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SHORT COMMUNICATION

Microsatellite characterisation and sex-typing in two invasive parakeet species, the monk parakeet *Myiopsitta monachus* and ring-necked parakeet *Psittacula krameri*

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Abstract

Invasive species can have wide-ranging negative impacts, and an understanding of the process and success of invasions can be vital to determine management strategies, mitigate impacts and predict range expansions of such species. Monk parakeets (*Myiopsitta monachus*) and ring-necked parakeets (*Psittacula krameri*) are both widespread invasive species, but there has been little research into the genetic and social structure of these two species despite the potential links with invasion success. The aim of this study was to isolate novel microsatellite loci from the monk parakeet and characterise them in both monk and ring-necked parakeets in order to facilitate future investigations into their behaviour and population ecology. Sex-typing markers were also tested in both species. Of the 20 microsatellite loci assessed in 24 unrelated monk parakeets, 16 successfully amplified and were polymorphic displaying between 2 and 14 alleles (mean = 8.06). Expected heterozygosity ranged from 0.43 to 0.93 and observed heterozygosity ranged from 0.23 to 0.96. Nine of the 20 loci also successfully amplified and were polymorphic in the ring-necked parakeet, displaying between 2 and 10 alleles. Suitable markers to sex both species and a Z-linked microsatellite locus were identified. A multiplex marker set was validated for monk parakeets. These novel microsatellite loci will facilitate fine and broad-scale population genetic analyses of these two widespread invasive species.

Keywords Population genetics · Microsatellite loci · Sex markers · Invasive species · Aves · Psittacidae

Abbreviations

PCR	Polymerase chain reaction
PIC	Polymorphic information content
H_O	Observed heterozygosity
H_E	Expected heterozygosity
HWE	Hardy–Weinberg equilibrium
LD	Linkage disequilibrium
EMBL-EBI	European Molecular Biology Laboratory-European Bioinformatics Institute
FDR	False discovery rate

Introduction

Invasive species are nonindigenous species that establish self-sustaining populations beyond their native range [1, 2]. The negative impacts of invasive species can be wide-ranging and include: extensive economic and environmental damage [3, 4], threats to biodiversity [5, 6] and damage to human health [7, 8]. Two such invasive species are the monk parakeet (*Myiopsitta monachus*) and the ring-necked parakeet (*Psittacula krameri*).

As a popular pet species, tens of thousands of monk parakeets have been exported from their native South America to meet the demands of the international pet trade [9–11]. Subsequent breaches in captivity during transit or from holding areas, together with accidental or deliberate release by owners facilitated multiple invasion events across four additional continents (e.g. [9, 10]). In Europe, monk parakeets are now among the invasive bird species with the potential to cause the most acute economic impacts [3]. For example, substantial crop damage caused by foraging monk parakeets has been identified in the agricultural belt surrounding the

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city of Barcelona, Spain [12]; while in North America, their communal nests built on power lines and in electricity substations cause power outages and safety concerns [13].

Ring-necked parakeets, native to Asia and Africa [9], are the world's most widespread invasive parrot species, with populations reported in at least 35 different countries [e.g. [14, 15]. Considered one of Europe's worst invasive species [3], ring-necked parakeets have wide-ranging negative impacts throughout their invasive range including outcompeting native cavity nesters for suitable nest-sites (e.g. nuthatches *Sitta europea*; [16]), killing native species through direct aggressive encounters (e.g. greater noctule bat *Nyctalus lasiopterus*; [17]), and causing severe economic damage [3].

Assessing the genetic structure of populations of invasive species can be key in understanding their origin and invasion history [18], investigating dispersal patterns [19], and determining eradication or management strategies [20]. Microsatellites are molecular markers that are regularly used in such studies, and polymorphic markers have already been published for both monk parakeets (12 markers) [21] and ring-necked parakeets (21 markers) [22]. Here we present the characterisation of novel polymorphic monk parakeet microsatellite loci and their cross-species utility in the ring-necked parakeet. These new microsatellites, when used in combination with the microsatellite markers previously published for use in these species [21, 22], will improve investigations into social and population genetic structure at a range of spatial scales, and help to examine the processes related to the invasion success of both species.

Materials and methods

Sampling and DNA extraction

Monk parakeet blood samples were collected in Barcelona, Spain (permit code: EPI 7/2015 (01529/1498/2015)) in May–July 2016 and 2017. Blood samples (maximum 100 µl) were taken from either the brachial or jugular vein of each individual, stored in 98% ethanol and kept at − 20 °C before DNA extraction.

DNA was extracted overnight using an ammonium acetate extraction protocol [23, 24]. DNA quality was assessed by gel electrophoresis and its concentration quantified using a fluorimeter (FLUOstar Optima, BMG LABTECH Ltd., Aylesbury, UK). The library was constructed using genomic DNA extracted from a single female monk parakeet sampled in Barcelona, Spain. Genomic DNA was digested with *MboI* and enriched for dinucleotide (AG, AC) and tetranucleotide (CTAA, CTTT, GATA, GTAA) repeat motifs; magnetic beads were used in the enrichment hybridisation (modified from [25, 26]). An Illumina paired-end library was generated using 1 µg of this repeat-enriched DNA. The NEBNext DNA Library Prep Kit for Illumina (New England Biolabs Inc.) protocol was followed and the DNA was sequenced using a MiSeq Benchtop Sequencer (Illumina Inc., San Diego, CA, USA). A total of 162 sequences that contained at least five tandem repeats were extracted from the data (EMBL-EBI accession numbers LR700312-LR700620). Twenty of these were selected and used to design primer sets.

Primer design and microsatellite evaluation

Primer pairs were designed using Primer3 v. 0.4.0 [27–29] in microsatellite flanking regions with a product size range from 100 to 270 bp. Further specifications for selecting primer pairs were: a melting temperature of 59–61 °C (optimum 60 °C, difference 0.5 °C), a length of 18 to 36 base pairs (20 bp optimum) the presence of a G/C clamp, a maximum poly-X of 3 tandemly repeating nucleotides (e.g. TTT), and all other parameters set to default. Forward primers were 5′-labelled with a fluorescent dye (HEX or 6-FAM). BLAST software [30] was used to assess and select unique sequences for primer design.

DNA from 24 monk parakeets (12 male and 12 female) was amplified using polymerase chain reaction (PCR) to assess microsatellite variability. Monk parakeets are sexually monomorphic [9], therefore genetic sex-typing was used to determine the sex of individuals and to enable the identification of sex-linked microsatellite loci. Of nine bird sexing markers tested in monk parakeets, five were successful: P2-P8 [31], P2D-P8 [32], Z002B [33], Z43B [34] and 2550F-2718 [35]. Two of these sex markers were used in the present study (P2-P8 [31] and Z002B [33]) to avoid any potential errors in sexing caused by misidentification of the Z and W alleles due to Z/W-polymorphism or the presence of heteroduplexes [36–38]. PCR amplification was performed using a DNA Engine Tetrad ®Thermal Cycler (MJ Research, Bio-Rad, Herts, UK) in 2 µl reaction volumes containing 10–50 ng of air-dried DNA, 1 µl QIAGEN Multiplex PCR mix (containing PCR buffer, HotStarTaq DNA polymerase, 1.5 mM $MgCl_2$ and 0.2 µm dNTPs; QIAGEN Inc.) and 0.2 µM of each primer. Initial denaturation stage was carried out at 95 °C for 15 min, followed by a PCR amplification of 35 cycles (94 °C for 30 s, 58 °C for 90 s and 72 °C for 60 s) and a final extension for 30 min at 60 °C. Sex-typing markers P2-P8 and Z002B were amplified using annealing temperatures 50 °C and 56 °C respectively. 1 µl of PCR product was diluted to a ratio of 1:2500–1:5000 (product:H_2O) and these products were then separated on an ABI 3730 48-capillary DNA Analyser using formamide and GeneScan™-500 ROX size-standard (Applied Biosystems, Warrington, UK). Alleles were scored using GENEMAPPER v 5 software (Applied Biosystems, California, USA).

Data analysis

Allele numbers, polymorphic information content (PIC), estimated null allele frequencies, and observed (H_o) and expected heterozygosities (H_E) were calculated using CERVUS v3.0.7 [39]. Linkage disequilibrium and any departures from Hardy–Weinberg equilibrium were calculated using GENEPOP web version 4.2 [40]. In order to correct for multiple testing, a false discovery rate control (FDR) [41] was applied to p-values obtained for linkage disequilibrium. ML-RELATE was used to estimate maximum-likelihood coefficients of relatedness for each dyad [42], confirming that the individuals used to characterise the microsatellite loci were unrelated ($r < 0.19$, mean ± SD = 0.02 ± 0.04).

Cross-species utility

Ring-necked parakeet blood samples were collected in November–March 2015–2017 in Barcelona, Spain (permit code: EPI 7/2015 (01529/1498/2015)). Blood samples (maximum 100 µl) were extracted from the jugular or brachial vein and stored at − 20 °C in 98% ethanol. An ammonium acetate extraction protocol was used for DNA extraction (see above for details), PCR amplification was conducted on DNA extracted from 18 ring-necked parakeets (11 females and 7 males; sexed using P2–P8 [31] and Z002B [33]), and microsatellite variability was then assessed as described for monk parakeets.

Results and discussion

Microsatellite characterisation

Of the 20 microsatellite loci tested, 17 successfully amplified and 16 were polymorphic in monk parakeets with allele numbers ranging from 2 to 14 with a mean of 8.06 alleles per locus (Table 1). H_O and H_E ranged from 0.23 to 0.96 and 0.43 to 0.93, respectively. PIC values ranged from 0.33 to 0.90 with 15 of the 16 microsatellite loci being highly informative ($PIC > 0.50$) and the other locus being reasonably informative ($0.50 > PIC > 0.25$; following [43]). One locus, MmonZ12, was heterozygous in some males (ZZ) yet homozygous in all 12 females (ZW) indicating it is sex-linked (Z-linked; Fisher's exact test, $p = 0.004$). All 15 other polymorphic loci amplified in both males and females, with no loci being homozygous in all females and all loci displaying heterozygotes in both males and females, and were therefore presumed to be located on the autosomes. For the Z-linked locus (MmonZ12), deviation from Hardy–Weinberg equilibrium was assessed in males only. Four loci (Mmon03, Mmon08, Mmon10 and Mmon13) exhibited significant deviations from Hardy–Weinberg equilibrium ($p < 0.05$), which may suggest the presence of null alleles. However, only one locus (Mmon08) possessed a high estimated null allele frequency (> 10%). Alternatively, deviations from Hardy–Weinberg equilibrium may result from population structure [44]. Following FDR control, no significant linkage disequilibrium was found between loci. Multiplex Manager 1.2 [45] was used to generate a multiplex marker set from these 16 loci, optimised in three plexes; these multiplexes were then validated by genotyping the same 24 individual monk parakeets that had been genotyped in single-plex (Table 1).

Cross-species utility

Nine of the 20 microsatellite loci also amplified and were polymorphic in the ring-necked parakeet (Table 2). One of the nine loci polymorphic in ring-necked parakeets (Mmon17, Table 2) was monomorphic in monk parakeets and therefore was not included in the multiplex set for monk parakeets (Table 1). Allele numbers in ring-necked parakeets ranged from 2 to 10 (mean 5.22), H_O and H_E ranged from 0.12 to 0.89 and 0.11 to 0.89, respectively, and PIC values ranged from 0.11 to 0.85 (Table 2), with five loci being highly informative ($PIC > 0.50$), and one locus being reasonably informative ($0.50 > PIC > 0.25$; [43]). MmonZ12 was also sex-linked in ring-necked parakeets (Fisher's exact test; $p < 0.001$), as in monk parakeets, but was more variable in ring-necked parakeets displaying a total of 10 different alleles in 18 individuals (Table 2). Z chromosome polymorphism was observed for both sex markers (P2-P8 and Z002B) in two out of 18 ring-necked parakeets (allele sizes: P2-P8 Z alleles 369 and 375 bp, W allele 403 bp; Z002B Z alleles 250 and 252 bp, W allele 234 bp) and was accounted for when assigning sex. Four loci (Mmon01, Mmon05, Mmon15 and MmonZ12) deviated significantly from Hardy–Weinberg equilibrium ($p < 0.05$) and two loci (Mmon03 and Mmon15) possessed a high estimated null allele frequency (> 10%) in ring-necked parakeets. Linkage disequilibrium was significant between three pairs of alleles ($p < 0.05$; Mmon03 and Mmon04, Mmon04 and Mmon15, Mmon05 and Mmon15). However, this may be due to the presence of relatives in the sample of ring-necked parakeets used to characterise these microsatellite loci. ML-Relate [42] indicated the presence of possible half-sibling (19/153) and full-sibling relationships (6/153) and one potential parent–offspring relationship among the 18 ring-necked parakeets sampled.

Table 1 Characterisation of novel monk parakeet (*Myiopsitta monachus*) microsatellite loci (Psittacidae, Aves)

Locus, Accession no.	Primer sequences (5'–3'); forward (F); reverse (R)	Repeat motif	Multiplex	Observed allele size range (bp)	n	No. of alleles	H_O/H_E	Est. null allele freq	PIC
Mmon01 LR700312	F: [HEX] CCCACA TGCTATGGT CCAG R: CTTCCAAGG ATGAGGCAGAG	$(TTAG)_9$	3	177–201	23	7	0.70/0.72	0.001	0.67
Mmon02 LR700313	F: [6-FAM] AATCTC TAAAGAGGTCCA CACTGC R: TGGATGTCT GAGGTGAACTCC	$(AC)_{16}$	1	141–173	24	10	0.96/0.89	−0.05	0.85
Mmon03 LR700314	F: [6-FAM] TTTGCA GTGACCTTCATT CTG R: CTACAGCCA GCCTACTGTGC	$(TG)_{18}$	1	227–268	24	11	0.71/0.86**	0.09	0.83
Mmon04 LR700315	F: [HEX] ATCCTG CCTGTGAACTCT GG R: CCTCCCTCACCA TTCCAAG	$(GT)_{20}$	1	220–243	24	10	0.75/0.85	0.06	0.81
Mmon05 LR700316	F: [HEX] TCCTGT CAAGGTGATGCT TG R: CTGTAGAAG ATGGGAGGTTAG AGTG	$(CTAT)_{18}$	1	166–202	24	11	0.67/0.80	0.09	0.76
Mmon06 LR700317	F: [HEX] GGGAAT TCAGTGGAAAGA GG R: CCCAAATCA GATTCTTGCTTC	$(CA)_{17}$	2	191–219	24	10	0.83/0.84	−0.002	0.80
Mmon07 LR700318	F: [6-FAM] TGGCAG TATGAAACATAC ACACAG R: GGAAGCCAC CAAGATTTCAG	$(ATCT)_{16}$	2	197–229	24	14	0.88/0.93	0.02	0.90
Mmon08 LR700319	F: [6-FAM] AAA CCCAATGGCAGT GTTTC R: ACCATGGAG CTGAGGAACAG	$(TGGA)_7$	3	240–250	22	4	0.23/0.61**	0.44***	0.54
Mmon09 LR700320	F: [HEX] ATCCAC AATCGTCAGATG GAG R: AAATGGGAA GTGAACCCAGAG	$(AC)_{13}$	3	136–148	24	6	0.71/0.70	−0.01	0.64
Mmon10 LR700321	F: [6-FAM] TCAGTC AAGATGTTCCCT TGG R: GAGACAACA GCTCATCTTCCT CTAC	$(TG)_{14}$	3	90–101	24	7	0.75/0.81**	0.03	0.77
Mmon11 LR700322	F: [6-FAM] TGCAGT AATGATTTGATG CATTG R: ACAAGCACA CCTCGCAAAC	$(TATC)_{11}$	3	153–173	24	6	0.71/0.72	0.01	0.67

Table 1 (continued)

Locus, Accession no.	Primer sequences (5'–3'); forward (F); reverse (R)	Repeat motif	Multiplex	Observed allele size range (bp)	n	No. of alleles	H_O/H_E	Est. null allele freq	PIC
MmonZ12 LR700323	F: [6-FAM] GCTTTC TCTGTGAAATCC ATCC R: AACATCATC TTAAGAACCATC CAAG	$(ATCT)_6$	1	103–107	12M	2	0.58/0.43	−0.17	0.33
			1	103–107	12F	2	0/0	NA	NA
Mmon13 LR700324	F: [6-FAM] CAGTAT ACCTATGGTTAA GGTTTCAGC R: CCTTGATTCAGA TGGTAATTAGAG AAG	$(TCTA)_{14}$	3	122–144	24	6	0.88/0.79**	−0.07	0.74
Mmon14 LR700325	F: [HEX] CTTTCT AACTCATTCCTA AGTGAGAGC R: GACTCTGTC TGACTCCTATTG CTG	$(GT)_{16}$	1	142–164	24	9	0.83/0.82	−0.02	0.77
Mmon15 LR700326	F: [HEX] TTAAAC AACAGTATTTGT GAGACCAAG R: TCCTTTCCAACC CTAACTATTCTG	$(AGAT)_{14}$	2	130–176	24	11	0.83/0.88	0.02	0.85
Mmon16 LR700327	F: [6-FAM] CAA ACAGTCTTCCCT TTGTGG R: AAACACAGG CCCATCTGC	$(AC)_{12}$	2	141–167	24	5	0.67/0.65	−0.03	0.57
Mmon17* LR700328	F: [HEX] AGGTCC TTTACAGCCCTA ACTG R: GTTGAACTT CCCAGCTTTCC	$(TG)_{26}$	NA	254	22	1	NA	NA	NA

EMBL-EBI sequence accession numbers LR700312-LR700328, number of individuals results are based on (n), observed and expected heterozygosities (HO and HE respectively)

**Loci with significant departures from Hardy–Weinberg equilibrium; estimated null allele frequency (Est. null allele freq.)

***Mmon08 possessed high estimated null allele frequency (> 10%), not applicable (NA); polymorphic information content (PIC)

Mmon17* was monomorphic in monk parakeets and not included in the multiplex set

Limitations

DNA samples from monk parakeets and ring-necked parakeets used to characterise these microsatellite loci were taken from invasive populations for which there is no detailed knowledge of introduction events. Therefore, it is possible that these individuals are descendants from small founding populations which may have had limited genetic variation. On the other hand, both species have very extensive native ranges across South America (monk parakeet) and Africa and Asia (ring-necked parakeet) [9] and if founders were drawn from across these ranges, genetic variation of invasive populations may be greater than in local populations within their native range.

Table 2 Cross-species utility of monk parakeet (*Myiopsitta monachus*) microsatellite loci in the ring-necked parakeet (*Psittacula krameri*)

Locus	n	No. of alleles	Observed allele size range (bp)	H_O/H_E	Est. null allele freq.	PIC
Mmon01	17	6	165–193	0.71/0.78**	0.003	0.72
Mmon03	18	2	216–218	0.22/0.29	0.11***	0.24
Mmon04	18	3	204–207	0.39/0.48	0.07	0.41
Mmon05	18	10	127–176	0.83/0.89**	0.02	0.85
Mmon07	18	8	189–217	0.89/0.84	− 0.04	0.79
Mmon09	17	3	142–148	0.18/0.17	− 0.04	0.16
MmonZ12	7 M	7*	201–245	1.00/0.88**	0	0.79
	11F	8*	205–245	0/0	NA	NA
Mmon15	17	6	138–155	0.35/0.80**	0.38***	0.74
Mmon17	17	2	218–226	0.12/0.11	− 0.02	0.11

Tested in 18 ring-necked parakeets; number of individuals results are based on (n)

*A total of ten different alleles were observed for MmonZ12, observed and expected heterozygosities (H_O and H_E respectively)

**Loci with significant departures from Hardy–Weinberg equilibrium; estimated null allele frequency (Est. null allele freq.)

***Mmon03 and Mmon15 possessed high null allele frequencies (> 10%), not applicable (NA); polymorphic information content (PIC)

Conclusions

These novel microsatellite loci, optimised in three multiplexes, provide a powerful tool for analyses of both fine and broad-scale population genetic structure, as well as for analyses of parentage and dyadic relatedness. Combining these markers with those previously published for use in both monk parakeets [21] and ring-necked parakeets [22] will facilitate detailed investigations into behavioural and population processes related to invasion success in these two widespread avian invaders. Such studies are likely to be particularly interesting in the case of monk parakeets given that they are highly social parrots, with unique compound nests made of sticks that may house many breeding pairs, often built in close proximity to other nests to form loose colonies [9, 46]. Furthermore, examination of population genetic structure at a range of spatial scales may aid in the design of effective management strategies, help to understand the history of invasive populations and to predict future range expansions in these species.

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Author contributions BJH, JCS and FSEDP designed the study. JCS supervised the field study and JCS and AOS collected the blood samples. BJH and JCS supervised the project. FSEDP and GJH conducted laboratory work at the NERC Biomolecular Analysis Facility, Sheffield, UK. FSEDP and DAD analysed the data. FSEDP wrote the paper with input from co-authors. All authors read and approved the final manuscript.

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Data availability The microsatellite sequences are available through EMBL-EBI and are accessible via the Accession Numbers: LR700312-LR700328.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval Birds were handled and blood samples taken with special permission EPI 7/2015 (01529/1498/2015) from Direcció General del Medi Natural i Biodiversitat, Generalitat de Catalunya, following Catalan regional ethical guidelines for the handling of birds. JCS received special authorization (001501-0402.2009) for the handling of animals in research from Servei de Protecció de la Fauna, Flora i Animal de Companyia, according to Decree 214/1997/30.07, Generalitat de Catalunya.

Informed consent All authors consent to publication.

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