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TECHNICAL NOTE

# Identification of polymorphic microsatellite loci for the endangered great bustard (*Otis tarda*) by high-throughput sequencing

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**Abstract** We used next-generation sequencing (NGS) techniques to develop 14 new microsatellite loci in the great bustard (*Otis tarda*), an endangered steppe bird. We genotyped 22 individuals of the species in order to quantify levels of polymorphism and found that the number of alleles per locus ranged from 2 to 11 (mean = 4.21), with observed heterozygosity values for each locus ranging between 0.16 and 0.82 (mean = 0.52). We found no linkage disequilibrium between marker pairs nor any departures from Hardy–Weinberg equilibrium after Bonferroni correction. This new set of markers will be useful for the study of genetic variation and structure in the great bustard and other members of the family Otididae, many of them highly vulnerable.

**Keywords** *Otis tarda* · Great bustard · Microsatellite · NGS

The great bustard (*Otis tarda*) is an endangered steppe bird with a formerly large Eurasian distribution that today comprises highly fragmented populations from western Europe through China (Palacín and Alonso 2008). Human activities represent the main threat to its long-term viability and the species is currently classified as vulnerable in the Red List of Threatened Species (IUCN 2012). Previous molecular work on patterns of genetic diversity and structure in the species focused on mitochondrial DNA markers (Alonso et al. 2009; Broderick et al. 2003; Martín et al. 2002; Pitra et al. 2000, 2011), and data from bi-parentally inherited markers remains limited. A previous study by Lieckfeldt et al. (2001) reported the development of 6 loci, three of which were used to assess genetic diversity in the Chinese subspecies *O. t. dybowskii* (Tian et al. 2006). Here we use a next-generation sequencing approach to develop additional loci so that a sufficient number of markers can be used to obtain robust estimates of diversity and structure across the species range.

We developed the microsatellite library with DNA samples from the Iberian population of great bustards, which comprises about 60 % of the current global population (Alonso and Palacin 2010), and tested for variation using 22 individuals from the Madrid region (central Spain). DNA was extracted from blood stored in Queen's buffer (Seutin et al. 1991) following the ammonium acetate protocol by Richardson et al. (2001).

Microsatellite identification was carried out with a 454 Life Sciences/Roche GS-FLX genome sequence system (Roche Applied Science; Margulies et al. 2005) in a partial run from a microsatellite-enriched genomic DNA following protocols by Santana et al. (2009). From a total of 376,323 sequence reads generated (104,769,722 base pairs), 2,719 contained a minimum of 5 tandem repeats in the following frequencies: 1,863 dinucleotides, 588 trinucleotides, 155 tetranucleotides, 99 pentanucleotides and 14 hexanucleotides. Out of these we selected 135 sequences that had sufficient flanking region for primer design with the PRIMER3 software (Rozen and Skaletsky 2000).

PCR amplification of loci was carried out with 10–250 ng DNA in a total reaction volume of 25 μL following TaKaRa Ex Taq protocol (Takara Bio Inc., Shiga, Japan) with the following PCR thermal cycle: initial denaturation at 98 °C for 5 min followed by 30 cycles of denaturation at 95 °C for

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**Table 1** Genetic variability and characteristics of the 14 microsatellite loci isolated from *Otis tarda*

| Locus | Primer sequence (5′–3′) | Motif | Size range (bp) | Na | $H_E$ | $H_O$ |
|---|---|---|---|---|---|---|
| Ot1 | F: CTTGTGCCTTCGTAGCTTCC | (AAT)7 | 240–243 | 2 | 0.20 | 0.14 |
| | R: TGTGCACTCCCAGAACAGTC | | | | | |
| Ot2 | F: ATGTCCAGGTAACTATGGCATTC | (ACAA)6 | 266–274 | 3 | 0.45 | 0.64 |
| | R: GTTCTCATGATCTCCTAGAAATCTCC | | | | | |
| Ot3 | F: AGCACCATTCCCTGAGTCAC | (TTTG)7 | 190–210 | 2 | 0.46 | 0.73 |
| | R: TGTGTACCCTCAGATACCACCA | | | | | |
| Ot4 | F: GACAGCGCTACAGACTTTGC | (ATT)7 | 150–165 | 5 | 0.70 | 0.77 |
| | R: GGAAGGACAATCCAAGCAGA | | | | | |
| Ot5 | F: ACTGTGGTGACCATGTGGAA | (TTTTA)7 | 259–294 | 7 | 0.82 | 0.82 |
| | R: TGCAACGTTATCAAATCATGG | | | | | |
| Ot6 | F: CTGGGAAAACTGTTGGAGGA | (AAAT)6 | 328–336 | 2 | 0.16 | 0.09 |
| | R: TGGTTCGTTGGAGTAAAGGA | | | | | |
| Ot7 | F: TGGGGAAACAAAGGTCTGAG | (ATA)7 | 196–241 | 5 | 0.57 | 0.32 |
| | R: TAAAAACACGACTGCGCTTG | | | | | |
| Ot8 | F: TTGGAAGCCAAATTTTACCC | (AACC)5 | 312–320 | 3 | 0.54 | 0.41 |
| | R: TGAGCCTCAGGAAAGAGCTA | | | | | |
| Ot9 | F: AAGGCAATGACCAAAAATGC | (TGACA)8 | 210–240 | 6 | 0.39 | 0.41 |
| | R: GACGTCAGATAAAAGTGAGTCCA | | | | | |
| Ot10 | F: AAGGCAGGACTGGGGTTAGT | (TCCA)7 | 173–177 | 2 | 0.27 | 0.32 |
| | R: GGGGAGACGTCAACACAGAG | | | | | |
| Ot11 | F: CTGGCAATTGAAGGACAAAA | (AGAATA)8 | 209–281 | 11 | 0.81 | 0.77 |
| | R: GCTTTCCCATCCCATTCTCT | | | | | |
| Ot12 | F: CGCTTATTTACAGGCGTGCT | (AC)10 | 199–205 | 3 | 0.58 | 0.64 |
| | R: GCCCGTGTGGTAAGTACCTG | | | | | |
| Ot13 | F: AAGGAGCAGGGACATCTTGA | (AC)11 | 176–186 | 4 | 0.67 | 0.68 |
| | R: ACTGTGAGTCGCTGCCTGT | | | | | |
| Ot14 | F: AAAAGGAAAACGCCTCCATT | (AT)10 | 150–158 | 4 | 0.56 | 0.5 |

$N_a$ number of alleles per locus, $H_E$ expected heterozygosity, $H_O$ observed heterozygosity, *F* Forward, *R* Reverse

20 s, annealing at 57 °C for 20 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 10 min.

Among the 135 candidate loci tested, 90 failed to amplify correctly in all samples. The remaining 45 loci were labelled with fluorescent-dye primers (VIC, NED, PET and FAM) and reamplified in all 22 samples. PCR products were run on an ABI 3130 Sequencer (Applied Biosystem) and resulting profiles were analysed using GeneMapper 4.0 (Applied Biosystems). A total of 14 loci were validated as sufficiently variable, whereas the other 31were monomorphic or produced irregular electrophoretic patterns (details available from the corresponding author upon request).

For these 14 validated loci, the 22 sample-set was analysed with Tandem 1.09 (Matschiner and Salzburger 2009) in order to automatically integrate allele binning from GeneMapper data. GENETIX V.4.05 (Belkhir et al. 2004) was employed to estimate the expected ($H_E$) and observed ($H_O$) heterozygosity as well as the number of alleles per locus ($N_a$). Linkage disequilibrium between locus pairs and Hardy–Weinberg proportions were calculated with GENEPOP v4 (Rousset 2008) applying Bonferroni corrections for multiple tests (Rice 1989).

Of the final 14 polymorphic microsatellite loci, one was an hexanucleotide, two were pentanucleotides, five tetranucleotides, three trinucleotides and three dinucleotides. These loci showed relatively high genetic diversity (Table 1): the mean number of alleles was 4.21 (SD = 2.51), the mean expected heterozygosity 0.51 (SD = 0.20) and the mean observed heterozygosity 0.52 (SD = 0.23). No linkage disequilibrium was found between pairs of loci, and no departures from Hardy–Weinberg equilibrium were detected after Bonferroni correction (Rice 1989). This set of markers provides an additional tool to explore patterns of genetic diversity and structure for conservation and population studies of the endangered great bustard.

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