Biochemical Systematics and Ecology 63 (2015) 109–112

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Characterization of 10 new tetranucleotide microsatellite markers for the European eagle owl, *Bubo bubo*: Useful tools for conservation strategies

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ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form 1 October 2015
Accepted 3 October 2015
Available online 23 October 2015

Keywords:
Raptors
Microsatellites
Genetic diversity
Conservation
Parentage

ABSTRACT

Bubo bubo is the largest owl in the world, showing a wide geographical distribution throughout the Palaearctic region. It underwent a demographic decline in many European countries during the last century and was considered "vulnerable" (Annex II of the CITES). Nowadays, it is classified as "Least Concern" according to IUCN.

Despite its ecological importance and conservation status, few polymorphic molecular markers are available to study its diversity and population genetics. We report on the isolation and development of 10 new microsatellites for the Eagle owl, *B. bubo*. All loci (10 tetra-nucleotide) are characterized by high polymorphism levels. Number of alleles ranged from 5 to 13 and expected heterozygosity varied from 0.733 to 0.840. These microsatellites would be very useful to assess the genetic diversity, connectivity patterns and parentage of *B. bubo*. This information will allow to establish new conservation strategies and improve the management of the species.

1. Introduction

The Eagle owl, *Bubo bubo* (Linnaeus 1758) (Cordata: Aves), is the largest owl in the world. It has a wide geographical distribution throughout the Palaearctic region, inhabiting a great diversity of habitats such as Mediterranean scrub lands and steppes, boreal forests, cliffs and deserts (Penteriani et al., 2010). It is prevalently a monogamous long-lived species (Penteriani et al., 2010), sedentary and territorial, playing a fundamental role in the ecosystem as top predator (Penteriani et al., 2008; Lourenço et al., 2011; Solonen, 2011). *B. bubo* is associated to high biodiversity ecosystems and is considered a keystone species (Sergio et al., 2004). It underwent an important demographic decline in many European countries during the last century (Penteriani et al., 2002), being completely eradicated in north-western Germany, south-western Switzerland and France (Dalbeck and Heg, 2006; Schaub et al., 2010). Consequently, the species was considered "vulnerable" (according to Annex II of the CITES, Convention on International Trade of Endangered Species of Wild Fauna and Flora and Annex I of the Council Directive 2009/147/EC on the conservation of wild birds, Birds Directive). Nowadays, it is classified as "Least Concern" according to IUCN criteria, although its populations are decreasing (IUCN, 2015).

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http://dx.doi.org/10.1016/j.bse.2015.10.003
0305-1978/

Despite the ecological importance and singularity of *B. bubo*, only one study using microsatellites has assessed its population genetics and connectivity patterns in south-eastern Spain (León-Ortega et al., 2014). This study used 7 loci markers, previously designed from captive individuals by Isaksson and Tegelström (2002), which showed low number of alleles and polymorphism. Therefore, the resolution of these 7 microsatellites is limited to assess the current genetic diversity and structure of *B. bubo* populations. Recently, Kleven et al. (2013) developed 8 new microsatellites for this species, using genomic DNA extracted from the feather calamus of 1 individual (a confiscated captive adult female eagle owl, CF68, and later tested them in 38 individuals from Luroy municipality, northern Norway). Most of these loci showed a low number of alleles (50% of the loci with 2 or 3 alleles, 2 loci with 5 alleles, and 2 loci with 6 and 8 alleles respectively).

In order to study connectivity, dispersal patterns and parentage on this species, more polymorphic genetic markers are required. To fulfil this need, we report here the isolation and development of ten new highly polymorphic microsatellites for *B. bubo*.

2. Material and methods

Genomic DNA of three wild *B. bubo hispanicus* adults from Murcia (SE Spain) was isolated following the protocol of Sambrook et al. (1989). Size-selected fragments from genomic DNA of each individual were enriched for simple sequence repeat (SSR) content by using magnetic streptavidin beads and biotin-labelled GATA and GTAT repeat oligonucleotides. The SSR-enriched library was analyzed on a Roche 454 platform using the GS FLX Titanium reagents. The total 74, 934 reads had an average length of 494 base pairs. From these, 11, 054 reads contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. Primer design was feasible in 8, 695 reads.

To determine polymorphisms of the newly developed microsatellite markers, the procedure originally described by Schuelke (2000) was used. In brief, an universal 18 base pair M13 tail was added (5′-TGTAAAACGACGGCCAGT-sequence-of-forward-primer-3′) to the 5′-end of each forward primers. During the first PCR cycles, such primers are incorporated into the PCR products. In subsequent cycles, these products function themselves as templates for the fluorophore-labelled universal M13 primer. Thus, labelled products can be analyzed on a laser detection system (i.e. sequencer). Polymerase chain reaction (PCR) was performed in 10 μl total volume including, 2–10 ng of DNA, 1.8 mM $MgCl_2$, 0.5 μM of forward primer, 0.5 μM of reverse primer, 200 μM dNTP's and 0.5 U HorsePower-Taq DNA polymerase (CANVAX). Cycling conditions consisted of an initial denaturation step of 15 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 45 s at annealing temperature to 56 °C, 45 s at 72 °C, followed by 8 cycles of 30 s at 95 °C, 45 s at 53 °C, 45 s at 72 °C and a final elongation step at 72 °C for 30 min. All PCR reactions were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems). PCR products from microsatellite amplification were visualized by gel (2.5% agarose) electrophoresis on a Molecular Imager Gel Doc XR system (Bio-Rad) and analysed on an ABI PRISM 3370 automated genetic analyzer (Applied Biosystems). Allele sizes were scored with STRAND (v. 2.4.59 http://www.vgl.ucdavis.edu/STRand).

The number of alleles per locus, observed (H_O) and expected heterozygosity (H_E), and linkage disequilibrium (10, 000 permutations) were calculated in GENETIX v. 4.05 (Belkhir et al., 2004). Deviations from Hardy–Weinberg equilibrium (HWE) across all samples were characterized and tested using exact test in the software GENEPOP v. 4.0.10 (Rousset, 2008). When the observed genotype frequencies deviated significantly from HWE ($p < 0.05$), the program Micro-Checker v.2.2.3 (van Oosterhout et al., 2004) was used to test the presence of null alleles.

3. Results and discussion

A total of 10 out of 24 primer pairs tested (Genbank accession numbers: KJ609140–KJ609149), were selected because they displayed the highest number of alleles (Table 1). These 10 loci were checked on a subset of 25 individuals from one wild population (northern Murcia, Spain), previously defined by León-Ortega et al. (2014).

The number of alleles per locus ranged from 5 (Bbu170 and Bbu225) to 13 (Bbu230). Allele richness of these microsatellites, was higher than that described for previous *B. bubo* microsatellites designed by Kleven et al. (2013) and Isaksson and Tegelström (2002). These authors tested their microsatellites in 38 individuals from Luroy (northern Norway) and in 66 individuals from a captive population of eagle owls in northern Sweden, respectively.

The observed differences on allele richness between microsatellites, could be due to different origins of DNA used to develop those markers: Kleven et al. (2013) and Isaksson and Tegelström (2002) utilized captive individuals, which should be showing lower genetic diversity than individuals belonging to wild populations. Also geographical origin of assessed populations could explain differences found on number of alleles and polymorphism. Considering the European distribution of *B. bubo*, populations from south-eastern Spain show the highest density and reproductive success (Penteriani et al., 2010); therefore it would be expected that, these populations have higher genetic diversity than other ones without those features. However, to truly compare our markers and that designed by Kleven et al. (2013) and Isaksson and Tegelström (2002), further genotyping has to be carried out in the same individuals.

Expected heterozygosity (H_E) oscillated from 0.733 (Bbu230) to 0.840 (Bbu256). The highest observed heterozigosity (H_O) values were observed in three loci: Bbu194, Bbu182 and Bbu210. Significant heterozygote deficiency was observed in 7 loci ($p < 0.05$), but considering a lower significant p-value ($p < 0.01$), only 3 loci (Bbu245, Bbu254 and Bbu225) showed significant deficiencies (Table 1). Such deviations from HWE may be due to the Wahlund effect, inbreeding, groupings of relatives, selection against heterozygotes or genotyping errors (null alleles and other scoring errors).

Table 1
Characterization of ten microsatellite loci in 25 individuals of *Bubo bubo hispanicus* (H_E: expected heterozigosity; H_O: observed heterozigosity; HWE: Hardy–Weinberg Equilibrium; *: 0.01 < p < 0.05; **: 0.001 < p < 0.01; ***: p < 0.001; n.s: non-significant).

Locus	Repeat motif	Primer sequences (5′–3′) and the fluorescent dye to mark the 5′ end of the forward primer	Nº Alleles	Size range (bp)	H_E	H_O	HWE	*p*
Bbu170	$(AGAT)_{13}$	F: HEX-CTTCATACTGTGCGAGGCTG R: GGCTAGCGGCTGAAAAATCC	5	170–186	0.778	0.640	0.180*	0.032
Bbu194	$(ATCT)_{12}$	F: 6FAM-CTAGTGGCTGGGGATGACAG R: CCCAGTTCATCGCAGTTGAC	9	180–222	0.800	1.000	−0.256n.s	1.000
Bbu256	$(ATAG)_{10}$	F: 6FAM-TGTCATGGCAACTCAGAAAGTC R: GCTACTGACAAAGCTGTGGG	10	232–262	0.840	0.680	0.193*	0.020
Bbu245	$(TATC)_{14}$	F: HEX-TTGCCTGTGCACCCATTATC R: TTAGTCCCACGGTGAGCAAG	7	245–261	0.796	0.680	0.148**	0.004
Bbu166	$(TATC)_{15}$	F: 6FAM-AGAAATGCGCGTTAGCTGTC R: GGTAAGAGGAGCTGGGATGG	11	128–168	0.803	0.560	0.307*	0.032
Bbu230	$(TATC)_{11}$	F: 6FAM-ACTGTTCTTCAGTGACCTCTTATTTAC R: GTTGGAGCTAGGGCATCCTG	13	214–266	0.733	0.522	0.293*	0.018
Bbu210	$(TCTA)_{15}$	F: HEX-CTCACCAGGTATCTGAACACAG R: GCAACGTCGTAAGTCATAATTCC	11	210–254	0.817	0.800	0.021n.s	0.642
Bbu182	$(TCTA)_{10}$	F: NED-AGCTGCATTATCAACAGCCAG R: GGATGCCATTTTGCCTTAGC	9	170–190	0.834	0.840	−0.007n.s	0.504
Bbu254	$(TCTA)_{10}$	F: HEX-CAGCCATGAATGTCAGCCAC R: AACACGCTGCCAATTTACCC	7	238–258	0.758	0.640	0.158*	0.006
Bbu225	$(AGAT)_{13}$	F: NED-ATGGGCACCTGGATGATTCG R: AGCACAGATTGGGACTGGTG	5	209–236	0.797	0.520	0.353**	0.0001

Only individuals belonging to one wild population (northern Murcia, SE Spain) were included on this analysis, therefore Wahlund effect should not be considered. Inbreeding could explain the heterozygote deficit because of the monogamy of *B. bubo*; non-random mating is likely in this species, such as it was described in another raptors (Roulin et al., 2000). Selection against heterozygotes can not be demonstrated from our results. However, these heterozygote deficits could be linked, unless in two loci (Bbu225 and Bbu245), with the presence of null alleles ($p < 0.001$). Only 1 pair of loci (Bbu245 and Bbu230) out of 45 tests, showed significant linkage disequilibrium ($p < 0.05$) after Bonferroni correction.

These new 10 microsatellite loci showed a high genetic diversity (number of alleles per locus, observed and expected heterozigosity) and suitability for studies of population connectivity and parentage, in combination with those previously published. Therefore, these microsatellites should serve as optimal molecular tools for projects concerning conservation strategies of the Eurasian eagle-owl.

Acknowledgements

We thank José Francisco Calvo Sendín and José Enrique Martínez Torrecillas for their comments. We are very grateful to Eloy Peréz and José Alfonso Lacalle for their help on field-work and Tomás García-Rubio and Maria José Ortega-Jiménez for their unconditional and constant support. Sergio León-Ortega and Aniceto León-Madrid collaborated with partial financial support. This study was carried out thanks to the Comunidad Autónoma de la Región de Murcia (Spain), with its permit and consent. Dr. Mercedes González-Wangüemert MGW was supported by Fundação para a Ciência e Tecnologia (FCT, Portugal) postdoctoral grant (SFRH/BPD/70689/2010) and later by FCT Investigator Programme-Career Development (IF/00998/2014).

References

Belkhir, K., Borsa, P., Goudet, J., Chicki, L., Bonhomme, F., 1996–2004. GENETIX 4.05, logiciel sous WindowsTM pour la génetique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000Université de Montpellier II, Montpellier France. Available on. http://www.univ-montp2.fr/genetix/genetix/genetix.htm.

Dalbeck, L., Heg, D., 2006. Reproductive success of a reintroduced population of Eagle Owls *Bubo bubo* in relation to habitat characteristics in the Eifel, Germany. Ardea 94, 3–21.

Isaksson, M., Tegelström, H., 2002. Characterization of polymorphic microsatellite markers in a captive population of the eagle owl (*Bubo bubo*) used for supportive breeding. Mol. Ecol. Notes 2, 91–93.

IUCN, 2015. IUCN Red List of Threatened Species. Version 2014.3. www.iucnredlist.org. Downloaded on 13 May 2015.

Kleven, O., Dawson, D.A., Gjershaug, J.O., Horsburgh, G.J., Jacobsen, K.O., Wabakken, P., 2013. Isolation, characterization and predicted genome locations of Eurasian eagle-owl (*Bubo bubo*) microsatellite loci. Conserv. Genet. Res. 5, 723–727.

León-Ortega, M., González-Wangüemert, M., Martínez, J.E., Calvo, J.F., 2014. Spatial patterns of genetic diversity in Mediterranean Eagle Owl *Bubo bubo* populations. Ardeola 61, 45–62.

Lourenço, R.F., Santos, S.M., Rabaça, J.E., Penteriani, V., 2011. Super predation patterns in four large European raptors. Popul. Ecol. 53, 175–185.

Penteriani, V., Gallardo, M., Roche, P., 2002. Landscape structure and food supply affect Eagle Owl (*Bubo bubo*) density and breeding performance: a case of intra-population heterogeneity. J. Zool. 257, 365–372.

Penteriani, V., Delgado, M.M., Bartolommei, P., Maggio, C., Alonso-Alvarez, C., Holloway, G.J., 2008. Owls and rabbits: predation against substandard individuals of an easy prey. J. Avian Biol. 39, 215–221.

Penteriani, V., Delgado, M.M., Pérez-García, J.M., Botella, F., García, S., Sanchez-Zapata, J.A., León-Ortega, M., Martínez, J.E., Calvo, J.F., Ortego, J., Penteriani, G., Melletti, M., Krugersberg, I., Mora, O., 2010. Sex allocation from an owl perspective: clutch order could determine brood sex to reduce sibling aggression. Ornis Fenn. 87, 135–143.

Roulin, A., Jungi, T.W., Pfister, H., Dijkstra, C., 2000. Female barn owls (*Tyto alba*) advertise good genes. Proc. Biol. Sci. 267, 937–941.
Rousset, F., 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol. Ecol. Res. 8, 103–106.
Sambrook, E., Fritsch, F., Maniatis, T., 1989. Molecular Cloning. Cold Spring Harbour Press, New York, EEUU, 545 p.
Schaub, M., Aebischer, A., Giménez, O., Berger, S., Arlettaz, R., 2010. Massive immigration balances high anthropogenic mortality in a stable eagle owl population: lessons for conservation. Biol. Conserv. 143, 1911–1918.
Schuelke, M., 2000. An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18, 233–234.
Sergio, F., Marchesi, L., Pedrini, P., Ferrer, M., Penteriani, V., 2004. Electrocution alters the distribution and density of a top predator, the Eagle Owl *Bubo bubo*. J. Appl. Ecol. 41, 836–845.
Solonen, T., 2011. Impact of dominant predators on territory occupancy and reproduction of subdominant ones within a guild of birds of prey. Open Ornithol. J. 4, 23–29.
van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. MicroChecker: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535–538.