Ann. Zool. Fennici 45: 211–216 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 26 June 2008 © Finnish Zoological and Botanical Publishing Board 2008

Low genetic diversity in the ground parrot (*Pezoporus wallicus*) revealed by randomly amplified DNA fingerprinting

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Received 9 May 2007, revised version received 9 Jan. 2008, accepted 9 Jan. 2008

Chan, K., Glover. D. R., Ramage, C. M. & Harrison, D. K. 2008: Low genetic diversity in the ground parrot (*Pezoporus wallicus*) revealed by randomly amplified DNA fingerprinting. — *Ann. Zool. Fennici* 45: 211–216.

The ground parrot (*Pezoporus wallicus*) is a vulnerable species that occurs in isolated pockets of heathland and sedgeland of Australia. This study used randomly amplified DNA fingerprinting (RAF) to examine genetic diversity in the eastern population of the ground parrot. The seven primers used produced an average of 68 markers per primer, and the number of unambiguous polymorphic markers per primer averaged 6.3 (9.2%). Overall genetic similarity was 0.978 ± 0.03. The low level of genetic diversity revealed by RAF is comparable to the lower end of diversity found in species that are declared endangered.

Introduction

The ground parrot (*Pezoporus wallicus*) is a vulnerable resident species endemic to Australia. It is unique in that it nests on the ground in fire-adapted heathland and sedgeland. Since European settlement to the east of the continent, populations of the eastern subspecies (*P. w. wallicus*) have experienced a significant reduction of their range; they now occur in isolated pockets corresponding to the fragmented nature of their specialist habitat (Higgins 1999). Conservation attempts have been limited; management practices are based almost solely on controlled burning leaving areas of varying post-fire age to maintain optimal habitat availability (McFarland 1991). Although translocation plans have been considered by state governments, too little about the bird's behavioural ecology is known for any program to be successful. Observational difficulties are related to the animal's elusiveness such that much behavioural information to date has been based on vocalisation recordings (Higgins 1999, Chan & Mudie 2004). Genetic analysis is an obvious alternative, though the only genetic study to date (Leeton *et al*. 1994) has been sequencing of cytochrome *b* to determine the ground parrot's genetic affinity with the related and extremely rare night parrot (*P. occidentalis*). Until now, nothing is known about the genetic variability of the ground parrot.

Species with small populations often have reduced genetic variability, making them susceptible to higher risk of inbreeding depres-

Fig. 1. Geographic distribution of the ground parrot in Australia (shaded) and sample collection sites (in insert).

sion and decreased fitness (Frankham *et al*. 2002). The genetics of such species are often difficult to study as prior knowledge of their genomic DNA sequence is usually non-existent, and suitable and powerful DNA techniques may be required to isolate useful genetic markers. Arbitrarily-primed DNA technologies require no prior knowledge of DNA sequence and therefore markers from any organism can potentially be identified quickly (Williams *et al*. 1990). This is one reason why randomly amplified polymorphic DNA (RAPD), which uses arbitrarily chosen decamer primers to generate DNA profiles, became a common protocol for determining genetic diversity in populations (Harris 1999). Unfortunately, RAPD profiles consist of only a small number of markers which are dominant, which are less susceptible to bias than codominant markers, and problematic reproducibility between laboratories has caused some concern (Jones *et al*. 1997, Pérez *et al*. 1998, Waldron *et al*. 2002). Improved dominant marker systems such as amplified fragment length polymorphism (AFLP) (Vos *et al*. 1995) have superior marker number and reproducibility, but are more technically demanding than RAPD and incur a much higher cost per assay.

Since the genome of ground parrot has not been analysed, we used randomly amplified DNA fingerprinting (RAF) to generate DNA profiles for our analysis of genetic diversity in the species. The technique is essentially a modified RAPD protocol, but has the advantage of generating dominant markers and producing a highly reproducible fingerprint (Peace *et al*. 2003), and that its profiles consist of a greater number of markers per primer comparable to those of AFLP (Waldron *et al* 2002). The RAF technique has been used successfully as a quick and robust dominant marker system for the generation of large numbers of markers in plants (Cunningham *et al*. 2002, Peace *et al*. 2003, Ramage *et al*. 2003, Dillon *et al*. 2005), and has proved useful for the study of insects (Schlipalius *et al*. 2001). The power of RAF was demonstrated by Ramage *et al*. (2003) who were able to distinguish minor mutations in the genome of a clonal plant species (*Garcinia mangostana*). Fingerprints from RAF are simpler to generate and require less genomic DNA than AFLP which necessitates adequate DNA to assess completion of restriction digestion of template DNA. Large volumes of blood can be difficult to obtain from small vertebrates in the field, particularly if the animals are to be released unharmed after handling. Low requirements of DNA quantities, and therefore low requirements of blood volume, make RAF an ideal tool for our preliminary genetic analysis of an elusive species whose genome is uncharacterised.

Material and methods

We targeted the Queensland population, which has been estimated to be as low as 2900 individuals (McFarland 1991). The resident bird is rarely seen, let alone captured. We were able to collect blood samples from 20 individuals at three breeding sites in south-east Queensland after a full year's effort in trapping by mist nets. One site was located in Cooloola National Park approximately 50 km from the other two sites, both of which were located at Noosa National Park (Fig. 1). The two Noosa National Park sites, Weyba and Woodland, were 7 km apart connected by a non-heath vegetated corridor. Noosa and Cooloola National Parks are separated by non-heath bushland and major urban infrastructure.

We collected blood samples (50–100 μl) taken from the brachial vein under the wing. The blood was stored in a Queens lysis buffer (0.01 M Tris base, 0.01 M NaCl, 0.01 M EDTA, and 1% *n*-laurolysarcosine pH 7.5) and placed

on ice immediately and then frozen at –80 °C upon reaching the laboratory. Genomic DNA was extracted in duplicate from each blood sample using DNeasy Tissue Kits (Qiagen) following manufacturer's instructions, with the exception that the amount of Proteinase K was doubled from 20 μl to 40 μl and the 10-min 70 °C digestion was repeated. This protocol was optimised using chicken blood. Genomic DNA extracts were quantified using a 0.7% (w/v) agarose gel in 1 × TBE (100 mM Tris, 100 mM Boric Acid, 2 mM EDTA, pH 8). Genomic DNA was loaded in a loading buffer consisting of 1 × TBE and 6× gel loading buffer (GLB) containing 0.25% (w/v) bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and 100 μg ml^{-1} RNase A (GibcoBRL) in sterile ultra-pure water and subjected to electrophoresis at 100 volts for one hour (5 V cm^{-1}). The DNA was visualised by staining the gel in a solution of ethidium bromide (0.5 mg ml^{-1} milliQ water) for 20–40 min. Non-degraded DNA samples were diluted to a concentration of 30 ng μl^{-1} for further PCR analysis.

The RAF protocol was performed as described by Waldron *et al*. (2002). We used 5.0 μM single 10-mer oligonucleotide primers (Operon Technologies) and 30 ng of genomic DNA template. Each of the seven primers selected for this study acted as both a forward and reverse primer (Table 1). The RAF amplification products were denatured at 94 °C for 3 min, cooled on ice and separated on large 4% (v/v) polyacrylamide sequencing gels containing 7.5M urea in 1-x TBE buffer (100 mM Tris Ultra pure, 100 mM boric acid, 2 mM EDTA, pH 8). Gels were subjected to electrophoresis in 1-x TBE buffer at 100 W for 2 hrs 15 min then transferred to gel blotting paper, dried for 1 hour at 80 °C with vacuum and exposed to X-ray film (Kodak Biomax MR) for 16–20 hours at room temperature.

RAF fingerprints generated from duplicate DNA extracts for each individual bird were transformed into a binary code — amplified DNA fragments (bands) for individual birds were scored as 1 if present and 0 if absent — and then subjected to clustering analysis for assessment. Scoring was considered positive only if present in both duplicate lanes. Genetic relationships were estimated in a hierarchical cluster analysis by the unweighted pair-group method, and an UPGMA dendrogram was obtained using the matrix of genetic distances following Nei and Li (1979). Standard error for the levels of genetic similarity was calculated by hand.

Results

As expected, appreciable differences in band positions was detected between ground parrot accessions and a chicken accession that was included for comparison. For the ground parrot, the seven primers produced a total of 476 markers, averaging 68 markers per primer. The minimum number of bands that can be scored from the primers used was 48, and the most was 92. The number of unambiguous polymorphic markers per primer averaged 6.3, or 9.2%, with one primer having just one polymorphic band from 82 scored (Table 2).

Table 1. Sequence of seven arbitrarily-chosen oligonucleotide primers employed to generate RAF profiles. The primer composition is five prime to three prime; each primer acts as both a forward and reverse primer.

Primer name	Primer sequence
K-01	5´-CATTCGAGCC-3´
K-02	5´-GTCTCCGCAA-3´
K-03	5´-CCAGCTTAGG-3´
K-04	5´-CCGCCCAAAC-3´
K-06	5´-CACCTTTCCC-3´
K-07	5´-AGCGAGCAAG-3´
K-08	5´-GAACACTGGG-3´

Table 2. Number and proportion of polymorphic bands generated by RAF.

Primer	Scoreable bands	Polymorphic bands	Polymorphic bands (%)
K-01	67	12	17.9
K-02	56	9	16.1
K-03	82	1	1.2
K-04	66	2	3.0
K-06	65	3	4.6
K-07	48	5	10.4
K-08	92	12	13.0
Total	476	44	9.2
Average	68	6.3	9.3

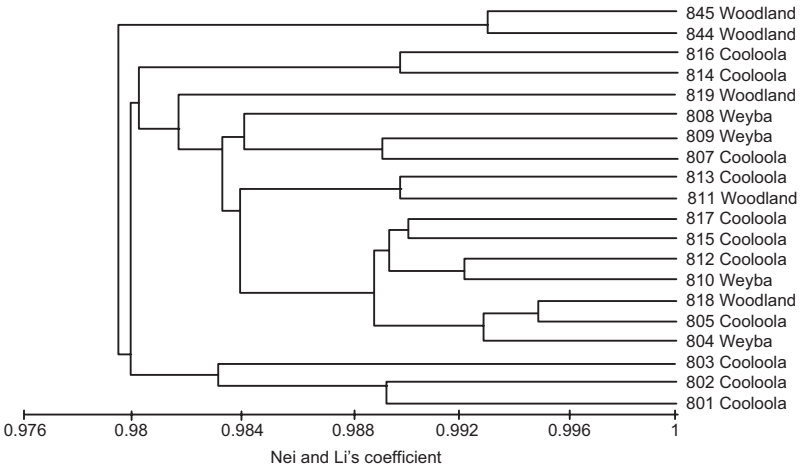

Fig. 2. UPGMA dendrogram based on Nei and Li's genetic distance. Each branch represents the identifier code for each ground parrot individual and its site of capture.

Average levels of genetic similarity (i.e. per cent band sharing) according to sample location was 0.982 ± 0.067 for Cooloola, 0.985 ± 0.100 for Weyba, and 0.981 ± 0.210 for Woodland. The overall average genetic similarity among all ground-parrot accessions was 0.978 ± 0.03. All ground-parrot accessions were distinguished by at least one polymorphic marker (i.e. all had unique RAF profiles). Cluster analysis revealed that individuals were not grouped according to sample location, although bird individuals 801, 802 and 803 from Cooloola did cluster at higher similarity as an outgroup (Fig. 2). Individuals 844 and 845, which are known siblings as they were found as nestlings occupying the same nest at Woodland, also clustered as an outgroup.

Discussion

The powerful RAF technique used in this study revealed low levels of genetic variation and high levels of genetic similarity in the eastern ground parrot. The 48–92 bands obtained are more than those produced by RAPDs and are comparable with those generated by AFLP. Although considered a valuable tool in providing an accurate and precise determination of genetic diversity in plants, as far as we are aware, RAF has not been used in vertebrate genetics. It is therefore not possible to compare our results with those of other avian species using the same technique. However, animal studies employing AFLP typically produce higher levels of polymorphism than that found in the ground parrot using RAF, averaging around 30% in birds, albeit with a wide range (Boere *et al*. 2006, Milot *et al*. 2007). For example, in the endangered southwestern willow flycatcher (*Empidonax traillii extimus*), Busch *et al*. (2000) found 197 polymorphic markers (27.8%) from a total of 708 produced from six primer combinations used. This compares with 44 polymorphic bands (9.2%) from a total of 476 generated by seven arbitrary primer combinations in our study. Avian studies that employed RAPD, a similar technique to RAF, commonly also produce much higher levels of polymorphism; examples include 25% for the greater rhea *Rhea Americana* (Bouzat 2001) and 31%–84% in avian genus *Vireo* (Zwartjes 2003). Even many threatened or endangered avian species have higher polymorphism; such as Iberian imperial eagle (*Aquila adalberti*, 59.7%) (Padilla *et al*. 2000), tufted titmouse (*Parus bicolour*, 45.7%) and marsh wren (*Cistothorus palustris*, 39.1%) (Bowditch *et al*. 1993). Exceptions include the light-footed clapper rail (*Rallus longirostris levipes*) with just 1.2% using RAPD (Nusser *et al*. 1996) and Amsterdam albatross (*Diomedea amsterdamensis*) with 2.1% using AFLP (Milot *et al*. 2007). Our results on genetic diversity for the eastern ground parrot are closer to that for the clapper rail and Amsterdam albatross than for most other endangered species. Generally, studies which compared RAPD and AFLP (e.g. Saliba-Colombani *et al*. 2000, Tosti & Negri 2002) found that RAPDs yield slightly higher proportion of polymorphic bands, but

AFLPs are more efficient in detecting polymorphism because of the higher number of bands generated per reaction.

Although the small sample size limited further analysis of genetic structure, there is sufficient evidence from this study which suggests low levels of genetic variation in the ground parrot in Queensland at least equal to those found in the lower end of diversity for endangered species. The bird's current national vulnerable status (Higgins 1999) may be justified at present, but caution needs to be exercised in ensuring genetic diversity is not reduced further through such pressures as loss or reduction in the heath habitat. The Weyba and Woodland sites are already encircled by urban development, and the high genetic similarity indicates susceptibility to inbreeding and further loss of genetic diversity.

In our study, the RAF DNA fingerprints were generated from duplicate DNA extracts for each individual animal by running paired reactions in adjacent lanes on polyacrylamide gel. A few duplicate lanes produced variable signal to noise ratio, non-reproducible results which were excluded from analysis. The non-reproducible results were likely the consequence of poor quality DNA templates rather than problems with the RAF technique, since usually each pair of all other duplicates produced identical results (except for polymorphic bands) from the same single-sequence primer used. Obviously inclusion of non-reproducible data would falsely inflate genetic diversity. Another potential problem is the manual scoring of band presence/absence. Unless the bands in the X-ray film are equally clear for the duplicate pair, viewing the autoradiograph can complicate the decision that a band is present or absent. This is important given measures of genetic diversity depend on the proportion of bands shared or not shared. A solution is to simply run the sequencing gel again in duplicates if any uncertainty (e.g. blur) occurs. The relatively low cost and effort of the protocol allow such re-runs of gels. When used properly, the RAF protocol is an efficient and reliable DNA marker technology that should be employed more often in animal research. Surprisingly, only a few studies employed the RAF approach, and a majority of these dealt with plants of major economic significance (e.g. tomato, macadamia, sugarcane, wheat). A possible reason for its rarity in animal research is that simple measures of band sharing may not mean much between populations and species because of the unknown loci element. RAF is not suitable for focus on a particular locus. A more common criticism of various random amplification methods is the lack of repeatability. This is not an issue with RAF. Furthermore, besides simpler operation, RAF requires less genomic DNA than the equally reliable AFLP which necessitates adequate DNA to assess completion of restriction digestion of template DNA. Collecting large volumes of blood from wild vertebrates can be difficult especially if the animals are small and need to be released unharmed after handling. Small sample volumes provide small primary DNA source and therefore low quantities of extracted genomic DNA, but is sufficient for the minimal template DNA requirement of RAF as long as genomic DNA is of high molecular weight. The latter requirement, however, may preclude the use of feathers and museum samples as these often lack good quality genomic DNA. The RAF technique is shown to be an excellent tool for determining genetic variability, especially in those species with low genetic diversity or low population size. It is less useful in answering other ecological questions such as sex-biased dispersal and mating systems which are better examined using techniques such as mtDNA and microsatellites (Frankham *et al*. 2002). RAF, when used in conjunction with other molecular techniques, can be a powerful tool for conservation genetics of vertebrates and other animals.

Acknowledgments

This study would not have been possible but for the effort of the late Diana Mudie who collected the blood samples under extremely difficult conditions. We also thank staff and students from the Agricultural Molecular Biotechnology Laboratory who assisted with the work. The University of the Sunshine Coast Animal Ethics Committee approved the study and Queensland Parks and Wildlife Service gave permission to collect blood samples.

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