Microsatellite markers for the study of paternity in Greater Flamingo (*Phoenicopterus roseus*) and Caribbean Flamingo (*P. ruber*)

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Abstract
DNA of Greater Flamingo (Phoenicopterus roseus) and Caribbean Flamingo (P. ruber) was amplified using PCR with cross-species primers amplifying polymorphic microsatellite loci in species belonging to order Pelecaniformes: great cormorant Phalacrocorax carbo and flightless cormorant Phalacrocorax harrisi; to order Ciconiiformes: great blue heron Ardea herodias, wood stork Mycteria americana, scarlet ibis Eudocimus ruber and roseate spoonbill Ajaia ajaja and to order Podicipediformes: red-necked grebe Podiceps grisegena.
From the whole number of 70 tested microsatellites we found 7 polymorphic microsatellites in Phoenicopterus roseus: WSμ 17, WSμ 19, PcD 6, PhD11, PhB2, PhB4 and 10 polymorphic microsatellites in Phoenicopterus ruber: WSμ 03, WSμ 17, WSμ 20, WS2, Eru03, Eru11, Aaju4, PcD 6, PhG8 and Ah 630. These microsatellite loci could be used for tests of paternity.

Key words: Flamingo, *Phoenicopterus roseus*, *Phoenicopterus ruber*, cormorant, heron, stork, ibis, spoonbill, grebe, microsatellite, cross-species amplification

Introduction

The flamingos are highly specialized, large long-legged water birds. Plumage is white, pink, red and black. Curved bill is specialized for filtration of feed from water or from bottom mud. They make mud-mound nests and lay only one egg (Brown *et al.,* 1982).

Although the flamingos are one of the oldest birds' family and their fossils are known from Oligocene (30 million years ago), their taxonomic situation is still unclear. Due to their anatomy and behavior, flamingos are separated to order Phoenicopteriformes, placed between Ciconiiformes and Anseriformes (del Hoyo *et al*., 1992). The most recent molecular (Tuinen *et al.,* 2001) and morphological (Mayr, 2004) studies place flamingos to connection with Podicipediformes. The order Phoenicopteriformes has only one family with 6 species (Brown *et al.,* 1982).

Up to date there have not been described any *de novo* or cross-species microsatellites to use them for the study of paternity. Microsatellites are hypervariable regions in DNA (Baker, 2000). Due to their variability and inheritance based on Mendelian laws, microsatellites are an important tool in paternity determination (Ellegren, 1992). To find the microsatellite loci from a new species usually requires testing cross-species PCR amplifications of their genomic DNA with primer pairs, which are known in other related species (Primmer *et al.,* 1996).

Material and methods

Blood samples were collected from *Phoenicopterus roseus* – 22 individuals and *P. ruber* – 30 individuals bred in the zoological garden in Dvůr Králové nad Labem (Czech Republic) during 2007-2008 and in zoological garden in Liberec (Czech Republic) 2008. Blood was venipunctured from tarsal part of leg and immediately stored in Queen's buffer (Seutin *et al.*, 1991). The DNA from blood in the storage buffer was isolated using the phenol - chloroform method, resolved in TE buffer and optimized to concentration of 20-50 μg/ml. 70 primer pairs were tested for the PCR amplification of 6 individuals of each species; these primer pairs were previously used to study the polymorphic microsatellite DNA loci in birds belonging to order Pelecaniformes: PcD 2, PcD 4, PcD 5, PcD 6, PcT 1, PcT 3 and PcT 4 from great cormorant *Phalacrocorax carbo* (Piertney *et al*., 1998) and PhB2, PhB4, PhB11, PhC11, PhD11, PhF12 PhG8 and PhG12 from

flightless cormorant *Phalacrocorax harrisi* (Duffie *et al*., 2008); to order Ciconiiformes: Ah 205, Ah 208, Ah 209, Ah 210, Ah 211, Ah 212, Ah 217, Ah 320, Ah 341, Ah 343, Ah 414, Ah 421, Ah 517, Ah 522, Ah 526, Ah 536 and Ah 630 from great blue heron *Ardea herodias* (McGuire & Noor, 2002), WS1, WS2, WS4, WS6, WSμ 03, WSμ 08, WSμ 09, WSμ 13, WSμ 14, WSμ 17, WSμ 18, WSμ 19, WSμ 20, WSμ 23 and WSμ 24 from wood stork *Mycteria americana* (van den Bussche *et al*.,1999; Tomasulo-Seccomandi *et al*., 2003), Eru02, Eru03, Eru04, Eru05, Eru06, Eru07, Eru08, Eru09, Eru10 and Eru11 from scarlet ibis *Eudocimus ruber* (Santos *et al*., 2006) and Aaju1, Aaju2, Aaju3, Aaju4, Aaju5 and Aaju6 from roseate spoonbill *Ajaia ajaja* (Sawyer & Benjamin, 2006) and to order Podicipediformes: PgAAT1, PgAAT3, PgAAT6, PgAAT8, PgAAT25, PgAAT34 and PgAAT41 from red-necked grebe *Podiceps grisegena* (Sachs & Hughes, 1999).

PCR amplification was performed in 10 μl reactions containing 1 μl of template DNA, 0.5 U of a*Taq* DNA Polymerase (Promega), 0.1 μl of dNTPs (20 mmol/l), 0.6 μl of $MgCl_2$ (25 nmol/l), 0.5 μl of each primer (10 μl/l), 1 μl of 10 x storage buffer A and 6.2 μl of deionized water. At first we used PCR conditions: initial denaturation at 94 ℃ for 5 minutes followed by 35 cycles at 94 ℃ for 30 seconds, annealing 50 ℃ for 30 seconds and 72 ℃ for 30 seconds. The final extension at 72 ℃ for 5 minutes followed the last cycle. Secondly, we tested higher annealing temperatures (max. 67 ℃) for primer pairs, which showed an unscorable multi-band pattern and lower annealing temperatures (min. 48 ℃) for primer pairs, which showed no product in PCR reaction. The amplified DNA fragments went through 6% denaturing polyacrylamide gel. The visualization of the DNA fragments was accomplished using silver treatment (Bassam *et al*., 1991).

Results and discussion

After the PCR amplification we excluded all the microsatellite loci, which produced ambiguous band patterns – no product, poor amplification, confusing stutter bands or multiple products. We have found 7 polymorphic microsatellites suitable for the paternity determination in *Phoenicopterus roseus*: WSμ 17, WSμ 19, PcD 6, PhD11, PhB2, PhB4 and Ah 630 and 10 polymorphic microsatellites in *Phoenicopterus ruber*: WSμ 03, WSμ 17, WSμ 20, WS2, Eru03, Eru11, Aaju4, PcD 6, PhG8 and Ah 630. Only three of them were common to the both flamingos' species: WSμ 17, PcD 6 and Ah 630. The most polymorphic microsatellite locus was WSμ 17: 5 alleles in *Phoenicopterus ruber* and 7 alleles in *P. roseus*. The average numbers of alleles per locus were 2.7 in *P. ruber* and 3.3 in *P. roseus*. The average observed heterozygosities (H_O) per locus were 0.33 in *P. ruber* and 0.62 in *P. roseus*.

WSμ 03, WSμ 17 and WSμ 19 were previously found polymorphic in *Mycteria americana* (3 alleles in WSμ 03 and 2 alleles in WSμ 17 and WSμ 19) – this is the first report about successful WSμ 03, WSμ 17 and WSμ 19 cross-species amplification. WSμ 20 was previously also found polymorphic in *Mycteria americana* (3 alleles) (Tomasulo-Seccomandi *et al*., 2003). This locus also showed a polymorphic product in *Pelecanus crispus* (2 alleles; high frequency of null allele) *P. onocrotalus* (3 alleles) and *P. rufescens* (3 alleles; high frequency of null allele) (Nádvorník *et al.*, 2008; Nádvorník, unpublished data). WS2 was previously found polymorphic in *Mycteria americana* (2 alleles) (van den Bussche *et al*., 1999) – this is the first report about the successful WS2 cross-species amplification.

Eru03 and Eru11 were previously found polymorphic in *Eudocimus ruber* (9 alleles in Eru03 and 3 alleles in Eru11) (Santos *et al*., 2006) – this is the first report about successful Eru11 cross-species amplification. Eru03 also showed a polymorphic product in *Pelecanus onocrotalus* (2 alleles) and *P. rufescens* (2 alleles) (Nádvorník *et al.*, 2008; Nádvorník, unpublished data).

Aaju4 was previously found polymorphic in *Ajaia ajaja* (2 alleles) (Sawyer & Benjamin, 2006) – this is the first report about successful Aaju4 cross-species amplification.

Ah 630 was previously found polymorphic in *Ardea herodias* (4 alleles), *A. alba* and *A. cocoi* (McGuire & Noor, 2002). Ah 630 also showed a polymorphic product in *Pelecanus onocrotalus* (5 alleles) and *P. philippensis* (2 alleles) (Nádvorník *et al.*, 2008; Nádvorník, unpublished data).

PcD 6 was previously found polymorphic in *Phalacrocorax c. carbo* (9 alleles), *P. c. novahollandiae* (4 alleles), *P. aristotelis* (2 alleles), *P. auritus* (2 alleles) and *P. atriceps* (3 alleles) (Piertney *et al*., 1998).

PhB2, PhB4, PhD11 and PhG8 were previously found polymorphic in *Phalacrocorax harrisi* (3 alleles in PhB2 and PhB4, 9 alleles in PhD11 and 4 alleles in PhG8) (Duffie *et al*., 2008) – this is the first report about the

successful PhB2, PhB4, PhD11 and PhG8 cross-species amplification.

Polymorphic microsatellite loci found in *Podiceps grisegena* failed in amplification in both *Phoenicopterus* species.

We can suppose very close kinship between Phoenicopteriformes, Pelecaniformes and Ciconiiformes due to successful PCR amplification of polymorphic microsatellite loci in Phoenicopteriformes, which were discovered in Pelecaniformes and Ciconiiformes. It is hard to predict a level of kinship between Phoenicopteriformes and Podicipediformes. Although all 7 loci from *Podiceps grisegena* failed in PCR amplification in *Phoenicopterus roseus* and *P. ruber*, it is not enough for presuming relationship between these two genera.

We suggest testing in both *Phoenicopterus* species microsatellite loci derived from crested ibis *Nipponia nippon* and successfully amplified in black-headed ibis *Threskiornis melanocephalus* (Ji *et al*., 2004), due to our successful experience with the cross-species PCR amplification of microsatellites derived from Ciconiiformes (*Ardea herodias*, *Mycteria americana*, *Eudocimus ruber* and *Ajaia ajaja*). Since Phoenicopteriformes are sometimes connected with Anseriformes (Sibley *et al.,* 1969), it is possible to try in *Phoenicopterus roseus* and *P. ruber* cross-species amplification of microsatellites derived from Anseriformes, which amplified polymorphic product in other Anseriformes taxa (Stain & Hughes, 2003; Guay & Mulder, 2005; etc.).

Conclusion

- PCR amplification of DNA of Greater Flamingo (*Phoenicopterus roseus*) and Caribbean Flamingo (*P. ruber*) with cross-species primers amplifying polymorphic microsatellite loci.
- Used primer pairs derived from taxa belonging to order Pelecaniformes: great cormorant *Phalacrocorax carbo* and flightless cormorant *Phalacrocorax harrisi*; to order Ciconiiformes: great blue heron *Ardea herodias*, wood stork *Mycteria americana*, scarlet ibis *Eudocimus ruber* and roseate spoonbill *Ajaia ajaja* and to order Podicipediformes: red-necked grebe *Podiceps grisegena*.
- Found 7 polymorphic microsatellites in *Phoenicopterus roseus*: WSμ 17 (7 alleles), WSμ 19 (2 alleles), PcD 6 (2 alleles), PhD11 (3 alleles), PhB2 (2 alleles), PhB4 (3 alleles) and Ah 630 (4 alleles) and 10 polymorphic microsatellites in *Phoenicopterus ruber*: WSμ 03 (2 alleles), WSμ 17 (5 alleles), WSμ 20 (3 alleles), WS2 (4 alleles), Eru03 (2 alleles), Eru11 (3 alleles), Aaju4 (2 alleles), PcD 6 (2 alleles), PhG8 (2 alleles) and Ah 630 (2 alleles).

Three of cross-species microsatellites common to the both flamingos' species: WSμ 17, PcD 6 and Ah 630.

- The average numbers of alleles per locus: 2.7 in *P. ruber* and 3.3 in *P. roseus*.
- The average observed heterozygosities (H_O) per locus: 0.33 in *P. ruber* and 0.62 in *P. roseus*.

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