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Combining biometrics and genetics to distinguish two subspecies of the Great Cormorant *Phalacrocorax carbo carbo* and *P. c. sinensis* at an inland lake in southeast Norway

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Abstract
Great Cormorants *Phalacrocorax carbo* are now regularly seen in inland watercourses in southeast Norway, after the *P. c. sinensis* subspecies first established breeding colonies in coastal south Norway in 1996. Although both the *sinensis* and *carbo* subspecies occur, the ratio between them is unknown. We tested the accuracy of subspecific identification based on biometrics and genetic analyses in 75 Great Cormorants that drowned in fishing nets in a lake in southeast Norway during the period 2009-2020. Primarily based on the gular pouch angle (GPA), we classified 40 individuals to the *carbo* subspecies and 35 individuals to the *sinensis* subspecies. Eight of the *carbo* individuals and 14 of the *sinensis* individuals were within the overlapping range of GPA and were therefore classified to subspecies by supplementary measurements (bill depth minimum and bill length). Genetic analyses were based on seven polymorphic microsatellite markers. Assuming one panmictic population, we performed Structure analyses to separate the genotypes into two assumed genetic clusters. The two clusters, *carbo* and *sinensis*, could only be separated when adding information about morphological subspecies identities for the *carbo*, *sinensis* and *carbo*/*sinensis* groups. The *sinensis* and the *carbo*/*sinensis* groups belonged almost entirely to one genetic cluster, whereas the *carbo* group consisted of individuals with varying proportions of mixed ancestry. Furthermore, we found significant genetic differentiation between the *carbo* and *sinensis* groups, and between the *carbo* and the *carbo*/*sinensis* groups, but no significant differentiation between the *sinensis* and the *carbo*/*sinensis* groups. Our results suggest that gene flow is more common from *sinensis* into *carbo* than vice-versa, and that the use of GPA < 73° has limitations for the identification of Great Cormorant subspecies in areas where both forms occur. We conclude that the numbers of *carbo* vs. *sinensis* individuals in our sample were 32 and 43, respectively.

INTRODUCTION

Reliable observations of the Great Cormorant *Phalacrocorax carbo* at inland watercourses in southeast Norway have been reported for at least 100 years. Previous observations were mainly of single subadults in the autumn migration period (Huitfeldt-Kaas 1917, Collett 1921, Haftorn 1971), and all historical observations lack subspecies information. Today, two subspecies of Great Cormorant are recognized in Europe, *P. c. carbo* and *P. c. sinensis*, and both forms occur naturally in Norway (Lorentsen 2014).

An emerging phenomenon during the last two decades has been the regular occurrence of foraging Great Cormorants at inland watercourses from spring to autumn (Lorentsen 2014, Andersen et al. 2018). The current status of the species in southeast Norway has clearly been influenced by the successful recovery of the *sinensis* subspecies in continental Europe (Bregnballe et al. 2014). The first documented breeding of the *sinensis* subspecies in Norway occurred in 1996. In 1997, the first breeding colony of *sinensis* was established on a

treeless islet in the Øra estuary, at the outlet of the river Glomma near Fredrikstad (Fredriksen & Johansen 1999, Figure 1). In the following years, the *sinensis* subspecies established new breeding colonies along the coastline of the southernmost part of Norway (Lorentsen 2014). Great Cormorants that have been ringed outside Norway and observed in the southernmost part of Norway during the last 30 years have mostly originated from populations on the coasts of Skagerak and Kattegat in Denmark and Sweden (Figure 2). Nearly 3,000 nestlings of the *sinensis* subspecies have been ringed in the Øra estuary since 1996, and most of the recoveries were reported from southern Scandinavia and the continental part of western Europe. Some individuals have been recovered as far south as Tunisia and Morocco (data from the Norwegian Bird Ringing Centre, Museum Stavanger, 5 December 2023). The westernmost countries in continental Europe are the most important wintering areas for cormorants breeding in southern Scandinavia (Bregnballe et al. 2021, Anonymous 2023). The same wintering areas are also predominantly used by cormorants breeding in Finland and southwestern parts of Europe (Bregnballe et al. 2013).

Bird records available at the Norwegian Species Observation Service (www.artsobservasjoner.no, searched 10 April 2023) indicate that the largest numbers of cormorants at inland watercourses have been observed in the autumn. The spawning migrations of coregonid whitefishes in the autumn seem to attract foraging cormorants, possibly of the *sinensis* subspecies. However, the *carbo* subspecies might also be registered along inland watercourses if inland migration routes are used. Most winter recoveries of Great Cormorants ringed in central Norway in the period 1970–1992 were from sites along the western coasts of Norway and Sweden and in the Skagerak and Kattegat (Mogstad & Røv 1997). Great Cormorants ringed in central Norway between 1993 and 2021 have severely dropped in number and the ring recoveries are therefore insufficient to show any significant changes in dispersal movements (data from the Norwegian Bird Ringing Centre, Museum Stavanger, 5 December 2023).

During the last decade, there have been several reports of flocks of 300–400 cormorants flying over the northern part of the lake Randsfjorden in late March (Geir Høitomt, pers. comm.). Such observations early in the spring may indicate migrating flocks of the *carbo* subspecies along their route to the coast of central Norway.

The regular presence of cormorants in inland southeast Norway has resulted in protests from members of angler organisations published in newspapers. The angler organisations have proposed implementation of bounties on the cormorants to reduce their numbers (GD.no 13.09.2017, OA.no 18.09.2017). Some biologists specialized in freshwater ecosystems have also expressed concerns related to an increase in the occurrence of cormorants in freshwater

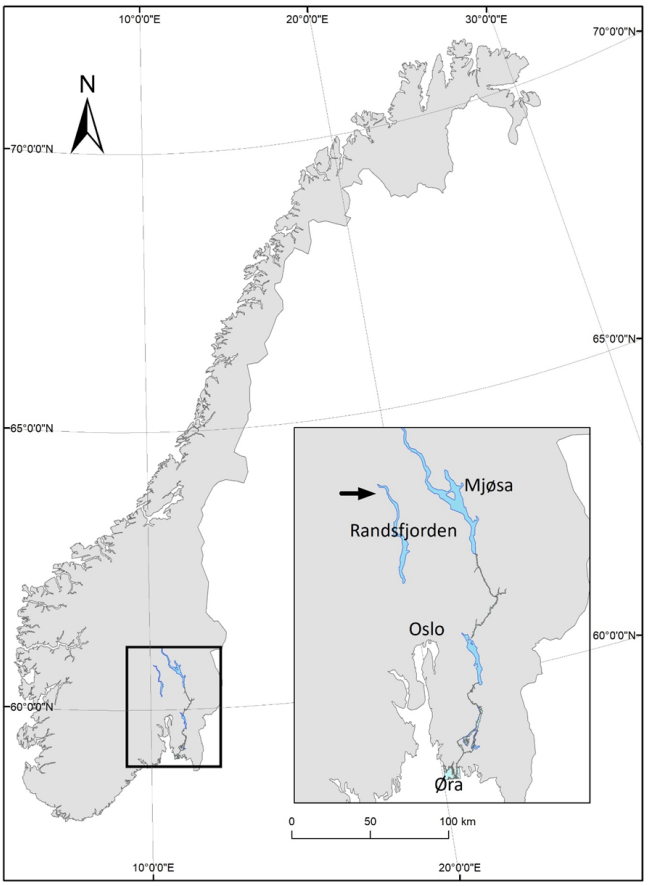

Figure 1. A map of with the study location at the lake Randsfjorden, the lake Mjøsa and the Øra estuary in southeast Norway. The sampling site in the northern part of the lake Randsfjorden is marked with an arrow.

systems as a potential threat to vulnerable populations of fish (GD.no 24.10.2015). There is no evidence that vulnerable populations of fish in inland watercourses in Norway are directly threatened by cormorants. The situation is different elsewhere in Scandinavia and continental Europe, where cormorants have denser populations with larger breeding colonies. In Danish rivers, vulnerable populations of sea-run brown trout *Salmo trutta* and grayling *Thymallus thymallus* are exposed to a high predation pressure from the *sinensis* subspecies (Jepsen et al. 2018, Källo et al. 2023).

The Great Cormorant is currently classified as Near Threatened on the Norwegian Red List (2021). Monitoring data shows that there has been a decline of 15–30% over the last three generations of the *carbo* subspecies in Norway (Stokke et al. 2021), which is a concern because mid- and north Norway remain the most important breeding areas for the *carbo* subspecies in Europe (Bregnballe et al. 2014, Anker-Nilssen et al. 2015). Based on ringing data, we assume that the majority of Great Cormorants in watercourses in inland southeast Norway are primarily of the Scandinavian population of *sinensis* (Figure 2). Andersen et al. (2018) collected 35 shot cormorants in 2017 from the lake Mjøsa and the connected river Gudbrandsdalslågen (Figure 1). Biometrical measurements showed that most

of the birds (91%) belonged to the *sinensis* subspecies. However, in the neighbouring lake Randsfjorden, biometrics indicated that the ratio of the two subspecies included a higher proportion of the *carbo* subspecies during the same period (Grøndahl et al. 2018).

Correct identification of the two subspecies is desirable when observation data for the Great Cormorant are registered in national databases for management and conservation objectives. Measuring gular pouch angles (GPA) is a cost-effective method for ready differentiation of *P. c. carbo* and *P. c. sinensis*. The GPA method can be used to identify the two subspecies of cormorants from observations or photos of live birds, and dead birds which were drowned in nets or shot. However, there is a range of angles which overlap between the two subspecies (Alström 1985, Newson et al. 2004, 2005). In the case of dead individuals with intermediate GPA values, the subspecies can also be identified using other biometrical measurements (Newson et al. 2004, 2005). However, potential hybridisation between the subspecies may weaken the accuracy of the gular pouch angle method.

In this paper, we used a combination of biometrics and genetic analyses for distinguishing *P. c. carbo* and *P. c. sinensis* from a sample of Great Cormorants which were collected as bycatch because they drowned in fishing nets in Randsfjorden during the autumns of 2009–2020. We tested the accuracy of a subspecies identification based on: 1) biometrical measurements, 2) microsatellite markers and 3) a combination of the two methods. We also investigated whether hybridisation between the two subspecies was indicated in the sample of birds. A third subspecies, *P. c. norvegicus*, suggested by Marion & Le Gentil (2006) was not considered in this study.

METHODS

A total of 75 cormorants were sampled. All the birds were recovered as bycatch after they drowned in fishing nets in the northern part of the lake Randsfjorden, close to the Dokka Delta Ramsar site. Randsfjorden is the fourth biggest lake in Norway (140 km^2) and is situated in Innlandet county (Figure 1). All sampling was completed in September and October in the 12-year period of 2009–2020. Most of the cormorants were stored in freezers until they could be measured in a fully thawed condition. All biometrical measurements, in addition to weight, sex and age determination, were completed according to a standardized protocol (Table 1). For a few individuals, some of the measurements were collected after the birds had been skinned or mounted as study specimens for a museum collection. Age was identified by plumage coloration. Adult cormorants differ from juveniles and immature birds by having fully black underparts. The underparts of juveniles and immatures are highly variable in coloration with a mixture of white and brownish feathers, but are never fully black (Camphuysen 1998).

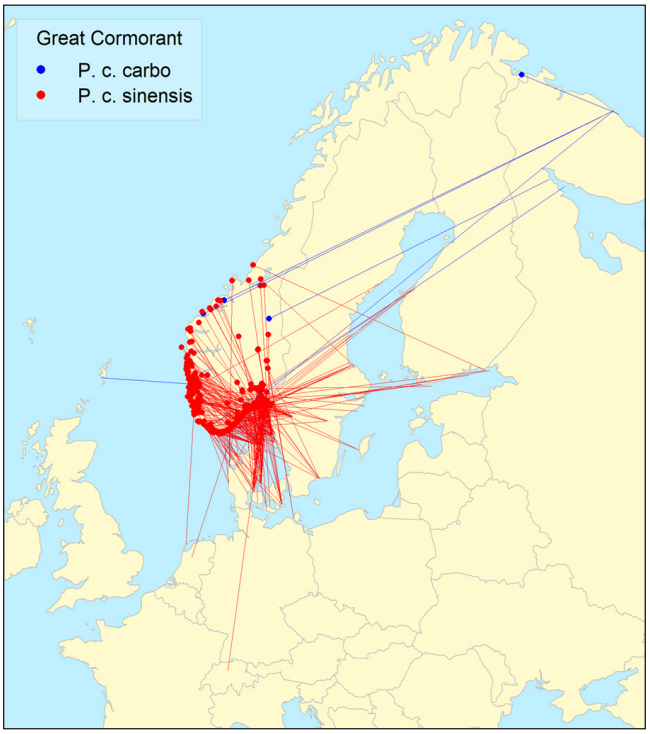

Figure 2. A map of the recoveries of foreign ringed Great Cormorants for the period 1991-2022 in Norway. Source: Håvard Husebø, The Norwegian Bird Ringing Centre 2023, Stavanger Museum.

In this study, all individuals with fully black underparts were classified as adults, and all individuals with light-coloured underparts (5–100%) as subadults. Biometrical data indicate minimal differentiation between adults and subadults (Koffijberg & van Eerden 1995, Bregnballe et al. 2012). In this study, age classification was conducted to evaluate the importance of inland watercourses for adults outside the breeding season. Sex was determined genetically and by dissection and gonadal inspection for all but one individual. All measurements were taken by the same person (Grøndahl). Tissue samples for genetic analyses based on microsatellites were taken from the pectoral muscle.

Newson et al. (2004, 2005) examined several morphological characteristics for distinguishing between the subspecies of *P. carbo* in Europe. The gular pouch angle was found to be significantly different between the *carbo* subspecies (GPA ≤ 65°) and the sinensis subspecies (GPA ≥ 73°), but individuals with intermediate morphology (GPA = 66–72°) could not be identified to subspecies with certainty. In this study, we have conservatively classified birds to subspecies based on thresholds for *P. c. carbo* with GPA ≤ 60°, and *P. c. sinensis* with GPA ≥ 80°. We slightly extended the overlapping zone to the interval ≥ 65°–≤ 75° because the use of a manual protractor could not measure angles with 1° accuracy. The gular pouch angle has some geographic variation, and decreases slightly with latitude for the carbo subspecies but increases slightly

Table 1. Table 1. A standardized protocol for identification of Great Cormorants to subspecies, sex and age-classes based on morphological and genetic information. Information recorded included the gular pouch angle, classification according to sex and age, weight and seven biometrical measurements. Individuals having damages or distortions which affected the biometrics were not included in this study.

Measurements	Description	References
Gular pouch angle (GPA)	The angle of the gular pouch is the shape of the area of bare flesh on the face behind the bill. In this study, the angle was measured to the nearest 5° using a non-digital protractor. The baseline from which the angle is measured is the "gape line" formed by the closed mouth below the eye, see figure 3. All measurements were taken whilst the pouch was in the normal position. All measured individuals were documented by photos.	Alström 1985, Newson et al. 2004, Newson et al. 2005
Sex	Dissection and gonadal inspection	Dalen & Nilsen 2002
	Genetic sex-determination	Fridolfsson & Ellegren 1999
Age	All individuals with fully black underparts were classified as adults. All individuals with light-feathered underparts (5–100%) were classified as subadults.	Camphuysen 1998
Body mass	The carcass mass after defrosting and drying of feathers, to the nearest g.	
Total body length	The distance from bill tip to the tip of the tail feathers, measured in mm.	Dalen & Nilsen 2002
Wing length	The distance from bend of wing (carpal joint) to tip of the longest primary feather, measured over the folded and extended closed wing, to the nearest mm.	Dalen & Nilsen 2002, Svensson 1992
Tarsus length	The distance from the front of the tarsometatarsal bone at the toe joint to the end of the bone below the ankle joint, to the nearest mm. In this study, the tarsus length was measured with digital callipers.	Dalen & Nilsen 2002, Svensson 1992
Bill length (BL)	The distance from the bill tip to the start of the forehead feathering, to the nearest mm. In this study, the bill length was measured with digital callipers.	Newson et al. 2005
Bill depth minimum (BD)	The bill depth just behind the gonys, measured at the narrowest point in the middle of the bill, to the nearest mm. In this study, the bill depth minimum was measured with digital callipers.	Camphuysen 1998
Length of tail feathers	The distance from the point where the central tail feathers emerge from the skin and to the tip of the longest feather, to the nearest mm.	Dalen & Nilsen 2002
Discriminant analysis	Males were classified as subspecies *carbo* if (0.92133 x BD) + (0.36504 x BL) – (0.50198 x GPA in degrees) was greater than 4.66583. Females were classified as subspecies *carbo* if (0.87159 x BD) + (0.56828 x BL) + (-0.61081 x GPA) was greater than 4.87236.	Newson et al. 2005

Figure 3. Examples of the measurement of the gular pouch angle by use of a baseline from the gape outwards. The red lines mark the correct angle of the individual. The top individual (no. 264) has a GPA of ca. 95° (*sinensis*), whereas the bottom individual (no. 300) has a GPA of ca. 50° (*carbo*).

eastwards for the *sinensis* subspecies (Newson et al. 2005). Newson et al. (2004, 2005) also found that the biometrical measurements of bill depth minimum and bill length raised the level of accuracy in distinguishing the subspecies for individuals of identifiable sex with overlapping GPA values. Thus, measurements of bill morphology were also included in this study. We classified our sample of cormorants into three groups based on the GPA measurements prior to the genetic analysis; the *carbo* group (1), the *sinensis* group (2), and a *carbo*/*sinensis* group for birds with intermediate measurements (3).

Molecular analyses

All tissue samples collected from Great Cormorants were archived for long-term storage in the DNA bank of the Natural History Museum, University of Oslo. DNA was isolated from the tissue samples using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek).

Molecular sexing was performed using the sex-specific markers 2550F/2718R (Fridolfsson & Ellegren 1999). Each 15 µl PCR reaction consisted of 0.3 µl dNTP's, 2.0 µl BSA (1g/l), 1.05 µl MgCL (25mM), 1.5 µl buffer (10x), 0.75 µl forward and reverse primer (10 µM), 0.15 µl AmpliTaq (Applied Biosystems™), 7 µl dH20 and 1.5 µl sample DNA. The thermal profile was as follows: initial denaturation step of 95°C for 3 min, followed by 35 cycles of 95 °C for 30 sec, 51°C for 30 sec and 72°C for 30 sec, with a final elongation step of 72°C for 5 min. PCR-products were visualised on a 2 % electrophoresis gel with a GeneRuler (Thermo Scientific™) 50bp ladder. Sexing was performed by visual inspection of the gel (Appendix 2), where heterozygous samples with two PCR fragments were scored as female (ZW), and homozygous samples with one fragment were scored as male (ZZ). Three of 75 samples did not amplify.

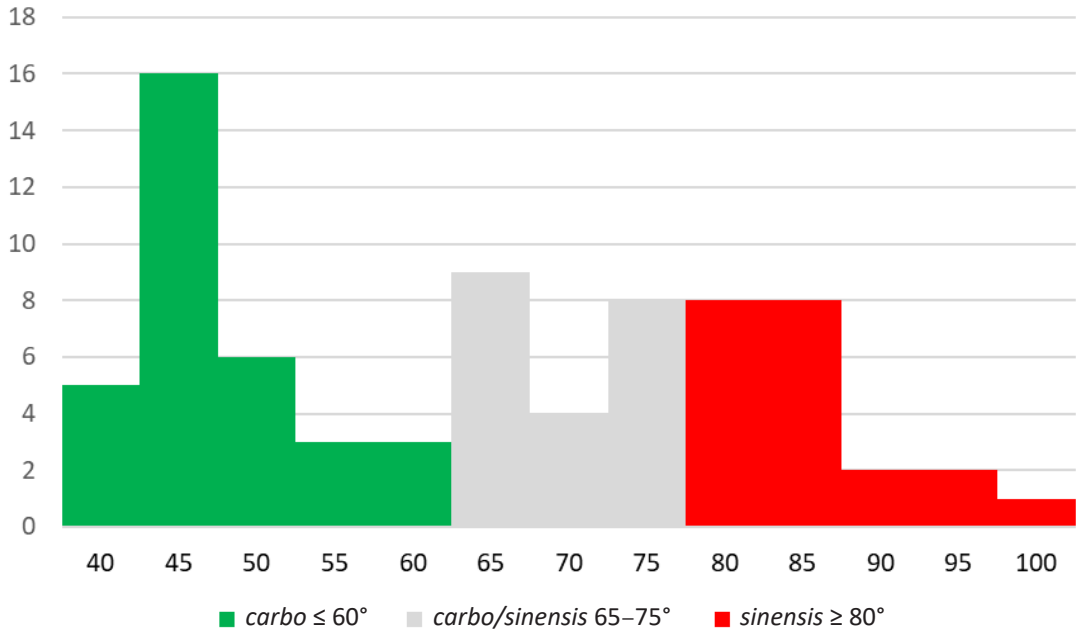

Figure 4. Classification of subspecies and distribution of gular pouch angles (range of 40–100°) in Great Cormorant from lake Randsfjorden, southeast Norway, 2009–2020 (n = 75).

Birds were genotyped with panel of seven polymorphic microsatellite markers developed for the Great Cormorant by Piertney et al. (1998). Markers were amplified using PCR in two panels (p1/p2): PcD2 (p2), PcD4 (p1), PcD5 (p1), PcD6 (p1), PcT1 (p2), PcT3 (p1), PcT4 (p2). Each 15 µl PCR reaction consisted of 7.5 µl 2x Type-it Multiplex PCR Master Mix (Qiagen), 1.5 µl 10X primer-mix, 4.5 µl dH2O and 1.5 µl DNA extract. The same PCR program was used for both panels of markers: 95°C for 5 min, then 28 cycles of 95°C for 30 sec, 55°C for 90 sec, 72°C for 30 sec, before a final elongation step of 60°C for 30 min. PCR products were sent to Macrogen Europe for fragment analysis and the resulting genotypes were analysed using Microsatellite analysis in the ThermoFischer cloud (ThermoFischer Scientific). Six of the seven markers gave a scorable product for all 75 individuals, while PcD5 could only be scored for 50 individuals (see appendix 3).

Using Structure 2.3.4 (Pritchard et al. 2000), we estimated the proportion of each multilocus genotype belonging to each of two assumed genetic clusters (K = 2, i.e. *carbo* or *sinensis*). Two different analyses were performed: (1) an analysis without information on the morphological subspecies designation and assuming that the sample was drawn from one panmictic population (admixture model), and (2) a second analysis with the birds classified by morphological subspecies designation (location prior with three levels, 1 = *carbo*, 2 = *sinensis* and 3 = *carbo*/*sinensis*) and again assuming that the sample was drawn from one panmictic population. For each set of analyses, we performed 10 iterations with a burn-in length of 50,000 and 1 million repetitions. We tested the assumption of two genetic clusters, using the method described in Evanno et al (2005), and found that K = 2 had the highest log-likelihood and the highest ΔK (see appendix 4). We also performed a principal component analysis in R v4.2.3 (R Core Team 2020), using the packages adegenet (Jombart 2008) and ade4 (Bougeard & Dray 2018), to test for clustering without any assumption about K. Genetic differentiation of the three morphological groups (1 = *carbo*, 2 = *sinensis* and 3 = *carbo*/*sinensis*) was estimated by the F_{ST} fixation index (Weir & Cockerham 1984), using FSTAT (Goudet 1995). Significance of differentiation between each pair of groups was determined with a permutation test based on 10,000 iterations.

RESULTS

A total of 75 Great Cormorants were recovered from lake Randsfjorden during 2009-2020. Based on GPA measurements, we identified 32 individuals to the *carbo* subspecies and 21 individuals to the *sinensis* subspecies (Table 2). A total of 22 individuals could not be separated into subspecies because they had intermediate values for gular pouch angle (Table 2, Figure 4). By using the bill depth minimum and bill length as supplementary biometrics in the discriminant analysis formula (Newson et al. 2004), we concluded that eight individuals belonged to the *carbo* subspecies whereas the other 14 birds belonged to the *sinensis* subspecies (Table 3). The measurements of bill depth minimum and bill length measurements and five additional biometrics taken in this study (Appendix 1) were all within the range of published values for Great Cormorants registered in Norway (Newson et al. 2004).

A majority of the 75 individuals (97%) had a high proportion of white feathers on their belly (5–100%), and were therefore aged as subadults. Moreover, 60% of the 75 birds were sexed as males (Table 3). A total of 72

Table 2. The gular pouch angle measurements for 75 collected Great Cormorants from the lake Randsfjorden in southeast Norway, after Newson et al. (2004)

	P. c. carbo n = 32	***P. c. carbo/sinensis*** n = 22	***P. c. sinensis*** n = 21
GPA	≤ 60°	65°–75°	≥ 80°
Mean ± SE	47.4 ± 1.00°	70.0 ± 0.99°	85.2 ± 1.27°
Range	40–60°	65–75°	80–100°

Table 3. The table presents the range of values for bill depth minimum and bill length of *P. c. carbo* and *P. c. sinensis* sub-species sampled from lake Randsfjorden (adults, immature- and juvenile cormorants, n = 74). One individual of unknown sex was not included in the table. Identification of subspecies was based on the gular pouch angle method (Ahlström 1985, Newson et al. 2004) and a discriminant analysis (Newson et al. 2004, 2005). Eight individuals showed a discrepancy in subspecies designation based on the discriminant analysis and the genetical analysis. The eight birds are marked x. Bill depth minimum and bill length were measured in mm.

Identifying method	**Subspecies**	**n**	**Sex**	**Bill depth minimum**	**Bill length**
Gular pouch angle	*sinensis*	9	M	12.84–13.56	62.98–74.09
(Ahlström 1995,	*sinensis*	12	F	10.86–11.93	60.52–65.31
Newson et al. 2004)	*carbo*	22	M	12.38–15.67	66.61–81.3
	carbo	9	F	11.92–13.47	64.1–73.89
Discriminant analysis	*sinensis*	9	M	12.22–14.37	66.47–74.00
(Newson et al. 2004)	*sinensis*	5	F	10.8–11.72	58.5–67.58
	carbo^{x}	5	M	12.51–14.9	71.65–73.08
	carbo^{x}	3	F	11.38–12.65	63.54–70.84

individuals were sexed genetically, and two individuals were sexed only by dissection. One individual could not be sexed by either method.

In the Structure analyses, all individuals showed an equal proportion of each of the two assigned genetic clusters when no information on subspecies was included and admixture was allowed (Figure 5a). However, when information on morphological subspecies was included in the analysis, the two clusters showed some separation according to subspecies. The group identified as *carbo*, consisted of individuals with varying proportions of mixed ancestry, while the groups identified as *sinensis* or of uncertain origin consisted of individuals belonging almost entirely to the same genetic cluster (Figure 5b). The model results indicated that the *carbo*/*sinensis* group consisted only of *sinensis* individuals, and the sample of birds thus consisted of 32 *carbo* individuals and 43 *sinensis* individuals. The conclusions from the genetic analysis thus differed from the discriminant analysis for the eight individuals mentioned above.

An overall test of genetic differentiation among the three groups showed an overall significant F_{ST}-value of 0.042 (95% confidence interval 0.012-0.088, $p < 0.001$). Pairwise comparisons also showed significant differentiation between *carbo* and *sinensis* ($F_{ST} = 0.049$, $p = 0.017$) and between *carbo* and *carbo*/*sinensis* ($F_{ST} = 0.069$, $p = 0.017$), but no significant differentiation between *sinensis* and *carbo*/*sinensis* ($F_{ST} = -0.002$, $p = 0.5$). Similarly, the principal component analysis supported the presence of two differentiated genetic clusters (Appendix 5).

DISCUSSION

The distribution and numbers of the subspecies of Great Cormorant in the inland watercourses of southeast Norway must be viewed in relation to changes in their population status in northwest Europe during the last fifty years. Protective measures were taken in many European countries in the period 1965–1981 and replaced a long period of persecution (van Eerden & Gregersen 1995). A successful recovery occurred where the *sinensis* subspecies showed a spectacular increase and recolonized former breeding areas in many parts of Europe. Expanding populations led to a shift in northwest Europe from a population dominated by the coastal *carbo* subspecies to a higher occurrence of the continental *sinensis* subspecies (Van Eerden & Gregersen 1995, Marion 2003, Bregnballe et al. 2014). By the second half of the 1990s, the bulk of the European populations of the *sinensis* subspecies were breeding in the Netherlands, Germany, Denmark and Sweden (Bregnballe et al. 2003). During 1998–2006, several studies based on population genetics documented the presence of two subspecies in the same breeding colonies in northwest France and southeast England (Goostrey et al. 1998, Winnie et al. 2001, Marion & Le Gentil 2006). Hybridisation between the *carbo* and the

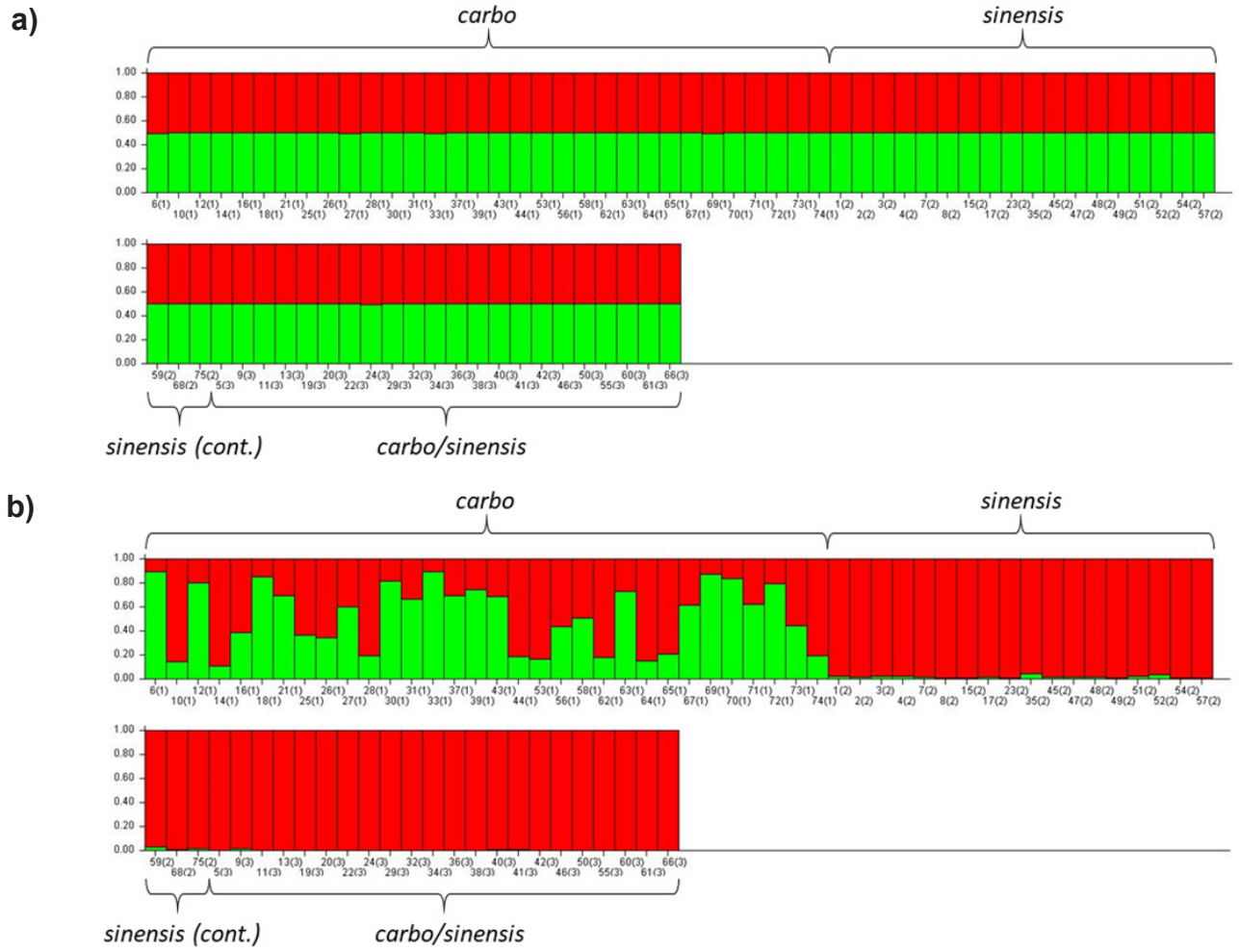

Figure 5. Assignment of 75 individual Great Cormorants to each of K = 2 genetic clusters, assuming admixture and (a) without, and (b) with inclusion of information on morphological subspecies identity. Each bar shows the proportional membership in each cluster for an individual on the y-axis, with individual number and morphological subspecies identity on the x-axis (1 = *carbo*, 2 = *sinensis* and 3 = *carbo/sinensis*, in parentheses).

sinensis subspecies was assumed to occur (Marion & Le Gentil 2006), and a high number of hybrids were documented twenty years later in breeding colonies in northwest France (Marion & Le Gentil 2021).

The main goal of this study was to identify the subspecies of 75 individuals of the Great Cormorant sampled in September and October during 2009–2020 in inland southeast Norway. Based on gular pouch angle measurements and supplemented by microsatellite analyses, we confirmed the presence of both *carbo* and *sinensis* subspecies, but with a surprisingly high number of the *carbo* subspecies. Subadults, of which a small majority were males, dominated the sample of birds. The age-composition had a small proportion of adults of either subspecies, even though the cormorants were sampled after the breeding season in September and October. *P. c. carbo* and *P. c. sinensis* were only distinguishable by the microsatellite markers when classifications of the subspecies identity from morphology were included. The level of genetic differentiation between individuals identified as two different subspecies based on gular pouch angles was therefore very small, which was also confirmed by the F_{ST}-analyses. A small genetic distance between the two subspecies has also been reported by Goostrey et al. (1998) and Marion & Le Gentil (2006). Nevertheless, the two subspecies were separated into two significantly different clusters by combining genetics with subspecific information based on the gular pouch angle. The *carbo* group consisted of individuals with mixed membership in the two genetic clusters, while the *sinensis* group consisted of individuals belonging almost exclusively to one of the clusters. The pattern indicates that gene flow is probably more common from *sinensis* into *carbo* than vice-versa. However, our findings based on microsatellites should be confirmed with additional genetic markers. We found a discrepancy between the biometrical data and the results from the genetical analysis for eight individuals. The occurrence of individuals with an uncertain subspecies identity is not unexpected if hybridisation occurs between the two subspecies.

In the future, we need additional genetic and ringing data for a better understanding of the origin of the mixed *carbo* individuals in this study. The most plausible origin of the mixed *carbo* individuals is a

hitherto unknown hybrid zone in southwest Norway. A distance of only ca. 45 kilometres separates the most northwesterly known breeding colony of the *sinensis* subspecies from the southernmost breeding colony of the *carbo* subspecies in Norway (Ankarstrand Larsen 2006, Byrkjeland 2015, Lorentsen et al. 2022). The potential for hybridisation also indicates that use of the gular pouch angle method < 73° for identification of Great Cormorants to subspecies could have some future limitations in areas where both of the two subspecies co-occur.

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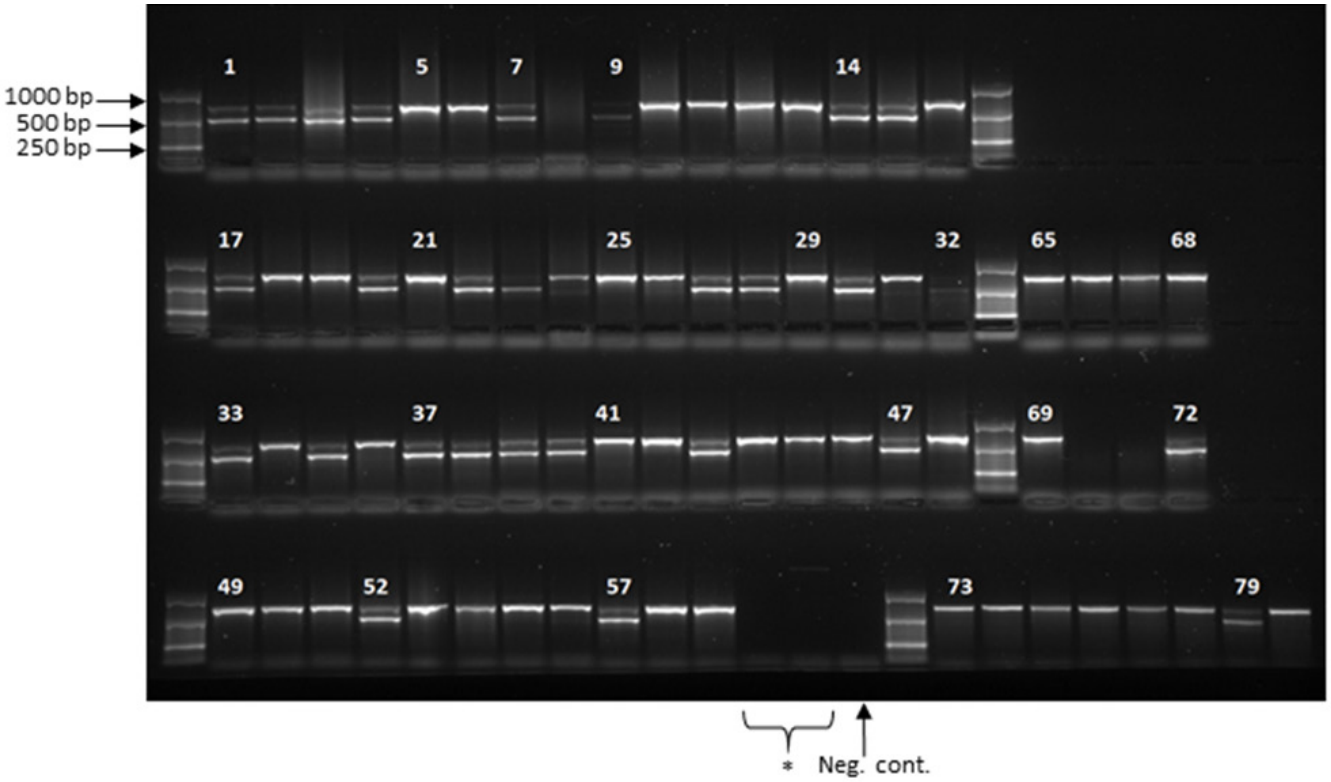
Appendix 1. Range of values for five additional biometrical measurements for two subspecies of Great Cormorant (*P. c. carbo* and *P. c. sinensis*) sampled at lake Randsfjorden, 2009-2020 (adults and sub-adults, n = 74). One individual of unknown sex was not included in the table. Identification of subspecies was based on the gular pouch angle method (Ahlström 1985, Newson et al. 2004) and a discriminant analysis based on morphological data (Newson et al. 2004, 2005). Eight individuals identified as *carbo* subspecies by the discriminant analysis differed from the conclusion of the genetical analysis (marked x). Body mass was measured in grams and linear biometrics were recorded in mm.

Identifying method	Subspecies	n	Sex	Body Mass	Total length	Tarsus length	Wing length	Tail feather length
Gular pouch angle (Ahlström 1985, Newson et al. 2004)	*sinensis*	9	M	2712–3644	820–875	79–85	335–350	180–200
	sinensis	12	F	2018–3540	750–900	73–80	290–345	141–210
	carbo	22	M	2276–3973	835–940	67–93	332–380	165–218
	carbo	9	F	2164–3008	810–860	79–83	335–353	188–205
Discriminant analysis (Newson et al. 2004, 2005)	*sinensis*	9	M	2532–3240	810–900	79–83	320–370	190–218
	sinensis	5	F	2002–2656	778–845	74–78	320–355	184–200
	carbo^{x}	5	M	2200–3120	850–892	80–83	342–365	190–215
	carbo^{x}	3	F	1890–2552	773–845	73–79	340–365	198–205

Appendix 2. Genetic sex-determination of Great Cormorants from lake Randsfjorden, 2009-2020. The sample numbers used in the sex-determination analysis (first column) were not identical to those in the Structure analysis (second column). The accession numbers are for the tissue samples in the DNA bank of the Natural History Museum in Oslo (n=75). The picture shows the electrophoresis gel, with some of the sample numbers indicated. There are six lanes with size ladders, the sizes of the most prominent bands being indicated for the ladder in the upper left corner. The negative control is indicated with an arrow (neg. cont.) and the * denotes two samples that were excluded from the final dataset.

Sample number for genetic sex-determination	Sample number from Structure analysis	Accession number	# bands	Sex
1	1	105439	Double	Female
2	2	105440	Double	Female
3	3	105441	Double	Female
4	4	105442	Double	Female
5	5	105443	Single	Male
6	6	105444	Single	Male
7	7	105445	Double	Female
8	8	105446		
9	9	105447	Double	Female
10	10	105448	Single	Male
11	11	105449	Single	Male
12	12	105450	Single	Male
13	13	105451	Single	Male
14	14	105452	Double	Female
15	15	105453	Double	Female
16	16	105454	Single	Male
17	17	105455	Double	Female
18	18	105456	Single	Male
19	19	105457	Single	Male
20	20	105458	Double	Female
21	21	105459	Single	Male
22	22	105460	Double	Female
23	23	105461	Double	Female
24	24	105462	Double	Female
25	25	105463	Single	Male
26	26	105464	Single	Male
27	27	105465	Double	Female
28	28	105466	Double	Female
29	29	105467	Single	Male
30	30	105468	Double	Female
31	31	105469	Single	Male
32	32	105470	Double	Female
33	33	105471	Double	Female
34	34	105472	Single	Male
35	35	105473	Double	Female
36	36	105474	Single	Male
37	37	105475	Double	Female
38	38	105476	Double	Female
39	39	105477	Double	Female
40	40	105478	Double	Female
41	41	105479	Single	Male
42	42	105486	Single	Male
43	43	105487	Double	Female
44	44	105488	Single	Male
45	45	105489	Single	Male
46	46	105490	Single	Male
47	47	105491	Double	Female
48	48	105492	Single	Male

Sample number for genetic sex-determination	Sample number from Structure analysis	Accession number	# bands	Sex
49	49	105493	Single	Male
50	50	105494	Single	Male
51	51	105495	Single	Male
52	52	105496	Double	Female
53	53	105497	Single	Male
54	54	105498	Single	Male
55	55	105499	Single	Male
56	56	105500	Single	Male
57	57	105501	Double	Female
58	58	105502	Single	Male
59	75	105519	Single	Male
65	59	105503	Single	Male
66	60	105504	Single	Male
67	61	105505	Single	Male
68	62	105506	Single	Male
69	63	105507	Single	Male
70	64	105508		
71	65	105509		
72	66	105510	Double	Female
73	67	105511	Single	Male
74	68	105512	Single	Male
75	69	105513	Single	Male
76	70	105514	Single	Male
77	71	105515	Single	Male
78	72	105516	Single	Male
79	73	105517	Double	Female
80	74	105518	Single	Male



Appendix 3. Data file for the Structure analysis, with sample number (Sample no), accession number in the DNA bank of the Natural History Museum in Oslo (Acc. no), morphological subspecies designation (Subsp.; 1 = *carbo*, 2 = *sinensis* and 3 = *carbo*/*sinensis*), and microsatellite genotype at each of the seven loci (a being first allele and b being second allele). Blank cells = missing data

Sample no	Acc. no	Subsp.	PcD2a	PcD2b	PcD4a	PcD4b	PcD5a	PcD5b	PcD6a	PcD6b	PcT1a	PcT1b	PcT3a	PcT3b	PcT4a	PcT4b
1	105439	2	192	194	164	166	212	214	183	183	349	397	272	342	221	241
2	105440	2	190	194	164	174	206	223	183	183	345	348	223	317	190	217
3	105441	2	194	211	164	168			183	183	326	345	269	331	221	245
4	105442	2	192	194	166	172			177	183	348	376	217	245	233	245
5	105443	3	194	211	164	166	220	223	183	183	343	359	249	276	210	253
6	105444	1	194	194	164	164	208	223	183	183	338	365	245	313	241	249
7	105445	2	192	194	164	164	214	220	183	191	351	398	245	276	206	229
8	105446	2	192	207	172	172	206	214	177	183	349	270	288	288	206	225
9	105447	3	194	194	164	174			177	183	323	345	237	309	225	269
10	105448	1	190	207	164	168	206	216	183	183	362	379	296	298	217	221
11	105449	3	194	198	164	168	214	225	177	177	372	379	284	292	253	306
12	105450	1	171	194	164	164	223	223	183	183	345	352	326	338	221	261
13	105451	3	190	192	164	164	222	227	183	185	326	372	268	346	241	400
14	105452	1	190	205	164	164	218	225	183	185	351	363	271	342	190	327
15	105453	2	190	190	166	168	223	227	183	183	369	398	227	302	210	285
16	105454	1	194	211	164	164	222	223	183	183	314	385	322	357	249	249
17	105455	2	171	194	164	166			183	183	329	349	227	249	285	327
18	105456	1	194	194	164	164	208	208	183	183	330	330	257	292	221	225
19	105457	3	190	211	166	166	212	214	183	183	329	336	285	331	230	241
20	105458	3	171	192	164	166			185	185	370	397	253	258	202	217
21	105459	1	194	194	164	164			183	183	314	338	255	346	214	225
22	105460	3	194	211	166	166	206	206	183	183	334	336	282	309	190	285
23	105461	2	205	211	154	174	214	216	177	177	323	397	300	318	202	237
24	105462	3	192	205	166	168	208	216	177	185	365	379	326	330	190	253
25	105463	1	194	205	164	168			183	183	345	365	241	317	221	245
26	105464	1	194	211	158	164	214	214	177	183	338	349	217	338	214	269
27	105465	1	194	194	164	166	223	223	183	183	329	345	276	334	217	225
28	105466	1	194	211	164	164			183	183	359	378	241	291	217	289
29	105467	3	190	211	164	166	223	227	183	183	352	362	217	276	217	225
30	105468	1	171	194	164	164	223	225	183	183	349	352	245	334	233	261
31	105469	1	194	194	154	164	223	225	177	183	330	347	292	346	225	233
32	105470	3	190	205	166	168	214	225	177	177	338	379	267	310	210	273
33	105471	1	194	194	164	164			183	183	338	338	245	313	221	265
34	105472	3	205	211	168	172			183	185	335	356	233	234	210	245
35	105473	2	194	194	164	166			177	183	323	345	245	309	186	257
36	105474	3	192	211	168	174			177	185	370	384	267	326	210	225
37	105475	1	194	194	164	164			177	183	330	345	249	296	225	347

Appendix 3, Continued.

Sample no	Acc. no	Subsp.	PcD2a	PcD2b	PcD4a	PcD4b	PcD5a	PcD5b	PcD6a	PcD6b	PcT1a	PcT1b	PcT3a	PcT3b	PcT4a	PcT4b
38	105476	3	171	194	158	174	206	206	177	183	349	394	253	297	217	225
39	105477	1	194	194	164	164			183	183	365	366	296	330	233	265
40	105478	3	194	194	164	166	214	223	183	183	314	346	252	294	233	253
41	105479	3	194	198	162	164	214	214	183	183	334	345	249	313	190	190
42	105486	3	194	211	158	172			185	185	343	349	215	249	217	229
43	105487	1	194	194	164	164			183	183	345	376	245	313	331	335
44	105488	1	194	211	164	168			177	183	328	347	285	323	217	261
45	105489	2	171	171	158	158			183	185	342	355	237	253	221	241
46	105490	3	171	211	164	164	214	214	183	183	311	349	264	305	225	241
47	105491	2	211	211	168	168			183	183	337	344	245	290	221	229
48	105492	2	194	205	166	168	206	208	183	183	312	336	253	288	217	221
49	105493	2	211	211	164	168			183	183	356	372	249	276	194	217
50	105494	3	194	211	158	158			183	185	359	375	217	300	194	245
51	105495	2	192	211	164	164			177	183	377	382	227	245	217	241
52	105496	2	194	194	164	168	208	225	183	183	344	349	257	267	202	245
53	105497	1	194	211	164	166	212	223	177	183	343	352	276	331	210	421
54	105498	2	171	192	164	174	214	223	183	183	311	391	280	326	217	293
55	105499	3	190	194	166	174			183	189	299	348	241	253	210	225
56	105500	1	194	194	164	164	223	227	183	185	338	345	309	353	245	339
57	105501	2	211	211	166	168	218	223	183	185	348	377	268	280	225	241
58	105502	1	171	194	164	164	223	223	183	183	337	345	300	334	230	277
59	105503	2	194	211	166	166	208	225	177	183	330	344	215	285	206	225
60	105504	3	192	194	164	174			183	183	318	362	233	276	217	225
61	105505	3	190	211	166	166	227	227	183	191	335	435	269	289	210	229
62	105506	1	194	211	164	164	216	223	183	183	359	363	249	273	198	257
63	105507	1	194	194	164	164	208	225	183	183	349	349	245	315	217	221
64	105508	1	171	194	164	172	214	222	183	191	362	373	276	302	241	257
65	105509	1	171	194	164	166	208	214	183	185	379	380	241	256	249	269
66	105510	3	194	211	170	174	206	214	183	183	311	376	253	317	190	217
67	105511	1	194	194	164	166	223	225	183	183	345	345	284	309	214	237
68	105512	2	171	198	158	174	216	223	191	191	348	362	317	326	217	217
69	105513	1	194	194	164	164	208	222	183	183	330	338	330	338	225	241
70	105514	1	194	194	164	164	208	223	177	183	330	352	313	321	241	241
71	105515	1	194	194	164	164	223	225	183	183	323	380	253	338	225	237
72	105516	1	194	194	164	164			183	183	330	330	305	313	241	253
73	105517	1	194	194	164	168	214	225	183	183	330	376	300	302	210	225
74	105518	1	192	209	164	168	214	218	177	183	349	352	219	268	214	233
75	105519	2	192	211	164	164	214	223	183	183	363	372	217	278	198	206

Appendix 4. Summary statistics used for evaluating the optimal number of genetic clusters, following the method described in Evanno et al. (2005). K = 2 shows the highest mean log-likelihood (L(K)) and the highest ΔK. L'(K) = L(K) - L(K-1) and |L''(K)| = the absolute value of L'(K + 1) - L'(K), are used in the calculation of ΔK = |L"(K)|/SD of L(K).

K	Number of iterations	Mean L(K)	SD of L(K)	L'(K)	|L"(K)|	ΔK
1	10	-2501.09	0.71			
2	10	-2476.49	5.37	24.6	34.12	6.35
3	10	-2486.01	9.74	-9.52	0.13	0.01
4	10	-2495.40	19.98	-9.39	9.39	0.47

Appendix 5. Results of a principal component analysis based on the microsatellite genotypes. The first two principal components are illustrated and the individual genotypes are coloured by the morphological subspecies designation: 1 = *carbo* (green), 2 = *sinensis* (light blue) and 3 = *carbo*/*sinensis* (dark blue). The ellipse for *carbo* shows relatively little overlap with *sinensis* and *carbo*/*sinensis*, while the latter two show a higher degree of overlap.