

Molecular verification of the subspecies status of the Mauritanian Spoonbill *Platalea leucorodia balsaci*

Theunis Piersma^{1,2,*}, Marco van der Velde¹, El-Hacen Mohamed El-Hacen^{1,3},
Tamar Lok^{1,2} & Otto Overdijk⁴

Piersma T., van der Velde M., El-Hacen E.M., Lok T. & Overdijk O. 2012. Molecular verification of the subspecies status of the Mauritanian Spoonbill *Platalea leucorodia balsaci*. *Ardea* 100: 131–136.

In 1974 R. de Naurois and F. Roux proposed that the distinct morphology of Eurasian Spoonbills *Platalea leucorodia* breeding on offshore islands in the Banc d'Arguin, Mauritania, in comparison with the sympatrically wintering northwest European breeding Spoonbills *Platalea leucorodia leucorodia*, justifies recognition as a separate subspecies *Platalea leucorodia balsaci*. This proposal is examined here on the basis of variation in nuclear DNA, microsatellites identified earlier for *P. minor* and *P. ajaja*. We show that there is significant variation between Spoonbills breeding in Mauritania ($n = 25$) and the sympatrically wintering conspecifics breeding in the Dutch Wadden Sea ($n = 105$). Of the total genetic variation among the 130 individuals, 6.3% is attributable to variation between the two breeding areas (93.7% of the variation is within breeding areas). Pairwise F_{ST} values showed low genetic differentiation (F_{ST} 's < 0.012) among breeding colonies within The Netherlands. The level of genetic differentiation indicates that the level of gene flow between The Netherlands and Mauritania is much lower ($\sim 4\text{--}5$ individuals/generation) than among the Dutch colonies on separate Wadden Sea islands. Field observations on individually colour-marked birds from The Netherlands indeed suggest extensive dispersal within northwest Europe, with some introgression of *leucorodia* genes into the *balsaci* population. The level of microsatellite distinctiveness between sympatric populations of the two subspecies is similar to that recorded for subspecies of other migrant birds, and as such justifies the subspecies status of the Mauritanian Spoonbill.

Key words: dispersal, genetic population structure, Mauritania, microsatellites, philopatry, phylogeography, subspeciation, Threshkiornitidae, Wadden Sea

¹Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, P.O. Box 11103, 9700 CC Groningen, The Netherlands;

²Department of Marine Ecology, NIOZ Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands;

³Parc National du Banc d'Arguin (PNBA), Avenue Gamal Abdel Nasser, B.P. 5355, Nouakchott, Mauritania;

⁴Werkgroep Lepelaar, Knuppeldam 4, 9166 NZ Schiermonnikoog, The Netherlands;

* corresponding author (Theunis.Piersma@nioz.nl)



The breeding distribution of Eurasian Spoonbills *Platalea leucorodia* along the East Atlantic seabords of northwest Africa and Europe is rather disjunct (Cramp & Simmons 1977). There is a cluster of colonies in northwest Europe centred in The Netherlands, there are breeding colonies 2000 km further south in southern Spain, and, again separated by a 2000 km gap, a concentration of colonies on Banc d'Arguin, Mauritania, on

the very verge of the Western Palearctic (Cramp & Simmons 1977). The Spanish and Dutch breeding birds have been assigned firmly to the subspecies *leucorodia*, while birds breeding on the offshore islands of Banc d'Arguin on the basis of their distinct morphology received recognition as a separate subspecies *balsaci* by de Naurois and Roux (1974). Mauritania-breeding birds are somewhat smaller, have less pronounced yellow

breast feathers in breeding plumage (de Naurois & Roux 1974, Cramp & Simmons 1977), and adults, but not immatures, have a uniformly black upper bill (O. Overdijk, unpubl. data).

Perhaps surprisingly, the proposal for this subspecies distinction has yet to be revisited in the ornithological literature. Here we examine whether the variation in microsatellites that were initially developed for Black-faced Spoonbills *P. minor* (Yeung *et al.* 2009) and Roseate Spoonbills *P. ajaja* (Sawyer & Benjamin 2006) is greater between Spoonbills breeding in Mauritania and The Netherlands than among Spoonbills from a range of breeding islands within the Dutch Wadden Sea. We will reflect on dispersal patterns documented for individually colour-marked birds to interpret the molecular findings.

METHODS

The long-term colour-marking project of Dutch Spoonbills (a population close to 4000 breeding pairs) that underlies the present study, is introduced in Lok *et al.* (2009, 2011). Blood samples were collected from *leucorodia* breeding colonies at four different Dutch Wadden Sea islands: Schiermonnikoog ($n = 26$), Terschelling ($n = 21$), Vlieland ($n = 40$) and Wieringen

Table 1. Polymorphic microsatellites for Eurasian Spoonbills. Ta = annealing temperature, N_A = number of alleles, and H_O and H_E = Observed and Expected Heterozygosity. Conc. primer = primer concentration in 10 μ l volume PCR reaction ($n = 130$).

Locus	Ta (°C)	Allele range (bp)	N_A	H_O	H_E	Conc. primer (μ M)
Panel-1:						
Aaj 1	60	161–204	12	0.84	0.79	0.3
Pm 2-16	60	317–377	13	0.85	0.76	0.6
Pm 2-29	60	223–276	12	0.81	0.81	0.1
Pm 3-20	60	228–240	4	0.57	0.51	0.015
Panel-2:						
Aaj 2	60	194–206	4	0.49	0.57	0.015
Pm 2-62	60	270–399	40	0.96	0.93	0.5
Pm 3-15	60	197–210	2	0.23	0.23	0.03
Pm 3-17	60	149–155	4	0.61	0.61	0.02
Panel-3:						
Pm 2-28	60	225–307	21	0.94	0.91	0.2
Pm 2-37	60	296–402	25	0.92	0.90	0.2
Pm 3-13	60	189–212	6	0.73	0.70	0.1
Pm 3-16	60	197–213	8	0.22	0.23	0.01

($n = 18$) in June 2011. Blood samples of the *balsaci* subspecies ($n = 25$), now consisting of under 1000 breeding pairs (O. Overdijk & E.M. El-Hacen, unpubl. data), were obtained at one of the four regular colonies on Banc d'Arguin, at Toufat in October 2007. In all cases we captured chicks before fledging in or near the breeding colonies, and this means that we cannot exclude the possibility that we occasionally sampled relatives. Blood was stored in 95% ethanol, and DNA was extracted from these blood samples using a salt extraction method (Richardson *et al.* 2001). As individual breeders may shift between colonies (O. Overdijk & E.M. El-Hacen, unpubl. data), the Toufat samples fairly represent the *balsaci* population.

To study genetic variation among the spoonbill samples, six primer sets from *P. ajaja* (Sawyer & Benjamin 2006) and 15 primer sets from *P. minor* (Yeung *et al.* 2009) were tested for amplification and polymorphism in eight samples (four *leucorodia* samples from Schiermonnikoog and four *balsaci* from Banc d'Arguin). Of these 21 microsatellites, 12 were found to amplify a PCR product and to be polymorphic (Table 1). Subsequently, all 130 DNA samples were analysed for these 12 microsatellite loci.

Microsatellites were amplified in multiplex PCRs of 3 separate panels (Table 1). PCR reactions were carried out in 10 μ l volume containing 20–50 ng DNA, 0.2 mM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.25 U Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), and a variable amount of each primer. See Table 1 for the amount of each primer in the PCR reaction. PCR program was as follows: 94°C for 1 min, 35 cycles of 94°C for 30 s, 60°C for 60 s and 72°C for 60 s, followed by 72°C for 2 min. Fluorescently labelled PCR products were separated on an AB3730 DNA analyser, and allele-lengths were determined using Genemapper 4.0 software.

Population genetic parameters were estimated using Arlequin 3.1 (Excoffier *et al.* 2005) and FSTAT 2.9.3 (Goudet 2001).

RESULTS

Microsatellites from *P. minor* and *P. ajaja* showed moderate to high levels of variation in *P. leucorodia*. Number of alleles (N_A) per microsatellite locus ranged from 2–40 and the expected heterozygosity (H_E) ranged from 0.23–0.93 (Table 1). Eleven loci showed no significant deviations from HardyWeinberg-equilibrium, the twelfth locus PM2–16 had a significant excess of heterozygotes in birds from the Banc d'Arguin after

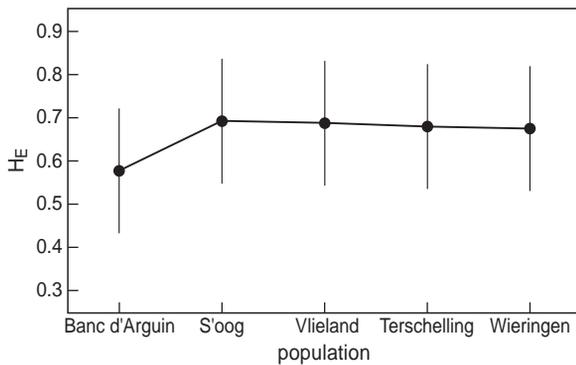


Figure 1. Level of genetic variation (mean expected heterozygosity, H_E) within the five sampled populations. Vertical bars denote 0.95 confidence intervals.

Bonferroni correction. The mean expected heterozygosity (H_E) was 0.57 for Banc d'Arguin birds and ranged from 0.67–0.69 for the Dutch colonies (Figure 1), even though the difference in H_E among breeding colonies overall was not significant (Kruskal-Wallis ANOVA, $P = 0.74$). A single locus, PM3–16, was monomorphic in Mauritania: it was fixed for allele 201 that had a frequency of 0.83–0.88 in the Dutch populations. Eleven loci showed (rare) alleles unique to either Mauritania or The Netherlands, but these alleles generally had a low frequency (<0.10).

A hierarchical Analysis of Molecular Variance (AMOVA) was used to partition the total genetic variation into two components: (1) variation between the source countries Mauritania and The Netherlands (i.e. between subspecies *balsaci* and *leucorodia*), and (2) variation within the breeding sites. The AMOVA revealed significant genetic differentiation between the breeding sites Mauritania ($n = 25$) and The Netherlands ($n = 105$, $F_{ST} = 0.063$, $P < 0.00001$). The remaining 93.7% of the variation was due to individual variation within breeding sites. In addition, tests of pair-wise genetic differentiation (F_{ST}) among all possible combinations of breeding colonies (Table 2), only

showed significant genetic differentiation between the single Banc d'Arguin and each of the Dutch colonies. Genetic differentiation among the Dutch populations was small and not significant.

DISCUSSION

The birds from Mauritania (*balsaci*) showed less genetic variation than the Dutch birds (*leucorodia*, Figure 1). Although this might suggest that the Banc d'Arguin population historically has gone through narrower population bottlenecks than the Dutch population, a more likely explanation is methodological: we probably sampled more families (and colonies) in The Netherlands than in Mauritania.

That there is little genetic structure within the *leucorodia*-spoonbills breeding on the Dutch Wadden Sea Islands is consistent with the considerable dispersal between the colonies on different islands. This can be illustrated by observations in the best studied colony on Schiermonnikoog in 1994–2009 of colour-ringed breeding birds born on islands with yearly colour-ringing of spoonbill chicks (Vlieland, Terschelling and, from 1997 onwards, Schiermonnikoog, see Lok et al. 2009; O. Overdijk, unpubl. data). During this period, 229 colour-ringed birds were observed breeding on Schiermonnikoog, of which 85 were born on Vlieland or Terschelling (dispersal), and 144 on Schiermonnikoog (philopatry). In only one case, a bird was known to have previously bred in another colony. Although the level of breeding dispersal is probably underestimated due to the lower observation effort in colonies other than Schiermonnikoog, it nevertheless suggests that natal dispersal is more common than breeding dispersal. Figure 2 shows that soon after establishment of the Schiermonnikoog colony in 1992 most breeding birds of 2–13 years old were born on Vlieland and Terschelling, whereas in recent years most recruiting birds were locally born individuals (but note that on Schiermonnikoog about

Table 2. Pairwise genetic differentiation (F_{ST}) between Spoonbill colonies and populations. Significant F_{ST} values are bold, $P \leq 0.005$ after Bonferroni correction.

	Banc d'Arguin	Schiermonnikoog	Vlieland	Terschelling
Banc d'Arguin	-			
Schiermonnikoog	0.071	-		
Vlieland	0.061	0.001	-	
Terschelling	0.066	-0.002	0.000	-
Wieringen	0.085	0.010	0.011	0.002

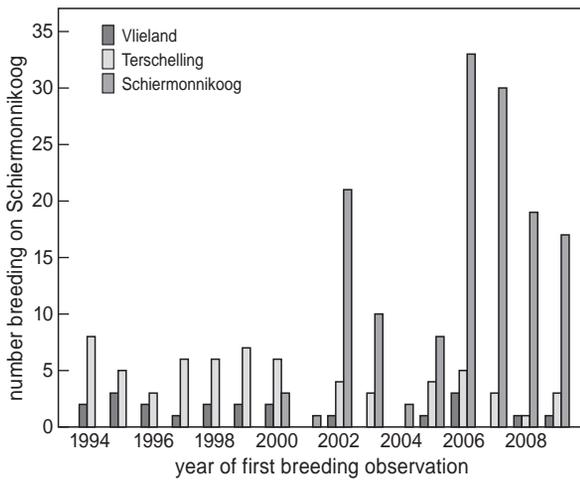


Figure 2. Total number of individually colour-marked Spoonbills observed breeding on Schiermonnikoog (first established 1992) per source colony and year of first breeding observation. Note that colour-ringing of chicks on Schiermonnikoog started in 1997.

twice as many chicks were colour-ringed each year compared to Vlieland and Terschelling). This suggests that gene flow is very high during the establishment of a colony, but strongly decreases during colony growth when locally born individuals start to recruit. Consequently, genetic differentiation among Dutch colonies may increase in the future.

We observed significant genetic differentiation between *balsaci* breeding in Mauritania and *leucorodia* breeding in The Netherlands. This suggests that gene flow between The Netherlands and Mauritania is much lower than the level of gene flow among the Dutch breeding colonies. Assuming that movements between

the two populations are now in equilibrium, and an island model of migration (Wright 1951; $F_{ST} = (1/4Nm + 1)$, the level of dispersers between Wadden Sea and Banc d’Arguin (or *vice versa*) should be about four of five individuals per generation. In fact, there is some equivocal observational evidence for such dispersal, albeit in a single direction only (Table 3). There are several cases of European-born Spoonbills copulating with *balsaci*-looking local breeders. In 2000 a Netherlands-born *leucorodia* was observed feeding chicks in a *balsaci*-colony on the island of Nair. However, the best documented example of dispersal from The Netherlands to Mauritania (but not necessarily subspecies-crossbreeding) is of a colour-ringed male Spoonbill born on Terschelling in 1997 that was breeding on Nair (probably in 2002 and certainly in 2010). When, on 17 May 2010, it was observed on its nest and on 30 June 2010, it was observed feeding chicks, it was paired with a *leucorodia*-looking bird probably of European origin (Figure 3).

Breeding colonies of the subspecies *leucorodia* are widely distributed across the Eurasian continent (Cramp & Simmons 1977). Therefore, genetic differentiation between geographic populations of *leucorodia*, at least in theory, could be larger than the estimate of divergence between the two subspecies based on Spoonbills breeding in Mauritania and The Netherlands. This question would need additional work, but the question of whether the genetic differentiation found is high enough to support a separation into two subspecies, *balsaci* and *leucorodia*, can be answered. As a gauge, we can compare the level of microsatellite differentiation between *leucorodia* and *balsaci* observed ($F_{ST} = 0.06$) with values found in other microsatellite

Table 3. Evidence for natal dispersal by *leucorodia* Spoonbills into the *balsaci* breeding population on the Banc d’Arguin, Mauritania, based on notes by O. Overdijk. In addition there are five cases of birds in *balsaci* breeding colonies showing the symmetric yellow spots on the upper bill characteristic of *leucorodia*.

Date	Breeding island	Details of the observations
24 April 2000	Zira	Bird ringed in The Netherlands in 1996 copulated with local breeder
2 May 2000	Nair	Bird ringed in The Netherlands in 1996 copulated with local breeder on nest
2 May 2000	Nair	Bird ringed in The Netherlands in 1996 fed chicks
19 June 2000	Nair	Bird ringed in The Netherlands in 1996 copulated with local breeder
17 May 2002	Cheddid	Bird ringed in The Netherlands in 1994 copulated with local breeder
21 May 2002	Arel	Bird ringed in The Netherlands in 1998 copulated with at least three different local breeders
4 June 2004	Zira	Bird ringed in The Netherlands in 2000 sharing nesting materials with <i>balsaci</i> female
17 May 2005	Cheddid	Male ringed in Spain in 2000 copulated with more than one local breeder
23 May 2002 & 18 May 2010	Nair	Bird ringed in The Netherlands (Terschelling) in 1997 copulated with more than one local breeder in 2002, was observed on a nest in May 2010 and nursing chicks with a <i>leucorodia</i> -looking partner in June 2010
30 June 2010		



Figure 3. A colour-ringed bird born on Terschelling in 1997 standing on its nest on Nair (Banc d'Arguin) on 17 May 2010. A month later it was observed feeding chicks. Its partner (sitting on the nest) has a yellow bill tip. Given that male spoonbills generally have larger bills than females, the standing bird is probably a male.

studies on avian subspecies. A sample of studies on migrant bird species, although generally using slightly smaller sets of microsatellite loci, showed rather comparable levels of differentiation; in order of increasing F_{ST} : Dunlin *Calidris alpina* (three subspecies, 7 loci, $F_{ST} \leq 0.010$; Marthinsen *et al.* 2007), Canada Goose *Branta canadensis* (two subspecies, 6 loci, $F_{ST} = 0.021$; Mylecraine *et al.* 2008), Bluethroat *Luscinia svecica* (seven subspecies, 7–11 loci, $F_{ST} = 0.042$; Johnsen *et al.* 2006), Reed Bunting *Emberiza schoeniclus* (two subspecies, 6 loci, $F_{ST} = 0.043$; Kvist *et al.* 2011), and Piping Plover *Charadrius melodus* (two subspecies, 8 loci, $F_{ST} = 0.104$; Miller *et al.* 2010). Thus, with $F_{ST} = 0.063$, the observed genetic differentiation between the two Spoonbill subspecies seems to fall in the upper range of values. On the basis of this comparison the subspecies status of *balsaci* seems entirely valid.

ACKNOWLEDGEMENTS

Jeltje Jouta, Petra de Goeij, Matthijs van der Geest, Carl Zuhorn, Oene de Jong, Leo Bot, Arjen Zonderland, Leon Kelder and Simen Kay helped with the blood-sampling of chicks. We thank Werkgroep Lepelaar for running the colour-ringing project and all the volunteers for contributing to it. The now-dissolved Netherlands Ministry of Agriculture, Nature and Fisheries, as well as the Vogeltrekstation, provided support and permissions to ring Spoonbill chicks, and Vereniging Natuurmonumenten and the Netherlands State Forestry Service (Staatsbosbeheer) provided permissions to work on their land. In addition, we thank the co-workers of the Parc National du Banc d'Arguin (PNBA) for logistic help, especially the crew at the field sites in Iouik and Mamghar. We thank the Foundation Working Group International Wader and Waterfowl Research (WIWO) and Adessium Foundation for financially supporting several of the Mauritania-expeditions. E.M.E. was supported by grants from Adessium Foundation, FIBA, and a personal grant from the Bleumink Fonds, and T.L. by a TopMaster scholarship of the University of Groningen and a Ph.D. grant of the Netherlands Organisation for Scientific Research (NWO-ALW grant 81701012 awarded to T.P.). The present study is part of the *Metawad1*-project supported by Waddenfonds. Yvonne Verkuil, three anonymous reviewers, and the editor provided constructive feedback.

REFERENCES

- Cramp S. & Simmons K.E.L. (eds) 1977. Handbook of the birds of Europe, the Middle East and North Africa: the birds of the Western Palearctic. Vol. 1: Ostrich-Duck. Oxford University Press, Oxford.
- de Naurois R. & Roux F. 1974. Précisions concernant la morphologie, les affinités et la position systématique de quelques oiseaux du Banc d'Arguin (Mauritanie). *L'Oiseau et la R.F.O.* 44: 72–84.
- Excoffier L., Laval G. & Schneider S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1: 47–50.
- Goudet J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from <http://www2.unil.ch/popgen/softwares/fstat.htm>.
- Johnsen A., Andersson S., Fernandez J.G., Kempnaers B., Pavel V., Questiau S., Raess M., Rindal E. & Lifjeld J.T. 2006. Molecular and phenotypic divergence in the Bluethroat (*Luscinia svecica*) subspecies complex. *Mol. Ecol.* 15: 4033–4047.
- Kvist L., Ponnikas S., Belda E.J., Encabo I., Martinez E., Onrubia A., Hernandez J.M., Vera P., Neto J.M. & Monros J.S. 2011. Endangered subspecies of the Reed Bunting (*Emberiza schoeniclus witherbyi* and *E. s. lusitanica*) in Iberian Peninsula have different genetic structures. *J. Ornithol.* 152: 681–693.
- Lok T., Overdijk O., Horn H. & Piersma T. 2009. The Spoonbill *Platalea leucorodia* population of the Wadden Sea islands: does population growth level off? *Limosa* 82: 149–157.
- Lok T., Overdijk O., Tinbergen J.M. & Piersma T. 2011. The paradox of Spoonbill migration: most birds travel to where survival rates are lowest. *Anim. Behav.* 82: 837–844.
- Marthinsen G., Wennerberg L. & Lifjeld J.T. 2007. Phylogeography and subspecies taxonomy of Dunlins (*Calidris alpina*) in western Palearctic analysed by DNA microsatellites and amplified fragment length polymorphism markers. *Biol. J. Linn. Soc.* 92: 713–726.
- Miller M.P., Haig S.M., Gratto-Trevor C.L. & Mullins H.D. 2010. Subspecies status and population genetic structure in Piping Plover (*Charadrius melodus*). *Auk* 127: 57–71.
- Mylecraine K.A., Gibbs H.L., Anderson C.S. & Shieldcastle M.C. 2008. Using 2 genetic markers to discriminate among Canada Goose populations in Ohio. *J. Wildl. Manage.* 72: 1220–1230.
- Richardson D.S., Jury F.L., Blaakmeer K., Komdeur J. & Burke T. 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles Warbler (*Acrocephalus sechellensis*). *Mol. Ecol.* 10: 2263–2273.
- Sawyer G.M. & Benjamin R.C. 2006. Isolation and characterization of microsatellite loci for parentage assessment in captive populations of Roseate Spoonbill (*Ajaja ajaja*). *Mol. Ecol. Notes* 6: 677–679.
- Wright S. 1951. The genetical structure of populations. *Ann. Eugen.* 15: 323–354.
- Yeung C.K.L., Hsu Y.-C., Yao C.-T. & Li S.-H. 2009. Isolation and characterization of 23 microsatellite loci in the Black-faced Spoonbill (*Platalea minor*) and amplification in other Ciconiiformes waterbirds. *Conserv. Genet.* 10: 1081–1084.

SAMENVATTING

In 1974 gaven de Franse vogelkundigen R. de Naurois en F. Roux aan de Lepelaars *Platalea leucorodia* die op de Banc d'Arguin in Mauretanië broeden de ondersoortnaam *balsaci*. De Lepelaars van de Banc d'Arguin zijn aanzienlijk kleiner dan de Europese broedvogels (ondersoort *leucorodia*), missen ze in hun broedkleed de gele borstveren die de Europese vogels kenmerken en is hun bovensnavel egaal zwart (zonder de gele vlekken op de lepel van de Europese soortgenoten). Op grond van deze verschillen werden de broedvogels van Mauretanië als een aparte ondersoort beschreven. Het aardige van *leucorodia* en *balsaci* is dat ze 's winters samen voorkomen op de Banc d'Arguin. Niemand is later op dit ondersoortvraagstuk teruggekomen. In dit artikel onderzoeken we met een gestandaardiseerde moleculaire methode in hoeverre het nucleaire (en 'neutrale' d.w.z. niet-coderende) DNA van Nederlandse en Mauretaanse Lepelaars van elkaar verschilt. Verreweg het grootste deel (93,7%) van de variatie in het voorkomen van verschillende lengtes DNA met repeterende basisvolgorden (de zogenaamde 'microsatellieten') is te vinden *binnen* de twee broedgebieden en 6,3% komt door variatie tussen de Lepelaars van de Waddenzee (*leucorodia*) en Banc d'Arguin (*balsaci*). Al lijkt die 6,3% misschien niet veel, het is zelfs groter dan wat wordt gevonden in de meeste andere studies van trekvogels waarbij ondersoorten worden vergeleken. Niettemin geven de verschillen en overeenkomsten in DNA aan dat er niet alleen sprake moet zijn van een grote 'gene flow' (uitwisseling van genen) tussen de kolonies in de Waddenzee onderling, maar ook een beetje tussen die van de Waddenzee en de Banc d'Arguin. Voor de eerste gedachte is direct bewijs voorhanden door de meldingen van vogels die op de Waddeneilanden van kleurringen zijn voorzien en later op 2–13 jarige leeftijd in andere kolonies zijn gaan broeden (de kolonie van Schiermonnikoog groeide mede door aanwas van vogels die op Vlieland en Terschelling waren geboren). Voor de tweede gedachte is veel minder bewijs voorhanden, maar toch zijn er verschillende waarnemingen van in Europa als kuiken gekleurde Lepelaars die copuleren, nestmateriaal delen of zelfs kuikens voeren in kolonies van *balsaci* op de Banc d'Arguin. Op grond van de gepresenteerde genetische waarnemingen schatten we dat er per generatie 4–5 individuen tussen de twee ver van elkaar gelegen broedgebieden switchen.

Corresponding editor: Ken Kraaijeveld

Received 17 February 2012; accepted 17 July 2012