D-loop deletion in the mitochondrial DNA of the Black Stork *Ciconia nigra*

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Abstract

The black stork *Ciconia nigra* is a migrant species which is poorly known and has a history of strong decline in the last century, with local extinctions and recolonizations in Central Europe. Few genetic studies have been reported for this species, and only a few conservative regions of DNA sequences are known. Using black stork samples from several European locations, we have been able to characterise a short variable region of *Ciconia nigra* mitochondrial D-loop. A 22 bp deletion was found, in comparison to available mitochondrial sequences from other stork species - *Ciconia ciconia* and *Ciconia boyciana*. This molecular data provides additional information on the unresolved phylogenetic relationships among storks of the genus *Ciconia*. This work is the first report on D-loop sequences from the black stork.

Key words: Black Stork, *Ciconia nigra*, mitochondrial DNA

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INTRODUCTION

The black stork *Ciconia nigra* nests throughout Eurasia from Iberia to eastern Siberia and China, and also in southern Africa (Del Hoyo et al 1992, Wetlands International 2002). The isolation of the Iberian black stork population may stem from an accentuated decline in numbers in Western and Central Europe during the 19th century, which seem to have led to local extinctions, and greater discontinuity in the European population (Del Hoyo et al 1992).

Cytochrome b and ribosomal regions of black stork mitochondrial (mt) DNA have been previously studied (Slikas 1997, Hedges & Sibley 1994, Van Tuinen et al 2000, 2001) but did not show strong differentiation among members of the genus *Ciconia*.

Aiming to highlight the intraspecific relationship between European black stork individuals, and based on previously available mitochondrial DNA sequences from *Ciconidae*, this work focuses on the characterisation of intraspecific polymorphisms in the variable d-loop region from black stork. No sequences from the mitochondrial control region were known for the stork species *Ciconia nigra*.

METHODS

Blood or feathers were obtained from 30 black stork individuals from different geographic European areas such as Iberia (Portugal and Spain), Latvia, Poland, and Belgium. DNA was extracted either from blood using the Puregene blood kit (Gentra) or from feathers using the Nucleospin tissue DNA extraction kit (Macherey-Nagel) with minor modifications.

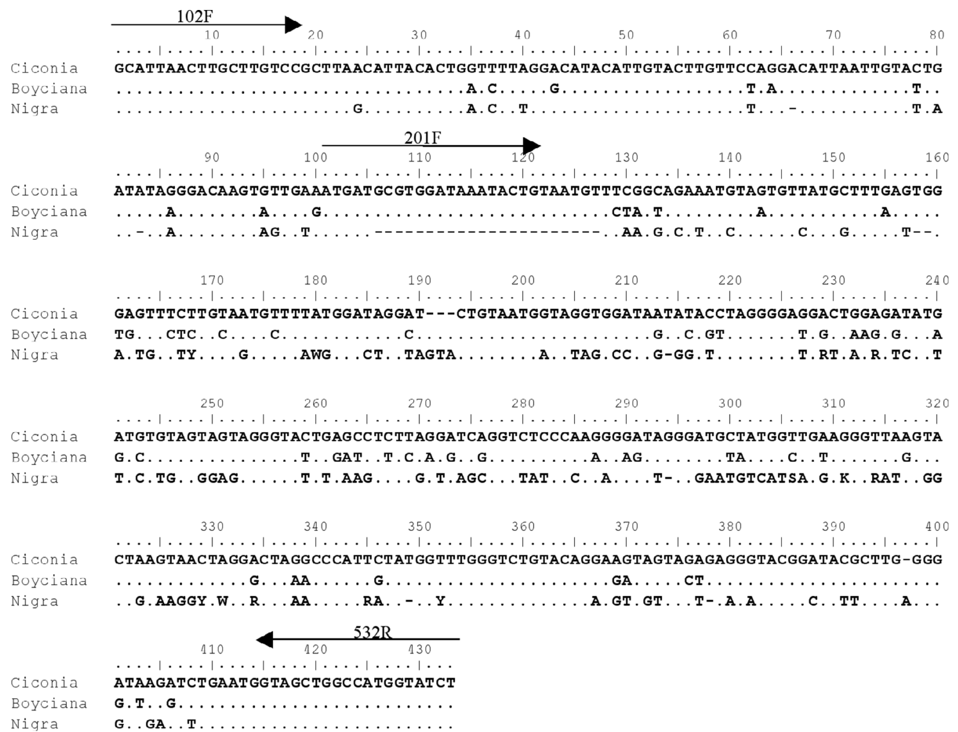
We designed three primers by alignment with homologous sequences from white stork - *Ciconia ciconia* (AB026818) and oriental stork - *Ciconia boyciana* (AB026193): two forward primers (102F-GCATTAACTTGCTTGTCC and 201F- ATGATGCGTGGATAAATACTG) and one reverse primer (532F-AGATACCATGGCCAGCTACC). Primers were designated according to the nucleotide position number in the *C. boyciana* D-loop sequence.

PCR amplification was carried out in a 25 µL reaction volume containing 1 mM dNTPs, 20 pmoles of each primer, 2.5 mM $MgCl_2$, 0.3 µg of BSA, and 1 U of Taq polymerase (Fermentas). The cycling parameters were 3 min at 94ºC, followed by 36 cycles of 95ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min and a final extension step at 72ºC for 3 min. Amplification products were separated by electrophoresis on a 1-2% agarose gel. As PCR products were absent when using primers 201F/532R, we used primers 102F/532R which amplified a multiplicity of fragments. Individual bands were picked from the gel and specifically PCR reamplified and cloned using a TA cloning vector (InsT/A cloning kit, Fermentas). Transformation of *Escherichia coli* DH5α was followed by recombinant plasmid purification. Recombinant plasmids were then sequenced using a 310 ABI Prism Genetic Analyser. The similarity between obtained sequencing data and *Ciconidae* mitochondrial sequences was confirmed using the nucleotide BLAST program (www.ncbi.nih.gov). Sequences from 13 black stork individuals and *C. ciconia* and *C. boyciana* were aligned with use of the Multialign program (Corpet 1988).

RESULTS AND DISCUSSION

By homology with the other *Ciconia* sequences, a PCR product of 425 bp was expected, but primers 102F/532R amplified several fragments ranging from 380 to 450 bp. This multi-band phenomenon has been observed in many vertebrate species, particularly birds, and is thought to result from the presence of nuclear copies of mtDNA. (Sorenson & Fleischer 1996). Sequencing of sub-cloned PCR products revealed one 405 bp amplified product corresponding to the *C. nigra* D-loop mitochondrial target. Another fragment around

Figure 1. DNA sequence comparison between a partial D-loop region from *Ciconia ciconia* (Ciconia) and *Ciconia boyciana* (Boyciana) and the determined sequences from *Ciconia nigra* (Nigra). Dots represent identical bases. Dashes represent deletions. In Nigra sequence base substitutions are represented by Y=C/T , W=A/T; R=A/G, S =C/G, K=G/T. Arrows represent primer positions, numbers above the arrows correspond to the base numbers from which primers were designed based on the *Ciconia boyciana* sequence (AB026193).



425 bp was found to be a forward-forward primer flanked product. From all 13 sequenced individuals, 5 haplotypes were found (submitted to GenBank, Acession Numbers: AY685122-AY685126). Sequence comparison is shown in Figure 1. There is high nucleotide identity with the *C. ciconia and C. boyciana* mitochondrial sequences (mainly from the 5′ forward direction) followed by a major 22 bp deletion. This deletion explains why the pair of primers 201F/532R did not amplify black stork samples, since the 201F primer was designed in the corresponding region of the *C. ciconia* and *C. boyciana* sequences.

The 5 identified haplotypes were derived from 13 individuals from different locations from the European black stork population: 3 individuals from Portugal, 3 from Spain, 4 from Poland, 2 from Belgium and one individual from Latvia. Despite the low sample size, the relatively high number of polymorphic sites supports the concept of a variable nature of the studied fragment, as previously hypothesised for the homologous sequences (Yamamoto et al 2000).
Multiple sequence alignment revealed eight deletions for black stork sequences: six 1 bp point deletions, one 22 bp deletion and one 2 bp deletion, as well as a 3

bp insertion (Figure 1). These variations show a clear differentiation between the black stork and the two other Ciconias. Accordingly, Slikas (1997) placed the black stork apart from the white stork and oriental stork. In that study the black stork was positioned closer to other African members of genus *Ciconia* on a consensus phylogenetic tree based on cyt-b sequences. The present work also shows that *Ciconia ciconia* and *Ciconia boyciana* are more similar to each other than Black Stork, their European congener.

Based on the sequence presented here, it is now possible to design primers for specific amplification of this short variable region of mitochondrial D-loop from *C. nigra*, bypassing the bias caused by the homologous primers used. This will enable faster mitochondrial specific PCR amplification and direct sequencing of PCR products for multiple individual analysis of black stork D-loop sequences. The variable mitochondrial region from *Ciconia nigra* revealed in this study can nevertheless be a valuable molecular marker for answering questions such as the unresolved phylogenetic relationships among members of *Ciconidae* family.

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