits were provided by the Peruvian and Australian authorities (N° 15PE000448/SP, N° PWS2015-AU-001508). We thank to the anonymous reviewers whose constructive suggestions and comments improved the quality of this paper.

References

Andreou D, Vacquie-Garcia J, Cucherousset J, Blanchet S, Gozlan RE, Loot G (2012) Individual genetic tagging for teleosts: an empirical validation and a guideline for ecologists. J Fish Biol 80:181–194. doi:10.1111/j.1095-8649.2011.03165.x

Baraloto C et al (2015) Effects of road infrastructure on forest value across a tri-national Amazonian frontier. Biol Conserv 191:674–681. doi:10.1016/j.biocon.2015.08.024

Beck NR, Peakall R, Heinsohn R (2008) Social constraint and an absence of sex-biased dispersal drive fine-scale genetic structure in white-winged choughs. Mol Ecol 17:4346–4358. doi:10.1111/j.1365-294X.2008.03906.x

Bridge ES et al (2011) Technology on the move: recent and forthcoming innovations for tracking migratory birds. Bioscience 61:689–698. doi:10.1525/bio.2011.61.9.7

Brightsmith DJ (2004) Effects of weather on parrot geophagy in Tambopata, Peru. Wilson Bull 116:134–145

Brightsmith DJ (2005) Parrot nesting in Southeastern Peru: seasonal patterns and keystone trees. Wilson Bull 117:296–305
Brightsmith DJ, Aramburú Muñoz-Najar R (2004) Avian Geophagy and soil characteristics in Southeastern Peru. Biotropica 36:534–543
Brightsmith DJ, Villalobos EM (2011) Parrot behavior at a Peruvian clay lick. Wilson Journal of Ornithology 123:595–602
Brightsmith DJ, Taylor J, Phillips TD (2008) The roles of soil characteristics and toxin adsorption in Avian Geophagy. Biotropica 40:766–774
Brouwer K, Jones ML, King CE, Schifter H (2000) Longevity records for *Psittaciformes* in captivity. Int Zoo Yearb 37:299–316
Bulut Z, Bragin EA, DeWoody JA, Braham MA, Katzner TE, Doyle JM (2016) Use of non-invasive genetics to assess nest and space use by White-tailed Eagles. J Raptor Res 50:351–362
Burger J, Gochfeld M (2003) Parrot behavior at a Rio Manu (Peru) clay lick: temporal patterns, associations and antipredator responses. Acta Ethol 6:23–34
Bush KL et al (2011) Population structure and genetic diversity of greater sage-grouse (*Centrocercus urophasianus*) in fragmented landscapes at the northern edge of their range. Conserv Genet 12:527–542. doi:10.1007/s10592-010-0159-8
Caparroz R, Miyaki CY, Bampi MI, Wajntal A (2001) Analysis of the genetic variability in a sample of the remaining group of Spix's Macaw (*Cyanopsitta spixii, Psittaciformes: Aves*) by DNA fingerprinting. Biol Conserv 99:307–311
Conover T (2003) Perú's long haul: highway to riches, or ruin? Natl Geogr 203:80–100
Dalén L, Götherström A, Meijer T, Shapiro B (2007) Recovery of DNA from Footprints in the Snow. Can Field-Nat 121:321–324
Forshaw JM (2011) Parrots of the world. vol Accessed from http://nla.gov.au/nla.cat-vn4839547. CSIRO Publishing, Collingwood
Frankham R, Ballou JD, Briscoe DA (2004) A primer of conservation genetics. Cambridge University Press, Cambridge
Gebhardt KJ, Brightsmith D, Powell G, Waits LP (2009) Molted feathers from clay licks in Peru provide DNA for three large macaws (*Ara ararauna, A. chloropterus*, and *A. macao*). J Field Ornithol 80:183–192
Groom C, Warren K, Le Souef A, Dawson R (2015) Attachment and performance of Argos satellite tracking devices fitted to black cockatoos (*Calyptorhynchus* spp.). Wildlife Research 41:571–583. doi:10.1071/WR14138
Hoffman JI, Trathan PN, Amos W (2006) Genetic tagging reveals extreme site fidelity in territorial male Antarctic fur seals *Arctocephalus gazella*. Mol Ecol 15:3841–3847. doi:10.1111/j.1365-294X.2006.03053.x
Horváth MB, Martínez-Cruz B, Negro JJ, Kalmár L, Godoy JA (2005) An overlooked DNA source for non-invasive genetic analysis in birds. J Avian Biol 36:84–88. doi:10.1111/j.0908-8857.2005.03370.x
IUCN (2014) The IUCN Red List of Threatened Species. Version 2014.2. http://www.iucnredlist.org/.
Jacob G, Debrunner R, Gugerli F, Schmid B, Bollmann K (2009) Field surveys of capercaillie (*Tetrao urogallus*) in the Swiss Alps underestimated local abundance of the species as revealed by genetic analyses of non-invasive samples. Conserv Genet 11:33–44. doi:10.1007/s10592-008-9794-8
Lee ATK, Kumar S, Brightsmith DJ, Marsden SJ (2010) Parrot clay-lick distribution in South America: do patterns of "where" help answer the question "why"? Ecography 33:1–4
Limiñana R, Arroyo B, Terraube J, McGrady M, Mougeot F (2015) Using satellite telemetry and environmental niche modelling to inform conservation targets for a long-distance migratory raptor in its wintering grounds. Oryx 49:329–337. doi:10.1017/S0030605313001075
Lynch M, Ritland K (1999) Estimation of pairwise relatedness with molecular markers. Genetics 152:1753–1766
Mantel N (1967) The Detection of disease clustering and a generalized regression approach. Cancer Res 27:209–220
Marques ARdO (2010) Caracterização da estrutura genética populacional das araras vermelhas Ara chloropterus e Ara macao (Psittaciformes, *Aves*). University of São Paulo, São Paulo
May DL (2001) Grey parrots of the Congo basin forest. PsittaScene 13:8–10
Meirmans PG (2006) Using the AMOVA framework to estimate a standardized genetic differentiation measure. Evol Int J Org Evol 60:2399–2402. doi:10.1111/j.0014-3820.2006.tb01874.x
Meirmans PG, Hedrick PW (2011) Assessing population structure: FST and related measures. Mol Ecol Resour 11:5–18. doi:10.1111/j.1755-0998.2010.02927.x
Miller CR, Joyce P, Waits LP (2005) A new method for estimating the size of small populations from genetic mark–recapture data. Mol Ecol 14:1991–2005. doi:10.1111/j.1365-294X.2005.02577.x
Mollet P, Kéry M, Gardner B, Pasinelli G, Royle JA (2015) Estimating population size for capercaillie (*Tetrao urogallus* L.) with spatial capture-recapture models based on genotypes from one field sample. PLoS ONE 10:e0129020. doi:10.1371/journal.pone.0129020
Moran-Luis M et al (2014) Demographic status and genetic tagging of endangered capercaillie in NW Spain. PLoS ONE 9:e99799. doi:10.1371/journal.pone.0099799
Munn CA (1992) Macaw biology and ecotourism, or "When a bird in the bush is worth two in the hand". In: Beissinger SR, Snyder NFR, Munn CA (eds) New world parrots in crisis: solutions from conservation biology. Smithsonian Institution Press, Washington, DC, pp 47–72
Nycander E, Blanco DH, Holle KM, del Campo A, Munn CA, Moscoso JI, Ricalde DG (1995) Manu and Tambopata: nesting success and techniques for increasing reproduction in wild macaws in southeastern Peru. In: Abramson J, Spear BL, Thomsen JB (eds) The large macaws: their care, breeding and conservation. Raintree Publications, Fort Bragg, California, pp 423–443
Olah G, Heinsohn RG, Espinoza JR, Brightsmith DJ, Peakall R (2015) An evaluation of primers for microsatellite markers in Scarlet Macaw (*Ara macao*) and their performance in a Peruvian wild population. Conserv Genet Resour 7:157–159. doi:10.1007/s12686-014-0317-2
Olah G, Butchart SHM, Symes A, Guzmán IM, Cunningham R, Brightsmith DJ, Heinsohn R (2016a) Ecological and socio-economic factors affecting extinction risk in parrots. Biodivers Conserv 25:205–223. doi:10.1007/s10531-015-1036-z
Olah G, Heinsohn RG, Brightsmith DJ, Espinoza JR, Peakall R (2016b) Validation of non-invasive genetic tagging in two large macaw species (*Ara macao* and *A. chloropterus*) of the Peruvian Amazon. Conserv Genet Resour 8:499–509. doi:10.1007/s12686-016-0573-4
Owens IPF, Bennett PM (2000) Ecological basis of extinction risk in birds: Habitat loss versus human persecution and introduced predators. Proc Natl Acad Sci 97:12144–12148. doi:10.1073/pnas.200223397
Palsboll PJ et al (1997) Genetic tagging of humpback whales. Nature 388:767–769
Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295. doi:10.1111/j.1471-8286.2005.01155.x
Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539. doi:10.1093/bioinformatics/bts460
Peakall R, Ebert D, Cunningham R, Lindenmayer D (2006) Mark–recapture by genetic tagging reveals restricted movements by

bush rats (*Rattus fuscipes*) in a fragmented landscape. J Zool 268:207–216. doi:10.1111/j.1469-7998.2005.00011.x

Petit E, Valiere N (2006) Estimating population size with noninvasive capture-mark-recapture data. Conserv Biol 20:1062–1073. doi:10.1111/j.1523-1739.2006.00417.x

Pires SF (2012) The illegal parrot trade: a literature review. Glob Crime 13:176–190

Pollock KH, Nichols JD, Simons TR, Farnsworth GL, Bailey LL, Sauer JR (2002) Large scale wildlife monitoring studies: statistical methods for design and analysis. Environmetrics 13:105–119 doi:10.1002/env.514

Powell LL, Powell TU, Powell GVN, Brightsmith DJ (2009) Parrots Take it with a Grain of Salt: Available Sodium Content May Drive Collpa (Clay Lick) Selection in Southeastern Peru. Biotropica 41:279–282

Prugnolle F, de Meeus T (2002) Inferring sex-biased dispersal from population genetic tools: a review. Heredity 88:161–165

Puechmaille SJ, Petit EJ (2007) Empirical evaluation of non-invasive capture–mark–recapture estimation of population size based on a single sampling session. J Appl Ecol 44:843–852. doi:10.1111/j.1365-2664.2007.01321.x

Renton K, Brightsmith DJ (2009) Cavity use and reproductive success of nesting macaws in lowland forest of southeast Peru. J Field Ornithol 80:1–8

Ringler E, Mangione R, Ringler M (2015) Where have all the tadpoles gone? Individual genetic tracking of amphibian larvae until adulthood. Mol Ecol Resour 15:737–746. doi:10.1111/1755-0998.12345

Rudnick JA, Katzner TE, Bragin EA, Rhodes OE, Dewoody JA (2005) Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan. Mol Ecol 14:2959–2967. doi:10.1111/j.1365-294X.2005.02641.x

Rudnick JA, Katzner TE, Bragin EA, DeWoody (2008) A noninvasive genetic evaluation of population size, natal philopatry, and roosting behavior of non-breeding eastern imperial eagles (*Aquila heliaca*) in central Asia. Conserv Genet 9:667. doi:10.1007/s10592-007-9397-9

Ruegg KC et al (2014) Mapping migration in a songbird using high-resolution genetic markers. Mol Ecol 23:5726–5739. doi:10.1111/mec.12977

Ruibal M, Peakall R, Claridge A, Murray A, Firestone K (2010) Advancement to hair-sampling surveys of a medium-sized mammal: DNA-based individual identification and population estimation of a rare Australian marsupial, the spotted-tailed quoll (*Dasyurus maculatus*). Wildlife Research 37:27–38. doi:10.1071/WR09087

Schmidt KL (2013) Spatial and temporal patterns of genetic variation in scarlet macaws (*Ara macao*): implications for population management in La Selva Maya, Central America. Columbia University, New York

Seabury CM et al. (2013) A multi-platform draft de novo genome assembly and comparative analysis for the Scarlet Macaw (*Ara macao*). PloS ONE 8:e62415–e62415

Segelbacher G (2002) Noninvasive genetic analysis in birds: testing reliability of feather samples. Mol Ecol Notes 2:367–369. doi:10.1046/j.1471-8286.2002.00180.x-i2

Sekino M, Saitoh K, Yamada T, Hara M, Yamashita Y (2005) Genetic tagging of released Japanese flounder (*Paralichthys olivaceus*) based on polymorphic DNA markers. Aquaculture 244:49–61. doi:10.1016/j.aquaculture.2004.11.006

Smith AL, Landguth EL, Bull CM, Banks SC, Gardner MG, Driscoll DA (2016) Dispersal responses override density effects on genetic diversity during post-disturbance succession. Proc R Soc Lond B. doi:10.1098/rspb.2015.2934

Smouse PE, Long JC (1992) Matrix correlation analysis in anthropology and genetics. Am J Phys Anthropol 35:187–213. doi:10.1002/ajpa.1330350608

Smouse PE, Peakall R (1999) Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. Heredity 82:561–573

Smouse PE, Long JC, Sokal RR (1986) Multiple regression and correlation extensions of the mantel test of matrix correspondence. Syst Zool 35:627–632. doi:10.2307/2413122

Symes CT, Hughes JC, Mack AL, Marsden SJ (2006) Geophagy in birds of Crater Mountain Wildlife Management Area, Papua New Guinea. J Zool 268:87–96. doi:10.1111/j.1469-7998.2005.00002.x

Valdéz-Peña RA, Ortiz-Maciel SG, Valdez Juarez SO, Enkerlin Hoeflich EC, Snyder NFR (2008) Use of clay licks by Maroon-fronted Parrots (*Rhynchopsitta terrisi*) in Northern Mexico. Wilson J Ornithol 120:176–180

Webster MS, Marra PP, Haig SM, Bensch S, Holmes RT (2002) Links between worlds: unraveling migratory connectivity. Trends in Ecol Evol 17:76–83. doi:10.1016/S0169-5347(01)02380-1

White GC, Burnham KP (1999) Program MARK: survival estimation from populations of marked animals. Bird Study 46:S120–S139 doi:10.1080/00063659909477239

Woods JG, Paetkau D, Lewis D, McLellan BN, Proctor M, Strobeck C (1999) Genetic tagging of free-ranging black and brown bears. Wildl Soc Bull 27:616–627 doi:10.2307/3784082

Wright TF, Rodriguez AM, Fleischer RC (2005) Vocal dialects, sex-biased dispersal, and microsatellite population structure in the parrot Amazona auropalliata. Mol Ecol 14:1197–1205. doi:10.1111/j.1365-294X.2005.02466.x

Wunderle JM Jr (1999) Pre- and post-hurricane fruit availability: implications for Puerto Rican Parrots in the Luquillo Mountains. Carib J Sci 35:249–264