A core set of microsatellite markers identified for use in population genetic studies of Purple Swamphen (*Porphyrio porphyrio*)

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Abstract

A core set of microsatellite markers identified for use in population genetic studies of Purple Swamphen (Porphyrio porphyrio).— Populations of Purple Swamphen have been declining in some parts of the world due to wetland degradation and poaching. Conservation efforts might benefit from population genetic studies using microsatellite markers. Here we describe the selection of microsatellite markers suitable for such studies. We tested the markers against the evidence of the null alleles and gametic disequilibrium. From fourteen candidate loci, ten displayed a null alleles frequency of less than 0.2 and had no significant linkage disequilibrium. The selected loci showed no deviation from HWE and had a mean polymorphic information content of 0.516. Based on the test, we suggest these loci are suitable for population genetic studies of the species.

Key words: Microsatellites, *Porphyrio*, Wetland, Indonesia.

Resumen

*Conjunto básico de marcadores de microsatélites identificados para el uso en estudios de genética de poblaciones en calamón común (*Porphyrio porphyrio*) en Java*.— Las poblaciones de calamón común han descendido en algunas partes del mundo debido a la degradación de los humedales y la caza furtiva. Los esfuerzos de conservación podrían beneficiarse de los estudios de genética de poblaciones que emplean marcadores de microsatélites. Aquí se describe la selección de los marcadores adecuados para tal estudio. Se han probado estos marcadores frente a la presencia de alelos nulos y desequilibrio gamético. De catorce loci candidatos, diez muestran una frecuencia de alelo nulo menor de 0,2 y no poseen desequilibrio de ligamiento significativo. Los loci seleccionados no presentaron desviación del equilibrio de Hardy–Weinberg (HWE) y tienen un contenido de información polimórfica (PIC) medio de 0,516. Sobre la base de esta prueba, se sugiere que estos loci deben ser incluidos en estudios de genética de poblaciones de esta especie.

Palabras clave: Microsatélites, *Porphyrio*, Humedal, Indonesia.

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Introduction

The Purple Swamphen (*Porphyrio porphyrio* Linnaeus, 1758) faces population decline due to wetland drainage and poaching in some parts of its distribution range (Taylor, 1996), although the bird is not listed as an endangered species globally (IUCN, 2011). In the Iberian Peninsula the population has been declining due to wetland loss, which has led to local protection efforts (Sánchez–Lafuente et al., 2001). In Asia, studies have reported population declines owing to wetland degradation and, as a result, population protection was requested (*e.g.* Lok & Subaraj, 2008; Menon, 2004). In Indonesia there are no reliable data on the current Purple Swamphen population, but more than 25 years ago McKinnon & Phillips (1983) roughly estimated that the bird was still commonly distributed in the greater Sundaland area, including Java Island. Taxonomy of the species is complex (Taylor, 1996) and there is possible taxonomical uncertainty of the Purple Swamphen inhabiting Java Island (Sangster, 1998).Taking into account the occurrence of wetland loss in Java (Whitten et al., 1996), it is likely that the bird population on Java Island has also been declining recently. Population genetic studies may contribute to conservation effortss by providing information on population dynamics and structure and assess potential losses of genetic diversity.

Population genetic studies of wild bird populations commonly rely on cross–species amplification as a cost–effective approach for developing microsatellite markers (Primmer et al., 1996). Cross–species amplification, however, can result in ascertainment bias. One way to solve this problem is by decreasing the annealing temperature and ensuring good DNA quality. Moreover, a thorough evaluation should be conducted against evidence of the null alleles, gametic disequilibrium and selective neutrality (Selkoe & Toonen, 2006). Here we report a set of microsatellite markers developed for *P. hochstetteri* that were found to be suitable for population genetic study of *P. porphyrio*.

Material and methods

Sample collection and DNA extraction

From February 2009 until January 2010, 50–100 µL of blood was taken from 88 individual birds inhabiting five wetlands on Java Island. The blood samples were taken via a wing vein using a syringe, and preserved in 1 ml ethanol (Arctander, 1988). After blood collection, the bird was marked with a small wing tag to avoid double sampling, and released.

We conducted DNA isolation using a kit (PureLink™ Genomic DNA; Invitrogen) for the majority of our samples, and fewer than 5% samples were isolated using phenol–chloroform technique. As an adjustment to the protocol provided by the kit manufacturer, we transferred 200–300 µL of the blood sample preserved in ethanol into a microtube containing 20 µL of Proteinase–K and incubated this at 55°C for 3 hours, as an increase in genomic DNA yield has been observed as compared to the same protocol without this step (De Volo et al., 2008). After isolation, the DNA was visualized on 1.8% agarose gel to assess the yield.

Microsatellite genotyping

Before DNA amplification, 14 potential microsatellite loci were selected from the literature. We selected four loci designed by Gregory & Quinn (2006) for *Porphyrio porphyrio* (PPOSNX18, PPOSNX19, PPOZAP04 and PPOZAP15) and ten loci designed by Grueber et al. (2008) for *P. hochstetteri* (Pho06, Pho12, Pho46, Pho60, Pho62, Pho74, Pho84, Pho90, Pho107 and Pho110). The selection was based on the reported number of alleles and expected heterozygosity.

We performed DNA amplification using a touch–down polymerase chain reaction (TD–PCR) technique and scored the PCR product using an automatic sequencer. TD–PCR was performed by involving 2.5–3 µL DNA template from 88 individuals, 0.4–0.6 µM primers, 7.5 µL pre–mixed PCR reagent (DreamTaq™ Green PCR Master Mix; Fermentas), and nuclease–free water up to the total volume (15 µL). The forward primer of each pair was labeled either with FAM (Pho06, Pho46, Pho60, Pho84, Pho90, Pho107, PPOSNX18, PPOSNX19, PPOZAP04, PPOZAP15) or HEX (Pho12, Pho62, Pho74, Pho110). After an initial denaturation step of 2 min at 95°C, 10 cycles of PCR were performed. Each cycle consisted of 30s denaturation at 95°C, and 30s of annealing starting at 63°C or 53°C and dropping by 1°C per cycle. After annealing, an extension of 20s at 72°C was performed. Additionally, we carried out a further 20 cycles with 30s denaturation at 95°C and 30s annealing at 53°C or 43°C, and an extension of 20s at 72°C. The reactions were completed by a final extension step of 5 min at 72°C, after which the temperature was kept constant at 4°C. The PCR product was visualized on a 6% polyacrylamide gel. Only primers that resulted in scoreable bands were selected to amplify the rest of the samples. The DNA fragment yielded from the amplification was visualized using an automated sequencer machine (ABI 3100 Prism; Applied Bioscience) and scored using GENEMAPPER v.4.0. Sequencing some of the alleles found in *P. porphyrio* would have been helpful to confirm the identity of alleles, but this was not done due to logistic constraints.

Statistical analysis

We checked for data typing errors, calculated the proportion of successful amplifications, identified two or more identical genotypes, and formatted the data for further analysis using MS–TOOLS (Park, 2001). We conducted loci selection based on absence of null alleles and significant linkage disequilibrium. Null allele, F (null), was calculated as a frequency using CERVUS 3.0 (Kalinowski et al., 2007). We excluded any locus from our list if they had a F(null) of more than 0.2 (Dakin & Avise, 2004) and coincidently indicated a heterozygosity deficit due to allele dropout or stuttering. We used MICROCHECKER

Table 1. Summary of characteristics of selected loci. No loci showed any linkage disequilibrium with each other and the frequency of null alleles was less than 0.2. The loci showed no deviation from HWE and had a mean PIC of 0.516: Ampl. Amplification success (%); A. Mean number of alleles; PIC. Polymorphic information content; bp. Base pair; H_e. Expected heterozygosity; H_o. Observed heterozygosity; F(null). Frequency of null alleles: * Heterozygote deficit due to allele dropout or stuttering.

*Tabla 1. Resumen y características de los loci selecionados. Ningún locus presentó desequilibrio de ligamiento con respecto a los demás, y la frecuencia de alelos nulos fue menor de 0,2. Dichos loci no presentaron desviación alguna del HWE y poseían un PIC medio de 0,516: Ampl (%). Éxito de amplificación en porcentaje; A. Número promedio de alelos; PIC. Contenido de información polimórfica; bp. Tamaño del alelo en pares de bases; H_e. Heterocigosidad esperada; H_o. Heterocigosidad observada; F (null). Frecuencia de alelos nulos: * Déficit de heterocigotos debido a su desaparición o su aparición esporádica.*

Locus	Ampl.(%)	A	PIC	Allele size (bp)	H_e	H_o	F(null)
PPOZAP15	83	3	0.42	178–200	0.60	0.51	0.09
Pho46	96.6	10	0.77	97–123	0.78	0.80	0.01
Pho60	97.7	6	0.45	91–123	0.43	0.53	0.11*
Pho84	96.6	8	0.71	149–171	0.71	0.75	0.02
Pho110	100	3	0.15	105–119	0.17	0.16	0.04
Pho12	98.9	3	0.58	129–135	0.58	0.66	0.06
Pho107	100	4	0.56	114–126	0.83	0.62	0.16
Pho06	80.7	2	0.08	138–140	0.09	0.08	0.01
Pho62	88.6	5	0.66	87–115	0.51	0.71	0.15*
Pho74	98.9	9	0.78	124–166	0.94	0.81	0.08
Mean	94.1	5.3	0.516		0.564	0.563	

(Van Oosterhout et al., 2004) to check the indication of the heterozygote deficit. We also discarded any candidate locus if it showed a significant linkage with other loci after testing with GENEPOP 4.0 (Rousset, 2008). Additionally, we conducted a Hardy–Weinberg Equilibrium test using the same program.

Using the selected loci, we explored the essential genetic diversity data of the population, including mean number of alleles per locus (A), allelic size, observed (H_o) and expected (H_e) heterozygosity, and polymorphic information content (PIC; Botstein et al., 1980). A, H_o, H_e, N, and PIC were calculated using CERVUS 3.0 (Kalinowski et al., 2007). Our loci selection showed that only 10 of the 14 loci selected from previous literature could feasibly be involved in the population genetic study. The four unsuitable loci were discarded due to a low rate of amplification success (PPOZAP04), monomorphism (Pho110 and Pho90) and a high frequency of the null alleles (PPOSNX18). Each of the remaining ten loci successfully amplified more than 80% of times, had a small (less than 0.05) to moderate (0.05–0.2) null allele frequency, and had no significant linkage between loci (Bonferroni correction; $P < 0.00022$). No loci showed any deviation from HWE and all had a mean PIC of 0.516. However, performance of each locus varied.

Results

Table 1 shows that loci Pho46, Pho74 and Pho84 had successful amplification rates of more than 95%, null allele frequency of less than 0.1, allelic richness of more than 7.5, and PICs of more than 0.7. These values were above average. Pho06, Pho110 and PPOZAP15 had below average allelic richness (less than 3.00) and PIC (less than 0.5), although Pho110 had a perfect amplification rate and low null allele frequency. The low performance of Pho06 may be attributable to sex linkage loci, as reported by Grueber et al. (2008) and Grueber et al. (2011). All three loci had a relatively high value of null allele frequency (> 0.1). Two of the loci, Pho60 and Pho62, show a heterozygote deficit due to allele dropout or stuttering. It may indicate presence of null alleles. However, we included both loci in our list because their null allele frequencies were less than 0.2.

To conclude, this study suggests that using pure DNA isolated from fresh blood and a wide range of annealing temperatures, as implemented in TD–PCR, supports the success of cross–species amplification (Primmer et al., 2005). Moreover, this study was also able to successfully select ten microsatellite loci that are potentially useful for a genetic structure analysis. Using those loci for population genetic studies may

produce reliable and valuable information for the conservation of the Purple Swamphen population in Java.

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