Ann. Zool. Fennici 50: 52–63 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 12 April 2013

Efficiency and accuracy of PCR-based sex determination methods in the European Phalacrocoracidae

Evanthia Thanou[1,*], Sinos Giokas[1], Vassilis Goutner[2], Vasilios Liordos[3] & Stella Fraguedakis-Tsolis[1]

[1)] *Section of Animal Biology, Department of Biology, University of Patras, GR-26501 Patras, Greece (*corresponding author's e-mail: ethanou@upatras.gr)*
[2)] *Department of Zoology, School of Biology, Aristotelian University of Thessaloniki, GR-54124 Thessaloniki, Greece*
[3)] *Hellenic Forest Service, 12 Minoas str., GR-19100 Megara, Attiki, Greece*

Received 19 Mar. 2012, final version received 9 Oct. 2012, accepted 19 Oct. 2012

Thanou, E., Giokas, S., Goutner, V., Liordos, V. & Fraguedakis-Tsolis, S. 2013: Efficiency and accuracy of PCR-based sex determination methods in the European Phalacrocoracidae. — *Ann. Zool. Fennici* 50: 52–63.

The applicability of simple PCR-based approaches for sex discrimination in the three European Phalacrocoracidae species was tested, using 93 individuals of known sex and two sets of primers (1237L/1272R and 2550F/2718R) for the amplification of the avian sex-specific chromo-helicase-DNA-binding protein gene. We evaluated the accuracy of each set of primers in providing the correct sex for each individual. The first primer set did not produce reliable results. The second provided a band pattern for each sex, easily distinguishable with agarose gel electrophoresis, which correctly identified all the individuals, even in samples of low DNA yield. The amplification products were sequenced and aligned revealing important nucleotide diversity among Phalacrocoracidae species. Compared with morphometric discriminant analysis and DNA-fingerprinting techniques previously applied, the PCR-based sexing with the 2550F/2718R primers is more accurate, less invasive and widely applicable to both adults and chicks, using a variety of DNA sources such as blood, tissue, feathers, egg shells and others.

Introduction

Information about an individual's sex is important in many studies of avian ecology, evolutionary biology and conservation, but also in poultry breeding, zoo-keeping and re-establishing natural populations. In birds, absence of juvenile sexual dimorphism often makes it difficult or even impossible to determine a chick's sex on the basis of external morphology. A similar problem exists for fully grown individuals of many species where adult sexual dimorphism is absent or at least not very pronounced (Fridolfsson & Ellegren 1999). On the other hand, some adult birds could be sexed by morphometric analyses, if the relationship between sex and body size or feather color was quantified. Yet, application of morphometric analyses becomes more complicated especially when the body size and feather color vary among geographical regions (Kahn *et al*. 1998, Shephard *et al*. 2004).

Usually, cytogenetic approaches like karyotyping or flow cytometry (Nakamura *et al*. 1990, De Vita *et al*. 1994) and various molecular

techniques are applied in sex identification of birds. Molecular techniques include two general strategies: hybridization with sex-specific DNA probes (Griffiths & Holland 1990, Longmire *et al*. 1991) and polymerase chain reaction (PCR) based techniques, such as random amplified polymorphic DNA (RAPD) (Griffiths & Tiwari 1993, Lessells & Mateman 1998), amplified fragment length polymorphism (AFLP) (Griffiths & Orr 1999), and amplification of microsatellite loci (Nesje & Røed 2000). The discovery of several introns in the chromo-helicase-DNA binding-protein (*CHD1*) gene, which vary in size between the male (Z) and the female (W) sex chromosomes, enabled the discrimination between sexes in most avian species in a simple, quick and reliable way (Griffiths & Korn 1997, Griffiths *et al*. 1998, Kahn *et al*. 1998, Fridolfsson & Ellegren 1999). After the PCR amplification of the *CHD1* gene, heterogametic (ZW) females are expected to have two products of different size while homogametic (ZZ) males should have two products of the same size. Using a standard agarose or polyacrylamide gel electrophoresis, one can visualize the sex of each bird, since females show two bands and males show one.

The *CHD1* gene amplification often produces unambiguous results, when one of three known primer pairs is used: 2550F/2718R (Fridolfsson & Ellegren 1999), 1237L/1272H (Kahn *et al*. 1998) or P2/P8 (Griffiths *et al*. 1998). However, no given primer-pair is in widespread use, due to the great nucleotide diversity across different avian species and the existence of polymorphisms in the male *CHD1Z* gene, which produce heterozygote (ZZ´) males (Dawson *et al*. 2001, Lee *et al*. 2002, Robertson & Gemmell 2006). Technical problems, such as PCR competition and non-specific primer-binding may also interfere with the correct sex determination (Griffiths *et al*. 1998). For example, PCR amplification of the *CHD1* introns has been reported to produce one PCR product in both sexes (Dubiec & Zagalska-Neubauer 2006, Ong & Vellayan 2008), two products of similar size (Dawson *et al*. 2001, Gheng *et al*. 2006, Han *et al*. 2009), many products that could not be easily discriminated (Dawson *et al*. 2001), or no products at all (Ito *et al*. 2003). As a result, the standard methods will have to be verified for each new species, using DNA samples from individuals of known sex.

In summary, there is no universally applicable technique for the sex identification of birds that can be considered reliable without thorough species-specific testing. In this context, we evaluated the accuracy of the classic PCR-based *CHD1Z*/*CHD1W* intron polymorphism analysis to determine the sex of the three European Phalacrocoracidae species: *Phalacrocorax pygmeus*, *P. carbo* and *P. aristotelis*. Sex determination is a useful tool in many studies of sea-bird ecology, evolutionary biology and conservation. Especially for the great cormorant, *P. carbo*, and the common shag, *P. aristotelis*, it has been shown that birds of different sexes may have different ecological needs and adaptations (Frederiksen & Bregnballe 2000), different foraging and hunting behavior (Grémillet *et al*. 1996), different survival and breeding rates (Graves *et al*. 1992, Childress & Bennun 2002). For example, experienced breeding female shags seem to affect the sex ratio of their hatchlings, according to food availability and clutch size (Potts *et al*. 1980, Velando *et al*. 2002). Moreover, both species are sexually dimorphic in size, with males being generally 20% heavier than females (Cramp & Simmons 1977, Potts *et al*. 1980), which may also affect their respective survival and reproduction.

Sex determination of the pygmy cormorant, *P. pygmeus*, has never been evaluated before, either with molecular or morphometric methods. The European race of the common shag, *P. aristotelis aristotelis*, has been included in the species originally tested with the P2/P8 pair of primers (Griffiths *et al*. 1998, Griffiths & Orr 1999), but "resisted any attempts at sex identification using the apparently ubiquitous avian CHD test". A more recent study (Kocijan *et al*. 2011) using three common shags of unknown sex from the Mediterranean race, *P. aristotelis desmarestii*, reported that the same set of primers produced a single band in both sexes, while primers 2550F/2718R produced two bands in two of the birds that were consequently considered females. Additionally, only two out of eight tested W-chromosome markers were found to amplify the *CHD1W* gene of the shag, which could be further used with the technically

demanding and time consuming PCR-AFLP protocol described in Griffiths and Orr (1999). So far, sex-related parameters of the common shag's ecology have been primarily studied with the use of morphometric discriminant analyses (MDA) (Calvo & Bolton 1997, Velando *et al*. 2000, Martínez-Abraín *et al*. 2006), where morphological measurements in birds of known sex and subsequent discriminant analyses have been used to provide reliable functions that would enable the correct prediction of sex. Molecular sexing of the common shag has been so far performed using the DNA fingerprinting technique, which is reported to be very accurate but highly time and effort consuming, since it involves several experimental stages including autoradiography which requires approximately three days (Graves 1992, Graves 1993, Velando *et al*. 2002). In the present study, we focus on the Mediterranean race of the common shag, in order to test a more rapid and simple protocol on birds of known sex. As for the great cormorant, MDA has also been used to enable correct sexing (Koffijberg & van Eerden 1995, Newson *et al*. 2004, Liordos & Goutner 2008). The ability of successfully sexing this species using a molecular approach has originally been tested by Fridolfsson and Ellegren (1999), but on a single pair of great cormorants. Here we include considerably more individuals of known sex, aiming not only to verify the reliability of the simplest PCR-based approaches to sex all three species but also the accuracy of the test in providing the correct sex for each individual. Finally, we compare the accuracy of the CHD sexing to other morphological and molecular methods available for the Phalacrocoracidae species.

Material and methods

Muscle tissue samples were obtained from 17 adult birds, found dead in fishery nets and deposited in the Zoological Museum of the University of Patras and the Natural History Museum of Crete. In these cases, sex was identified by gonadal inspection during necropsy. A blood sample was also collected from the brachial vein (wing) of one additional female common shag. This individual had previously been ringed and its sex was identified during egg-laying, in an extensive survey of a Greek shag colony (author's unpubl. data). In total, we used seven great cormorants (five males and two females), two pygmy cormorants (one male and one female) and nine common shags (six males and three females). Moreover, we included muscle or kidney tissue samples from 81 great cormorants (34 males and 47 females, based on gonadal inspection) that were previously used by Liordos and Goutner (2008) in a morphometric analysis of sexual dimorphism. Tissue samples were stored at –80 °C or in 95% alcohol and blood was stored either at –20 °C or in 95% alcohol. Tissue and blood samples stored in alcohol were thoroughly washed with TE9 (500 mM Tris-HCl, 20 mM EDTA, 10 mM NaCl, pH 9.0) and PBS (50 mM KH2PO4, 150 mM NaCl, pH 7.2) prior to DNA extraction.

Total DNA was extracted using the DNeasy Extraction Kit (QIAGEN) and used in two independent PCR protocols to amplify introns from the *CHD1Z* and W genes, both upstream with primers 2550F/2718R (Fridolfsson & Ellegren 1999) and downstream with primers 1237L/1272H (Kahn *et al*. 1998). The alternative downstream pair of primers P2/P8 (Griffiths *et al*. 1998) was considered ineffective according to previous studies in other bird species and particularly in *P. aristotelis aristotelis* (Griffiths *et al*. 1998, Griffiths & Orr 1999, Kocijan *et al*. 2011). Each 10 μl reaction contained 4–8 μl (8–14 ng μl^{-1}) of genomic DNA, 1.0 mM of each primer and 2.5 U of Taq DNA Polymerase (Finnzymes) in the manufacturer's buffer, including 1.75 mM $MgCl_2$ and 0.25 mM of each dNTP. In the reaction profile of each pair of primers, an initial denaturing step at 94 °C for 2 min was followed by 45 cycles of 94 °C for 30 s, 48 °C for 45 s (for the first primer pair)/57 °C for 1m (for the second) and 72 °C for 45 s, then a final step at 72 °C for 10 min. PCR products were visualized at first in 1%–4% agarose gels, stained with ethidium bromide, under UV light, run for up to 2.5 h at 120 V, in order to provide sufficient resolution to differentiate between the resulting *CHD1Z* and W fragments. In some cases the pattern of bands seen in agarose gels gave questionable results. We then used one radioactive, labeled primer for each

pair (^{32}P-2550F and ^{32}P-1237L) during PCR, followed by electrophoresis on 16% polyacrylamide gels, run for approximately 4 h.

In order to verify the origin of our PCR products and estimate the nucleotide diversity between species, we amplified *CHD1Z* and *CHD1W* fragments using the 2550F/2718R primer pair for 10 individuals of known sex: two males and two females for the great cormorant and the common shag, and one of each sex for the pygmy cormorant. PCR products were run on a 1% agarose gel and the resulting DNA fragments were excised directly from the gel. The isolated fragments were purified using the NucleoSpin Extract II Kit (Macherey-Nagel) and sequenced bi-directionally with an ABI PRISM 3100 capillary sequencer (VBC Biotech, Austria) using both primers of the amplification procedure. Sequences were aligned with the program CLUSTAL-W ver. 1.4 (Thompson *et al*. 1994) in a BIOEDIT Sequence Alignment Editor ver. 5.0.9 (Hall 1999), along with the nucleotide sequence data of *CHD1Z* and *CHD1W* of the Japanese cormorant, *P. capillatus*, deposited in the GenBank database with accession numbers AB080660 and AB080661 (Inoue-Murayama *et al*. 2002). Sequences obtained in this study have been deposited in GenBank (accession numbers JX901065–JX901070, *see* Fig. 1).

Results

Comparison of the two primer-sets used for molecular sex discrimination

The 1237L/1272H primer pair was tested in 18 *P. carbo*, *P. aristotelis* and *P. pygmeus* samples, using the PCR protocol designed by Kahn *et al*. (1998). Four samples of low quality could not be amplified. The remaining 14 samples gave *CHD1W* and Z products of similar sizes (approx. 300 bps and 280 bps, respectively) differing by less than 30 bps from the 100 bp size-standard (Fermentas) (Fig. 2a). Therefore, observing the difference between the band pattern of male and female individuals was difficult using a low concentration agarose gel (< 3%). At higher agarose gel concentrations (3% and 4%) and after running electrophoresis for up to 2.5 h, the two bands corresponding to females could be visible, but heating of the agarose gel produced a smear that also confused positive sex determination. Modifications of the PCR protocol, involving different PCR annealing temperatures and different magnesium ions concentrations, did not produce a clearer band-pattern. In fact, even slight changes in the protocol resulted in multiple bands for both sexes. As a result, four females were misidentified as males. Thus of the 14 samples amplified, 10 were correctly assigned to each sex and the accuracy of the test was estimated to be 71% (Table 1). The use of polyacrylamide gels was expected to provide sufficient resolution to discriminate between Z and W products. When this was tested, all but two female common shags were correctly identified (12 of 14 samples or 86%), although several additional bands were observed in all samples, for both sexes.

The primer pair 2550F/2718R, used according to the protocol of Fridolfsson and Ellegren (1999) for the same 18 birds, revealed one band for males and two bands for females. The female bands were well distinguished even by low-concentration (1%–2%), short-time (45 min) agarose gel electrophoresis, since they differed by approx. 200 bps, and revealed no other PCR by-products (Fig. 2b). Amplification of both Z and W products was achieved, even in PCR of low productivity, for example in those samples for which the extracted DNA yield was relatively low and could not be amplified with the 1237L/1272H primer pair. Additionally, using the 2550F/2718R pair of primers, all the individuals tested were correctly assigned to the respective sex (Table 1). Cases of males that were incorrectly considered females were not found, and heterozygous males (ZZ´) were not detected for either primer set, even with polyacrylamide electrophoreses.

Of the 81 great cormorants studied by Liordos and Goutner (2008), six samples did not provide a DNA yield. Samples of the remaining 75 birds were amplified with both primer pairs and sex was visualized on an agarose gel, under the best conditions for each respective primer pair, as described above. The 1237L/1272H pair failed to produce any results in 17 individuals and misidentified 29 females. The accuracy of the test was estimated at 29 of the 58 samples amplified or 50%. The 2550F/2718R pair cor-

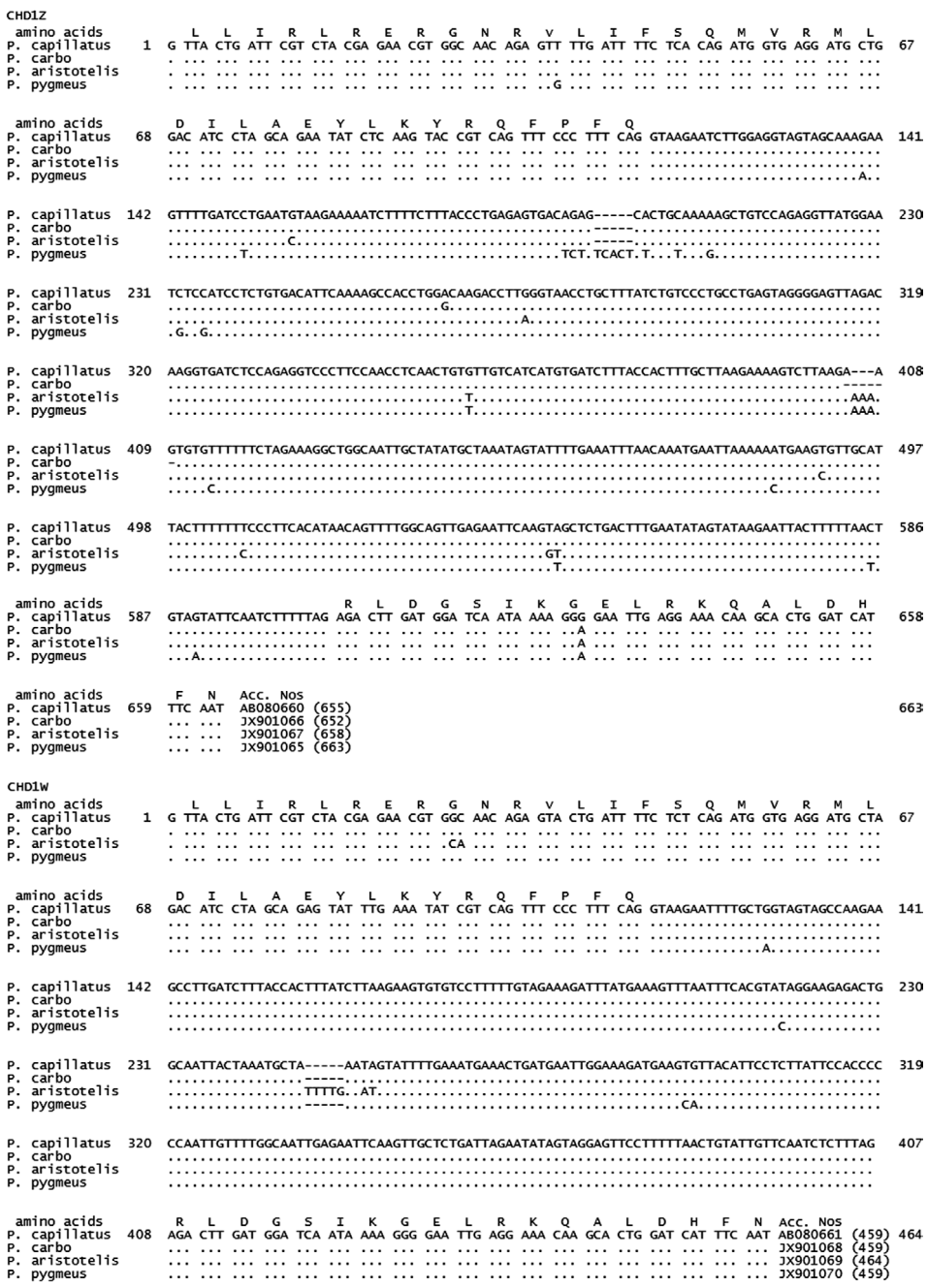

Fig. 1. Chromo-helicase-DNA-binding (*CHD1*) gene sequences of the Z (above) and W (below) sex chromosomes, amplified with the 2550F/2718R set of primers, for the three European Phalacrocoracidae species, *Phalacrocorax carbo*, *P. aristotelis* and *P. pygmeus* aligned with the respective sequences of *P. capillatus* (Inoue-Murayama *et al.* 2002). Nucleotide substitutions among the four Phalacrocoracidae species are indicated by the respective nucleotide. Dots represent similar nucleotides as in *P. capillatus*. The regions corresponding to the exon sequences are organized in codons and the deduced amino-acids are shown above each codon. GENBANK accession numbers (Acc. Nos.) and lengths (in parentheses) are provided at the end of each sequence. Although exon sequences of both sex-chromosomes are similar in length and nucleotide composition, enabling their alignment, the intron sequences are differentiated in both aspects and cannot be unambiguously aligned. In this sense, the *CHD1Z* and *CHD1W* sequences are presented here in two distinct alignments.

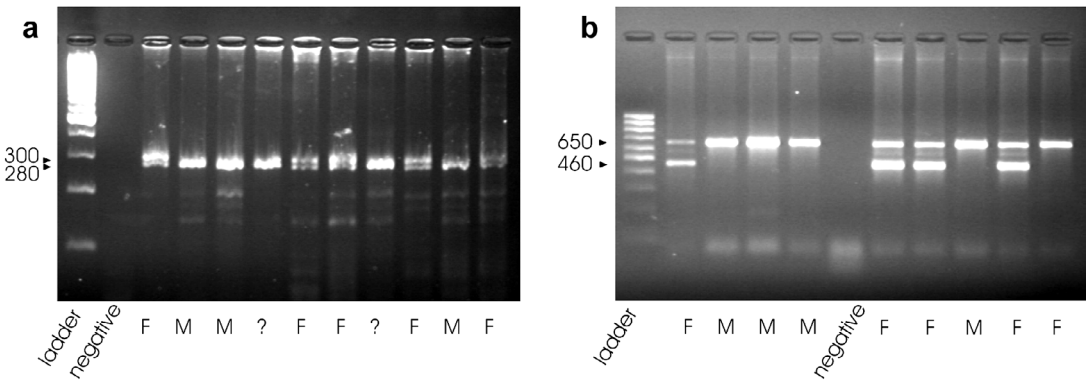

Fig. 2. The female (F), male (M) and ambiguous (?) band patterns and the respective length (bps) of each band, as shown in agarose gel electrophoresis. The respective set of primers and electrophoretic conditions are: (**a**) 1237L/1272H, run for 2.5 h on a 4% agarose gel, and (**b**) 2550F/2718R, run for 45 min on a 1% agarose gel. The length of each band is determined in comparison to a 100 bp size-standard (ladder). A negative control, 25 μl PCR mix without DNA, was included.

rectly identified sex in all the cases (100%) (Table 1) and produced clear band patterns even in samples of low DNA concentration.

Nucleotide variation between sexes and among species

In the case of the primer pair 1237L/1272H, polyacrylamide gel electrophoresis revealed several differences in band size both between sexes and among the three species studied. Many PCR products sized between approx. 100–280 bps were amplified, but these products varied in size and were characteristic of specific individuals regardless of their given sex or species. The most prominent band corresponded to the *CHD1Z* downstream intron which varied slightly in size (1–5 bps) between the three species. The great cormorant and the common shag females produced an additional band of the same size for the *CHD1W* downstream intron, but the respective product for the pygmy cormorant female was distinctively smaller by approx. 10 bps (Fig. 3). These products could not be excised as "clear" bands from an agarose gel, thus it was not possible to sequence them and study their nucleotide composition.

On the contrary, the upstream introns of *CHD1Z* and *CHD1W* were sequenced with the 2550F/2718R primer pair for two males and two females, except for the pygmy cormorant, for which one individual per sex was sequenced. The resulting sequences were unambiguously aligned (Fig. 1) with the corresponding sequences of the Japanese cormorant (Inoue-Murayama *et al.* 2002). No double peaks were observed in the *CHD1Z* sequences that might indicate heterozygous males. The exon and intron parts of the studied species were determined in correspondence to the Japanese cormorant, as well as the exon-deduced amino acid sequence. The size differences between *CHD1Z* and *CHD1W* for each of the three species studied were due to insertions or deletions found within the intron parts.

Nucleotide substitutions of the *CHD1Z* and *CHD1W* sequences produced with the 2550F/2718R primer pair are summarized in Table 2. In all examined Phalacrocoracidae species, the *CHD1W* and *CHD1Z* exon sequences were very similar, except for a few nucleotide substitutions that did not alter the translation product in any case. As for the intron sequences of the *CHD1Z* and *CHD1W* genes, they were very similar between the great and Japanese cormorants and most divergent between the pygmy cormorant and the common shag (25 substitutions or 3.8% and 13 substitutions or 2.8%, for each respective gene). Including exon and intron sequences of all four species, similarity in *CHD1Z* and *CHD1W* sequences ranged between 96.2%–99.2% and 97.2%–100%, respectively.

Table 1. Number of the samples tested, amplified and correctly sexed for the three Phalacrocoracidae species, with the two sets of primers used in this study. The accuracy of each method is given (percentage of correctly identified samples), as estimated in this and previously conducted studies. MDA = Morphometric Discriminant Analysis.

Sex identification test	Species	*N* samples (Male/Female)	*N* amplified (Male/Female)	*N* correct sex (Male/Female)	Accuracy (%)	Source
1237L/1272H primers						
polyacrylamide gel electrophoresis	*P. aristotelis*	9 (6/3)	7 (5/2)	5 (5/0)		Kahn *et al.* (1998),
	P. carbo	7 (5/2)	5 (4/1)	5 (4/1)		this study
	P. pygmeus	2 (1/1)	2 (1/1)	2 (1/1)		
	all	18	14	12	86	
2550F/2718R primers						
agarose gel electrophoresis	*P. aristotelis*	9 (6/3)	9 (6/3)	9 (6/3)		Fridolfsson & Ellegren (1999),
	P. carbo	7 (5/2)	7 (5/2)	7 (5/2)		this study
	P. pygmeus	2 (1/1)	2 (1/1)	2 (1/1)		
	all	18	18	18	100	
1237L/1272H primers						
polyacrylamide gel electrophoresis	*P. carbo**	75 (30/45)	58 (24/34)	29 (24/5)	50	Kahn *et al.* (1998), this study
2550F/2718R primers						
agarose gel electrophoresis	*P. carbo**	75 (30/45)	75 (30/45)	75 (30/45)	100	Fridolfsson & Ellegren (1999), this study
MDA	*P. carbo** (Greece)	81 (34/47)			82–96	Liordos & Goutner (2008)
	P. carbo (Netherlands)	116 (60/56)			87–96	Koffijberg & van Eerden (1995)
	P. carbo (Scotland)	261 (156/105)			98	Newson *et al.* (2004)
MDA	*P. aristotelis* (Spain)	43 (25/18)			98–100	Velando *et al.* (2000)
	P. aristotelis (Spain	114 (38/76)			90	Martínez-Abraín *et al.* (2006)
	P. aristotelis (United Kingdom)	484 (149/335)			93	Calvo & Bolton (1997)
DNA fingerprinting	*P. aristotelis*	158 (84/74)			100	Graves (1992), Graves (1993)

* Samples used by Liordos and Goutner (2008).

Table 2. Nucleotide substitutions among four Phalacrocoracidae species, of the CHDW1 and CHDZ1 sequences amplified with the 2550F/2718R set of primers. Numbers above columns indicate the respective nucleotide positions in which these nucleotide substitutions were found, for each gene aligned according to the respective *P. capillatus* sequence (Inoue-Murayama *et al.* 2002).

CHD1W	28	29	127	218	248	249	250	251	252	255	256	295	296
capillatus	G	C	G	T	–	–	–	–	–	T	A	T	G
carbo	G	C	G	T	–	–	–	–	–	T	A	T	G
aristotelis	C	A	G	T	T	T	T	T	G	A	T	T	G
pygmeus	G	C	A	C	–	–	–	–	–	T	A	C	A

CHD1Z	37	139	151	157	191	192	193	195	196	197	198	299	201	205	209	232	235	265	275	357	404	405	406	407	408	409	414	484	490	507	545	546	585	590	630
capillatus	T	G	C	G	A	G	A	–	–	–	–	–	A	C	A	C	C	A	G	G	A	–	–	–	A	G	T	A	T	T	T	A	C	G	G
carbo	T	G	C	G	A	G	A	–	–	–	–	–	A	C	A	C	C	G	G	G	–	–	–	–	–	–	T	A	T	T	T	A	C	G	A
aristotelis	T	G	C	C	A	G	A	–	–	–	–	–	A	C	A	C	C	A	A	T	A	A	A	A	A	G	C	A	C	C	G	T	C	G	A
pygmeus	G	A	T	G	T	C	T	T	C	A	C	T	T	T	G	G	G	A	G	T	A	A	A	A	A	G	T	C	T	T	T	T	T	A	A

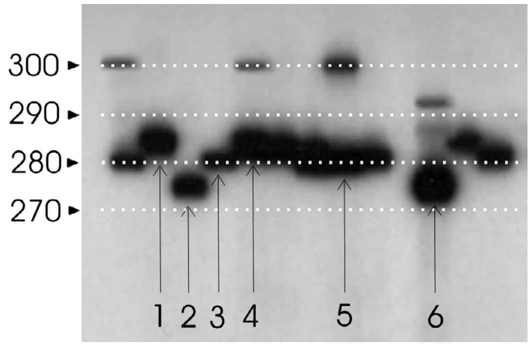

Fig. 3. The band pattern after PCR with the 1237L/1272H set of primers, as shown in a polyacrylamide gel electrophoresis, for the three Phalacrocoracidae species: *P. carbo* male (1) and female (4), *P. pygmeus* male (2) and female (6), and *P. aristotelis* male (3) and female (5). The approximate length (bps) of each band is also shown.

Discussion

Assessing the accuracy of a CHD test for sexing birds is a two-part process: (i) assessing the sex-specific nature of the test, and (ii) assessing the accuracy of the test to correctly sex individuals (Lessells & Mateman 1998, Griffiths 2000). The first step involves the selection of a molecular marker that provides different results in males and females and relatively few known-sex individuals are needed (e.g., four males and four females, Griffiths 2000). Nevertheless, both upstream and downstream sexing primers should be initially tested in order to identify those best suited to the studied species. The second step requires considerably more individuals to ensure veracity (Lessells & Mateman 1998, Griffiths 2000). Uncertainty remains in PCR sexing because most researchers disregard the second step. Typically, the demonstration of the sex-specific nature of the test becomes a surrogate for the accuracy of each sex subsequently identified (Robertson & Gemmell 2006).

In this study, we adressed both issues, using as many individuals of known sex as possible from the three European species of the Phalacrocoracidae family. This is the first study that provides results on the molecular sexing of known-sex individuals for the common shag and the pygmy cormorant. Sacrificing several birds in order to determine sex anatomically is not

desirable, particularly for the later two species, which live in fragmented, declining populations in Greece. Consequently, the majority of the known-sex samples belonged to the great cormorant. Although only one representative per sex was used for the pygmy cormorant, all three species provided similar results and band patterns with all methods and protocols used in this study. In this sense, we discuss the accuracy of each applied sex-test in all three species combined.

The most important outcome of this study is the high accuracy of the 2550F/2718R primers in sexing the Phalacrocoracidae species. Both sets of primers, as used here, were able to amplify the expected DNA fragments, but PCR competition and non-specific primer binding of the 1237L/1272H primers produced many products and made sex determination ambiguous, using agarose gel electrophoresis. Nevertheless, applying the Kahn *et al*. (1998) protocol, followed by polyacrylamide electrophoresis, provided a respectively accurate (89%) one-way method to discriminate both between sexes and among the three European species of the Phalacrocoracidae, simultaneously. This could be useful for museum collections, zoos, conservationists, or even field enthusiasts who may need to identify a given sample of unknown or questionable origin in terms of sex and species (e.g., carcasses or feathers that cannot be identified otherwise).

Regarding the reliability of avian CHD-based molecular sexing protocols, a major point of concern comes from several studies reporting two bands observed in males as the result of a polymorphism in the Z chromosome. Especially with the use of downstream *CHD1*-intron primers 1237L/1272H (Kahn *et al*. 1998) or P2/P8 (Griffiths *et al*. 1998), Z chromosome polymorphisms result in the amplification of two products (Z and Z´), which are seen as two bands in agarose gel electrophoreses, causing males to be misidentified as females. The formation of ZZ´ heteroduplex molecules during the PCR process may also lead to their misidentification as females (Casey *et al*. 2009). A capillary electrophoresis system *vs*. agarose gel may reveal the presence of heterozygotic males and heteroduplex molecules (Dawson *et al*. 2001). In this study, polyacrylamide gel electrophoresis was performed for both primer pairs, and did not reveal heterozygotic males or heterodublex molecules. The heterozygotic males are relatively rare as compared with homozygotic ones: for example only four heterozygotic males were found among 90 upland sandpipers, *Bartramia longicauda* (Casey *et al*. 2009). Despite the high total number of cormorants and shags used in our study, the possible existence of heterozygotic males cannot be ruled out, especially for the common shag and the pygmy cormorant, for which fewer specimens were tested.

The Fridolfsson and Ellegren (1999) method, as applied in this study, provides accurate discrimination between sexes, even in the case of heterozygotic males. With this method, the W and Z products (Z and Z´) cannot be confused, since the W one is significantly smaller (about 250 bps) and Z-chromosome polymorphisms usually induce small alterations in length (about 30 bps in Casey *et al*. 2009). So even if two bands would appear in a heterozygotic male, misidentification of its sex is highly improbable because both bands would be longer than the W band amplified in true females. In cases of templates with low DNA concentrations, only one band may be seen in females, as a result of PCR competition between the two primers. This band is the one corresponding to the W chromosome and is smaller in length than the Z-chromosome band seen in normal males, or the two Z and Z´ bands that could be amplified in heterogametic males. Conclusively, it is safe to assume that the only possible error for molecular sex assignment with 2550F/2718R primer pair could be the false identification of females as males. This would probably mean that an allelic dropout in the *CHD1W* exon, prevented or drastically reduced the amplification of the W chromosome (Arnold *et al*. 2003, Robertson & Gemmell 2006). The probability of a substitution that would alter the primer-binding area in the W chromosome is rather small, considering that no such error was detected in the 58 females amplified in this study.

When the same sample of great cormorants was tested, our analysis was more accurate than MDA applied by Liordos and Goutner (2008), who correctly classified 82.2%–95.1% of the birds. Similar discriminant functions for the sex

determination of Dutch and English great cormorants showed accuracies of 89.7%–96.1% and over 98%, respectively (Koffijberg & van Eerden 1995, Newson *et al*. 2004). The reduced accuracy of MDA as compared with that of a molecular analysis can be mainly attributed to the partial overlap of body size between the sexes, especially when immature individuals and/or robust females and weak males are included in the sample. In the extensive survey of Velando *et al*. (2002) on a Spanish shag colony, the sexing of chicks was performed with two methods: the DNA fingerprinting technique (Graves 1992, 1993) and MDA (Velando *et al*. 2000), which were both reported to be 100% accurate, although the accuracy of MDA decreases when chicks are less than 30 days old (Velando *et al*. 2000, 2002). In another study, morphological measurements were taken in adult common shags of known sex and subsequently discriminant functions enabled the correct prediction of sex in 92.6% of British and 90% of Spanish birds, due to the differences in size between the two populations (Martínez-Abraín *et al*. 2006). According to our study, molecular sexing with the Fridolfsson and Ellegren (1999) method is more effective and involves an easier and less invasive procedure, since it requires a small blood sample (this study) or few feathers (Kocijan *et al*. 2011) that can be taken once from adults and chicks soon after their hatching. The MDA approach of Velando *et al*. (2000) needs several repeatedly taken measures and requires frequent handling of each chick.

The Fridolfsson and Ellegren (1999) primers provided an interesting insight into the nucleotide variation between species and sexes. Especially intron sequences revealed several nucleotide substitutions among the four species. As expected, the Z-linked intron was more polymorphic than the W-linked one, a characteristic that might be associated with higher ecological adaptation of males (Montell *et al*. 2001, Elgvin *et al*. 2011). More similar were the sequences belonging to the great and Japanese cormorants, which share a sister-species relationship (Siegel-Casey 1988, Kennedy *et al*. 2000). The highest degree of dissimilarity was detected between the common shag and the pygmy cormorant. The first two cormorants are members of the subfamily Phalacrocoracinae, while the common shag is placed in a different genus (*Strictocarbo*) (Kennedy *et al*. 2000), and the pygmy cormorant has recently been ascribed to the genus *Microcarbo* (Christidis & Boles 2008). The degree of genetic divergence between *P. carbo*, *P. pygmeus*, *P. aristotelis* and *P. capillatus*, as it was revealed by the analysis of *CHD1*-intron sequences, seems to depict their phylogenetic relationships (Siegel-Casey 1988, Kennedy *et al*. 2000). In this sense, the 2550F/2718R primers could be used to amplify the *CHD* gene as an indicative nuclear marker for the phylogenetic study of these families, subfamilies and genera.

In concluson, the Fridolfsson and Ellegren (1999) protocol was the most accurate, cost- and time-effective of the procedures tested. Another important advantage of PCR-based molecular sexing is that both DNA fingerprinting and morphometric analyses have to be re-evaluated when applied to new populations different than the ones originally tested, due to genetic or morphological geographic variation (Graves 1992, 1993, Velando *et al*. 2002, Martínez-Abraín *et al*. 2006, Liordos & Goutner 2008). The method applied in this study provides a very similar band pattern for sex discrimination of all three Phalacrocoracidae species, taking advantage of the conserved sequence of the *CHD1* gene, so primer binding is expected to show the same accuracy and sex-descriptive capability when applied to different populations of the same species. Especially for the pygmy cormorant, the small number of available samples requires further testing of known-sex individuals prior to the general use of this method in long-term field studies. Nevertheless, we can predict that sexing errors are highly unlikely in all three species and for both sexes.

Acknowledgments

The authors would like to thank Aristeidis Christidis (Fisheries Research Institute — FRI/NAGREF) and Petros Lymberakis (Vertebrate Curator, Natural History Museum of Crete — NHMC) for providing specimens. We are also grateful to Dr. Antonis Augustinos for his help with the polyacrylamide gel electrophoreses and Dr. Manos Koutrakis (FRI/NAGREF) for his support and hospitality.

References

Arnold, K. E., Orr, K. J. & Griffiths, R. 2003: Primary sex ratios in birds: problems with molecular sex identification of undeveloped eggs. — *Molecular Ecology* 12: 3451–3458.

Calvo, B. & Bolton, M. 1997: Sexing shags *Phalacrocorax aristotelis* from external measurements using discriminant analysis. — *Ringing and Migration* 18: 50–56.

Casey, A. E., Jones, K. L., Sandercock, B. K. & Wisely, S. M. 2009: Heteroduplex molecules cause sexing errors in a standard molecular protocol for avian sexing. — *Molecular Ecology Resources* 9: 61–65.

Childress, R. B. & Bennun, L. A. 2002: Sexual character intensity and its relationship to breeding timing, fecundity and mate choice in the great cormorant *Phalacrocorax carbo lucidus*. — *Journal of Avian Biology* 33: 23–30.

Christidis, L. & Boles, W. E. 2008: *Systematics and taxonomy of Australian birds*. — CSIRO Publishing, Victoria, Australia.

Cramp, S. & Simmons, K. E. L. 1977: *The birds of the Western Palearctic*. — Oxford University Press, Oxford, UK.

Dawson, D. A., Darby, S., Hunter, F. M., Krupa, A. P., Jones, I. L. & Burke, T. 2001: A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. — *Molecular Ecology Notes* 1: 201–204.

De Vita, R., Cavallo, D., Eleuteri, P. & Dell'Omo, G. 1994: Evaluation of interspecific DNA content variations and sex identification in Falconiformes and Strigiformes by flow cytometric analysis. — *Cytometry* 163: 46–50.

Dubiec, A. & Zagalska-Neubauer, M. 2006: Molecular techniques for sex identification in birds. — *Biology Letters* 43: 3–12.

Elgvin, T. O., Hermansen, J. S., Fijarczyk, A., Bonnet, T. E., Borge, T., Sæther, S. A., Voje, K. L. & Sætre, G.-P. 2011: Hybrid speciation in sparrows II: a role for sex chromosomes? — *Molecular Ecology* 20: 3823–3837.

Frederiksen, M. & Bregnballe, T. 2000: Evidence for density-dependent survival in adult Cormorants from a combined analysis of recoveries and resightings. — *Journal of Animal Ecology* 69: 737–752.

Fridolfsson, A. K. & Ellegren, H. 1999: A simple and universal method for molecular sexing of non-ratire birds. — *Journal of Avian Biology* 30: 116–121.

Gheng, Y.-H., Kuo, T.-F., Weng, C.-F. & Lee, D.-N. 2006: Sex identification of the black-faced spoonbill (*Platalea minor*). — *Zoological Studies* 45: 104–113.

Graves, J., Hay, R. T., Scallan, M. & Rowe, S. 1992: Extra-pair paternity in the shag, *Phalacrocorax aristotelis* as determined by DNA. — *Journal of Zoology* 226: 399–408.

Graves, J., Ortega-Ruano, J. & Slater, J. B. 1993: Sex ratio of chicks in the shag *Phalacrocorax aristotelis* determined by a female-specific band in DNA fingerprinting. — *Ibis* 135: 470–472.

Grémillet, D., Argentin, G., Schulte, B. & Culik, B. M. 1996: Flexible foraging techniques in breeding cormorants *Phalacrocorax carbo* and shags *Phalacrocorax aristotelis*: benthic or pelagic feeding? — *Ibis* 140: 113–119.

Griffiths, R. 2000: Sex identification using DNA markers. — In: Backer, A. J. (ed.), *Molecular methods in ecology*: 295–321. Blackwell Science Oxford, UK.

Griffiths, R., Double, M. C., Orr, K. & Dawson, R. J. G. 1998: A DNA test to sex most birds. — *Molecular Ecology* 7: 1071–1075.

Griffiths, R. & Holland, P. W. H. 1990: A novel avian W chromosome DNA repeat sequence in the Lesser Black-backed Gull (*Larus fuscus*). — *Chromosoma* 99: 243–250.

Griffiths, R. & Korn, R. M. 1997: A *CHD1* gene is Z chromosome linked in the chicken *Gallus domesticus*. — *Gene* 197: 225–229.

Griffiths, R. & Orr, K. 1999: The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. — *Molecular Ecology* 8: 671–674.

Griffiths, G. & Tiwari, B. 1993: The isolation of molecular genetic markers for the identification of sex. — *Proceedings of the National Academy of Sciences USA* 90: 8324–8326.

Hall, T. A. 1999: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. — *Nucleic Acids Symposium Series* 41: 95–98.

Han, J.-I., Jang, H.-J. Cheong, S., Kim, S., Park, S.-R. & Na, K.-J. 2009: Sex determination by PCR-RFLP in the oriental white stork *Ciconia boyciana*. — *Zoological Studies* 48: 619–624.

Inoue-Murayama, M., Ueda, Y., Yamashita, T., Nishida-Umehara, C., Matsuda, Y. Masegi, T. & Ito, S. 2002: Molecular sexing of Japanese cormorants used for traditional fishing on the Nagara River in Gifu City. — *Animal Science Journal* 73: 417–420.

Ito, H., Sudo-Yamaji, A., Abe, M., Murase, T. & Tsubota, T. 2003: Sex identification by alternative polymerase chain reaction methods in Falconiformes. — *Zoological Science* 20: 339–344.

Kahn, N. W., St. John, J. & Quinn, T. W. 1998: Chromosome-specific intron size differences in the avian *CHD* gene provide an efficient method for sex identification in birds. — *The Auk* 115: 1074–1078.

Kennedy, M., Gray, R. D. & Spencer, H. G. 2000: The phylogenetic relationships of the shags and cormorants: can sequence data resolve a disagreement between behavior and morphology? — *Molecular Phylogenetics and Evolution* 17: 345–359.

Kocijan, I., Dolenec, P., Inko, T. Š., Nenadić, D. D., Pavoković, G. & Dolenec, Z. 2011: Sex-typing bird species with little or no sexual dimorphism: an evaluation of molecular and morphological sexing. — *Journal of Biological Research, Thessaloniki* 15: 145–150.

Koffijberg, K. & van Eerden, M. R. 1995: Sexual dimorphism in the cormorant *Phalacrocorax carbo sinensis*: possible implications for differences in structural size. — *Ardea* 83: 37–46.

Lee, P. L. M., Brain, P. F., Forman, D. W., Bradbury, R. B. & Griffiths, R. 2002: Sex and death: *CHD1Z* associated with high mortality in moorhens. — *Evolution* 56:

2548–2553.

Lessells, C. M. & Mateman, A. C. 1998: Sexing birds using random amplified polymorphic DNA (RAPD) markers. — *Molecular Ecology* 7: 187–195.

Liordos, V. & Goutner, V. 2008: Sex determination of great cormorants (*Phalacrocorax carbo sinensis*) using morphometric measurements. — *Waterbirds* 31: 203–210.

Longmire, J. L., Maltbie, M., Pavelka, R. W., Smith, L. M., Witwe, S. G., Ryder, O. G., Ellsworth, D. L. & Baker, R. J. 1993: Gender identification in birds using microsatellite DNA fingerprint analysis. — *The Auk* 110: 378–381.

Martínez-Abraín, A., Oro, D., Velando, A., Genovart, M., Gerique, C., Bartolomé, M. A., Sarzo, B. & Villuendas, E. 2006: Morphometric similarities between central and peripheral populations of the European shag *Phalacrocorax aristotelis*. — *Marine Ornithology* 34: 21–24.

Montell, H., Fridolfsson, A.-K. & Ellegren, H. 2001: Contrasting levels of nucleotide diversity on the avian Z and W sex chromosomes. *Molecular Biology and Evolution* 18: 2010–2016.

Nakamura, D., Tiersch, T. R., Douglass, M. & Chandler, R. W. 1990: Rapid identification of sex in birds by flowcytometry. — *Cytogenetics and Cell Genetics* 53: 201–205.

Nesje, M. & Røed, K. H. 2000: Sex identification in falcons using microsatellite DNA markers. *Hereditas* 132: 261–263.

Newson, S. E., Hughes, B., Russell, I. C., Ekins, G. R & Sellers, R. M. 2004: Sub-specific differentiation and distribution of great cormorants *Phalacrocorax carbo* in Europe. — *Ardea* 92: 3–10.

Ong, A. H. K. & Vellayan, S. 2008: An evaluation of *CHD*-specific primer sets for sex typing of birds from feathers. — *Zoo Biology* 27: 62–69.

Potts, G. R., Cossulson, J. C. & Deans, I. R. 1980: Population dynamics and breeding success of the shag, *Phalacrocorax aristotelis*, on the Farne Islands, Northumberland. — *Journal of Animal Ecology* 49: 465–484.

Robertson, B. C. & Gemmell, N. J. 2006: PCR-based sexing in conservation biology: wrong answers from an accurate methodology? — *Conservation Genetics* 7: 267–271.

Shephard, J. M., Catterall, C. P. & Hughes, J. M. 2004: Discrimination of sex in the whitebellied sea-eagle, *Haliaeetus leucogaster*, using genetic and morphometric techniques. — *Emu* 104: 83–87.

Siegel-Casey, D. 1988: Phylogeny of the Phalacrocoracidae. — *The Condor* 90: 885–905.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994: CLUSTAL-W — Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. — *Nucleic Acids Research* 22: 4673–4680.

Velando, A., Graves, J. & Freire, J. 2000: Sex-specific growth in the European shag *Stictocarbo aristotelis*, a seabird with size dimorphism. — *Ardea* 88: 127–136.

Velando, A., Graves, J. & Ortega-Ruano, J. E. 2002: Sex ratio in relation to timing of breeding, and laying sequence in a dimorphic seabird. — *Ibis* 144: 9–16.