

Detecting female precise natal philopatry in green turtles using assignment methods

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Abstract

It is well established that sea turtles return to natal rookeries to mate and lay their eggs, and that individual females are faithful to particular nesting sites within the rookery. Less certain is whether females are precisely returning to their natal beach. Attempts to demonstrate such precise natal philopatry with genetic data have had mixed success. Here we focused on the green turtles of three nesting sites in the Ascension Island rookery, separated by 5–15 km. Our approach differed from previous work in two key areas. First, we used male microsatellite data (five loci) reconstructed from samples collected from their offspring ($N = 17$) in addition to data for samples taken directly from females ($N = 139$). Second, we employed assignment methods in addition to the more traditional F -statistics. No significant genetic structure could be demonstrated with F_{ST} . However, when average assignment probabilities of females were examined, those for nesting populations in which they were sampled were indeed significantly higher than their probabilities for other populations (Mann–Whitney U -test: $P < 0.001$). Further evidence was provided by a significant result for the mAI_C test ($P < 0.001$), supporting greater natal philopatry for females compared with males. The results suggest that female natal site fidelity was not sufficient for significant genetic differentiation among the nesting populations within the rookery, but detectable with assignment tests.

Keywords: AI_C , *Chelonia mydas*, F_{ST} , microsatellites, nest site fidelity, sex-biased gene flow

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Introduction

Natal philopatry, defined as situations where individuals return to reproduce in their place of birth, is well documented for many species (Greenwood 1980). Where there are high fitness costs in finding new breeding sites, natural selection will favour natal philopatry (Bensch *et al.* 1998; Perrin & Mazalov 2000). If many individuals are faithful to their natal sites, then groups of individuals that reproduce at the same sites will constitute discrete populations with limited genetic exchange between them. In contrast, gene flow, the exchange of genetic material between populations, can occur through dispersal, which is the movement/immigration of an individual from the natal population to a different population in which reproduction subsequently occurs (Greenwood 1980). Gene flow without dispersal can

also occur where populations are mixed for a period of time (Bowen *et al.* 2005). Behaviours promoting gene flow between populations may reduce local mate competition (Hamilton 1967) or local resource competition (Clarke 1978). Natal philopatry will promote the genetic distinctiveness of populations, whereas genetic exchange between populations will homogenize them. Thus, natal philopatry and gene flow between populations both contribute to shaping the genetic structure of populations. Also, if natal philopatry varies among the sexes, understanding the resulting complex population genetic structure poses a challenge for research (Bowen *et al.* 2005).

Many species undertake long-distance migrations between feeding and breeding grounds. Among migratory organisms, sea turtles are large marine reptiles that have been intensively studied for many reasons, including their conservation importance (Bowen *et al.* 1993; Avise 1998; Bowen *et al.* 2005). Tagging females with external flipper tags or internal PIT tags has shown that most individuals

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for various species regularly return to the same area to nest even across several years (Plotkin 2003). Genetic studies on green turtles have further indicated that both males and females migrate back to their general region of birth to breed (FitzSimmons *et al.* 1997). The reproductive populations at distinct breeding grounds are described as 'rookery stocks', and are commonly regarded as the management units for sea turtle conservation (Bowen & Karl 1997; Avise 1998; Formia *et al.* 2006). A rookery, however, can consist of a number of different nesting beaches within close proximity, and females still have to choose between sites. We therefore make a distinction between migrations to natal breeding grounds and precise natal site fidelity, because even nonmigratory species can display the latter (Freedberg *et al.* 2005). It has been argued that if female sea turtles were returning to nest on the same beach where they had been hatched ('precise natal site fidelity') then these should represent distinct female lineages within a rookery (Schroth *et al.* 1996). However, whereas molecular evidence for long-distance migration from feeding areas back to natal breeding grounds is now well established for sea turtles (Bowen *et al.* 1989; Meylan *et al.* 1990), similar evidence for precise natal site fidelity is less clear-cut as there have been mixed results from various studies.

Several investigations of sea turtles have indeed shown strong genetic evidence for precise natal philopatry (Peare & Parker 1996; Schroth *et al.* 1996; Hatase *et al.* 2002), although others have failed to do so (Peare & Parker 1996; Dutton *et al.* 1999; Moore & Ball 2002; Chassin-Noria *et al.* 2004; Shanker *et al.* 2004) (see Discussion for brief details of these). A possible reason is that even though the philopatry displayed by male sea turtles appears to be similar to that of females in terms of the instinct to migrate back to the rookery of birth (FitzSimmons *et al.* 1997), the promiscuous mating system means that males invest heavily in competition for females and are thus likely to mate whenever the opportunity arises. This mating system has been described as 'scramble-competition polygamy' (Jessop *et al.* 1999), where competitive mate searching is the most important strategy for males (Thornhill & Alcock 1983; Schwagmeyer 1988). If both male and female sea turtles were migrating back to their natal rookery to breed, and males would mate with any female they could locate in the rookery (as predicted by scramble-competition polygamy) whereas females would tend to nest on natal beaches within the rookery (as predicted by natal philopatry) then sex-biased genetic exchange between nesting sites may therefore be expected. Detecting this is difficult because even if only one sex carries genetic material between populations, it would be transmitted to both sexes in the following generation. This will reduce the overall degree of differentiation between populations, and comparisons of male and female genotypes may not have much statistical power under high levels of gene flow (Goudet *et al.* 2002).

The problem is further exacerbated by limitations in genetic analyses. The most widely implemented methods for describing population genetic structure are based on Wright's F_{ST} (Wright 1951) or similar approaches. These have equilibrium assumptions, and aim to describe long-term genetic processes. Contemporary events occurring on shorter timescales that may be relevant to ecological studies are 'noise' in such models (Whitlock & McCauley 1999; Paetkau *et al.* 2004). For F_{ST} , genetic differentiation among populations caused by drift will only occur if there are fewer than one effective migrant per generation (Wright 1931; Wang 2002). Hence, gene flow mediated by only one sex could result in populations characterized as genetically uniform under these models. One solution has been to compare markers with different inheritance modes. Discrepancies in F_{ST} values estimated for maternally and biparentally inherited genetic markers have been useful (see review by Bowen *et al.* 2005). For sea turtles, disparate F_{ST} values estimated from mtDNA and microsatellite markers have been interpreted as resulting from females returning faithfully to rookeries, with some male-mediated genetic exchange between rookeries occurring in shared migration corridors (Bowen *et al.* 2005). However, mtDNA in turtles evolves far more slowly than for other vertebrates (Avise *et al.* 1992; Bowen *et al.* 1993) so at smaller geographical scales, such as within a sea turtle rookery, mtDNA haplotype variation becomes limited. For green turtles, for example, there are usually not more than seven haplotypes within turtle rookeries (Luke *et al.* 2004; Formia *et al.* 2006). Extremely low haplotype diversity will hinder the detection of fine-scale natal site fidelity (Freedberg *et al.* 2005) and it is not surprising that the use of mtDNA markers to demonstrate sea turtle population genetic structure between closely adjacent nesting locations has been only successful in a few cases (Schroth *et al.* 1996; Hatase *et al.* 2002).

An alternative approach is to use methods less sensitive to gene flow than F_{ST} (see Pearse & Crandall 2004 for suggestions). Here, we adopted an approach new to studies of sea turtle natal site fidelity in examining 'assignment probabilities' estimated with individual-based tests (Paetkau *et al.* 1995; Waser & Strobeck 1998; Davies *et al.* 1999; Campbell *et al.* 2003; Paetkau *et al.* 2004; Piry *et al.* 2004; Evanno *et al.* 2005). Assignment tests provide results on a par with field-based techniques such as mark-and-recapture, even for populations with high rates of dispersal (Berry *et al.* 2004). A key impetus for this study is to attempt detecting genetic signals of female precise natal site fidelity for sea turtles by means of the simple but powerful assignment approach.

Another suggestion to detect sex-biased gene flow is to directly compare male and female genetic data (Favre *et al.* 1997; Goudet *et al.* 2002). For sea turtles, females are easily sampled when they are on beaches to lay eggs. Males

however, can only be sampled by capture in the ocean, which is not easily performed and only one study has so far managed this (FitzSimmons *et al.* 1997). Also, it is impossible to know on which beach the offspring of male sea turtles, if any, are subsequently born. We solved this problem by using male parental genotypes reconstructed from microsatellite genotypes of hatchlings sampled at known nest locations (Pearse *et al.* 2001). This gives us better data than direct sampling of males since it provides the genotypes of 'effective' males and the nest locations for their offspring.

We collected microsatellite data for green turtles (*Chelonia mydas*) of Ascension Island and used assignment approaches to test the hypothesis of precise natal site fidelity by females within this rookery. As the island is an isolated peak on the mid-Atlantic ridge (7°57'S, 14°22'W), the Ascension rookery is not expected to receive migrants from other rookeries (Bowen *et al.* 1989; Meylan *et al.* 1990; Bowen *et al.* 1992; Formia *et al.* 2006). Satellite telemetry has demonstrated the migratory behaviour of these turtles (Luschi *et al.* 1998; Luschi *et al.* 2003), and they have been tracked to foraging areas around the coast of Brazil (Luschi *et al.* 1998), where they join mixed feeding aggregations as far north as the West Indies (Luke *et al.* 2004). Some rookeries contain multiple beaches that share common breeding grounds (Limpus 1993), but most mating observed for the Ascension rookery occurs within a kilometre of nesting beaches, where males gather to intercept females just prior to the start of the nesting season (Godley *et al.* 2002). It is fairly well established that sea turtle mating occurs mainly adjacent to the nesting beaches (Miller 1997). Females are only receptive to males for a short period prior to nesting and hence mating on distant foraging grounds is not thought to occur. Furthermore, foraging grounds are often very large and so the chances of male/female encounters are massively reduced compared to at the nesting grounds. Early tracking and tagging studies showed that within the same breeding season, individual females can repeatedly nest on the same beach in Ascension (Mortimer & Portier 1989). Fine-scale nesting site fidelity has therefore been demonstrated, but whether females are returning to their natal beach, i.e. the one they hatched from themselves, is unknown. If females were faithful to natal nesting sites within the rookery, they should show genetic cohesion at nesting locations. If males only mated in the mating area adjacent to their own natal beaches, then the genetic patterns between and within beaches should be similar for both sexes. Under scramble polygamy, however, males could be mating with any female in the rookery, so an alternative prediction is that the fathers of hatchlings of the same beaches should not show genetic clustering. A possible way by which males could be mating with females of different natal beaches is by intercepting them further out at sea away from the areas where most mating activity is observed. To assess if this happens, we employed offshore

boat surveys because the detailed spatial pattern of mating intensity in the vicinity of nesting beaches is unknown, with most studies being limited to shore-based observations of mating within a few hundred metres of beaches (e.g. Godley *et al.* 2002).

Methods and materials

Terminology

We define 'nesting population' as the group of females that nest at a particular beach location within the Ascension rookery. This is appropriate because nest site choice within Ascension is not a random decision (Mortimer & Portier 1989). Thus, the 'nesting population' has spatial limits even though the members of nesting populations may travel from various foraging grounds, or indeed mix in the waters around the rookery when not nesting. Whether 'nesting populations' have genetic limits is the subject of this study. Males however, cannot be considered members of the 'nesting population' because male green turtles do not participate in nesting and they do not have a direct choice in where their offspring are born. Male samples, including genotypes reconstructed from hatchlings of known beaches, are considered of unknown origin. However, because we know where these males' offspring were laid, we can still ask if the nesting population for that location (defined by the female adults that nest there) is also likely to be the population they (the fathers) had themselves been born from. 'Gene flow' is the exchange of genetic material between the nesting populations. This can occur if females are nesting on beaches that are not their natal sites ('female-mediated gene flow'), and/or males are fathering offspring in the nesting populations of beaches that are not their natal sites ('male-mediated gene flow'). Note however, that male-biased genetic exchange may also occur through female movements: for example if females visit other beaches and mate with males there but subsequently nest on their natal beach.

Data sets

We used microsatellite data for five loci Cm3, Cm58, Cm84, Cc7 and Cc117 (FitzSimmons *et al.* 1995; FitzSimmons 1998) collected in a previous study (Lee & Hays 2004). Samples were for two breeding seasons (1999 and 2000), and three locations: Long Beach (LB), Northeast Bay Beach (NB) and Southwest Bay Beach (SB) (Fig. 1a). These are separated by only a few kilometres (5–15 km) and host approximately half of all nests on Ascension. Data for the two seasons were pooled for larger sample sizes, as there were no significant differences in assignment probabilities for either year (results not shown). See the previous study (Lee & Hays 2004) for sample and data collection, and

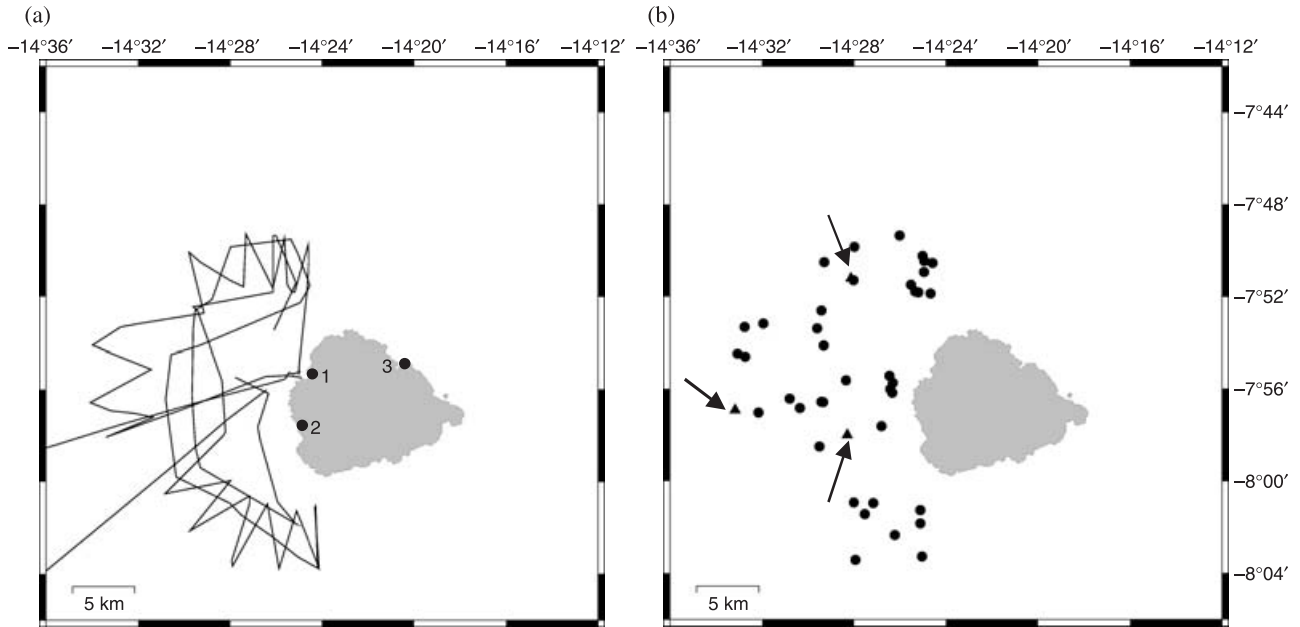


Fig. 1 Maps of boat survey work and results of surveys. (a) The surveys conducted in January 2004. One survey extended west of $14^{\circ}36'W$ (about 25 km from the island), but for clarity is not shown. The map also shows the locations of beaches: 1, LB; 2, SB; 3, NB (b) Solid circles are solitary turtles sighted at least 2 km from the island; sighted mating pairs are indicated with solid triangles and arrows. Fifty-three turtles at least 2 km from land, in very deep water (100 s–1000 s of meters) were observed, including three mating aggregations (mating pairs with attendant males) totalling 12 turtles (23% of all turtles seen in deep water).

genotyping methods. Samples taken directly were almost entirely from adult females ($N = 139$), except for two males captured at sea.

The previous study (Lee & Hays 2004) established the microsatellite genotypes for 402 offspring of 18 clutches as well as those of their respective mothers. This enabled estimation of the minimum number of fathers per clutch (see Lee & Hays 2004 for methods). One method, DADSHARE (www.zoo.cam.ac.uk/zoostaff/amos/), established the full-sib offspring groups per putative father. With this method, the degree of relatedness between individuals within a clutch was estimated (Queller & Goodnight 1989), and sib groups then identified by UPGMA clustering (Blouin *et al.* 1996). When maternal alleles were eliminated from the offspring, paternal alleles could be inferred (Pearse *et al.* 2001). Where data for several offspring were available for a particular father, there was a better chance for all paternal alleles to be detected. However, aside from ensuring that several offspring represented each paternal genotype (six was arbitrarily chosen as a cut-off point), the confidence in the DADSHARE-reconstructed parental genotypes could not be assessed. So a second method, GERUD2.0 (Jones 2005), was also carried out.

GERUD2.0 (Jones 2005) performs an exhaustive search of the minimal set of parents necessary to explain a set of offspring or 'progeny array' known to have a single mother. If the solution, which is the combination of paternal

genotypes explaining the progeny array, is not exact, and there are multiple possible solutions, each one is assigned a probability score. These are relative probabilities based on Mendelian segregation and allele frequencies (Jones 2005). Since scores cannot be interpreted except in comparing solutions within the same progeny array, it may be useful to count the frequency of each particular genotype across all solutions in the array (A.G. Jones, personal communication). This can be carried out by examining GERUD's solutions output and counting the number of times the genotype in question occurs in all solutions.

Simulations (GERUDSIM2.0) to evaluate the efficacy of GERUD2.0 for each particular progeny array were also carried out with 1000 iterations, and included the known maternal genotype, allele frequencies for the population, the minimum number of fathers estimated for the progeny array, the number of offspring sampled, and the number sired by each putative father. GERUDSIM2.0 then reports the frequency of iterations for which the correct paternal genotypes were found in the simulation. If the solution found by GERUD, given the conditions of the data, is to be considered robust, then the frequency of iterations for which the correct paternal genotypes were found in the GERUDSIM simulation should be high. Only if the same paternal genotypes were found with both GERUD2.0 and DADSHARE, and if they were supported with less than 10% error in simulations, were they included in further analyses.

A potential source of error occurs if paternal alleles were not sampled in the offspring. If paternal alleles had been missed, there would be null alleles in the male reconstructed data set. If such errors are more likely to arise from this approach compared with genotyping directly from samples, then a higher frequency of null alleles might be expected when comparing the male data set with the female data set. MICRO-CHECKER (van Oosterhout *et al.* 2004) was used to check for null alleles in the data sets.

Analyses of population structure

GENEPOP (Raymond & Rousset 1995) and SPAGEDI (Hardy & Vekemans 2002) were used to estimate F_{ST} values (Weir & Cockerham 1984). The P values of 2-sided tests for the F_{ST} values were estimated with 10 000 random permutations in SPAGEDI. For analyses of population structure, female and male data sets were treated separately. GENEPOP was also used to check for deviations from Hardy–Weinberg equilibrium, and to describe allele and genotype distributions across populations.

Assignment tests

GENECLASS (Piry *et al.* 2004) was used to calculate individual assignment probabilities for each of the three nesting populations. A Bayesian-based method for computation (Rannala & Mountain 1997) and a resampling algorithm for probability computation with 1000 simulated individuals (Paetkau *et al.* 2004) were chosen. This method tests the null hypothesis that an individual was born in the population where it was sampled by estimating the probability of drawing that individual's multilocus genotype from the allele distributions observed in a series of study populations. When compared to other assignment methods, the Bayesian method (Rannala & Mountain 1997) performs best for any value of F_{ST} (Cornuet *et al.* 1999). It also performs better than STRUCTURE (Pritchard *et al.* 2000) with increasing problems of high gene flow, small numbers of loci or small sample sizes (Waples & Gaggiotti 2006). Statistical thresholds for confidence in excluding a population as the origin of an individual were established with Monte Carlo resampling methods with $P < 0.01$ for excluding populations (Paetkau *et al.* 2004). Before the tests were carried out, the data were examined with the Genetic Algorithm-based Feature Selection (GAFS) (Topchy *et al.* 2004) to determine the optimal set of loci. Selection of a subset of loci can improve the accuracy of assignment testing because some loci may not provide suitable discriminatory information due to lack of polymorphism, measurement errors or correlation with other variables (Guinand *et al.* 2004). GAFS was carried out in 10 experiments, with population sizes of 100 and 100 generations per run (other parameters were left as default), and with

cross-validation in 10-folds to provide accuracy estimations for various combinations of loci. This showed four loci, excluding CC117, as the best performing combination. In GENECLASS (Piry *et al.* 2004) assignment of females did indeed improve by 2.1% to 52.5% correctly assigned, and quality index was higher at 40.94% compared with 40.61% if all loci were used. The four loci combination was subsequently used for all GENECLASS assignment tests.

For GENECLASS tests, female genotypes were used as the adult female data set. Each individual's assignment probability for each nesting population was then estimated against the reference data set. We used adult females as the reference because only females can be considered members of a nesting population; males were treated as unknown (see *Terminology*). The 'leave-one-out' procedure (Efron 1983) was used to control for error caused by assigning females to populations defined by the female data set. This eliminates any bias by excluding individuals from their own population during computation. Individual assignment probabilities were then compared among groups of samples using nonparametric tests (SPSS for WINDOWS, Release 12).

To test for sex-bias in gene flow between different nesting populations, we used the method suggested by Favre *et al.* (1997) as implemented in FSTAT (Goudet *et al.* 2002). For this test, an assignment index for each individual is first estimated. Comparing all male assignment indices with all female assignment indices across different populations could suffer from population effects, so log-transformed indices were corrected by subtracting the population means to give AI_c values (Favre *et al.* 1997). AI_c values average zero for each population and individuals with negative values are those with lower probability than average to have been born in that population. F_{ST} approaches can also be used (Fontanillas *et al.* 2004) so sex-biased gene flow was tested with F_{ST} as well as with mean AI_c (mAI_c) and variance of AI_c (vAI_c) (Goudet *et al.* 2002). In FSTAT a randomization approach with 10 000 permutations tested whether these statistics differed significantly between the sexes. One-tailed tests with females expected to be more philopatric were implemented.

Boat surveys

To examine the distribution of turtles off shore from Ascension Island we conducted boat surveys in January 2004. This corresponds with the start of the nesting season at Ascension Island (Godley *et al.* 2002). Boat surveys were conducted from an 11-m long big game charter fishing boat, *Harmattan*, from which three people watched out for turtles. Boat speed during the surveys was 10 knots, except when turtles were sighted, when the boat was slowed down to allow more detailed observations. On each survey, we recorded the position of any turtles sighted,

whether turtles were mating and, where possible, whether individuals were males or females, as distinguished by the long tail of males. Boat surveys were conducted on 9, 13, 15 and 16 January 2004. Durations of surveys were 5.5 h, 6.9 h, 6.5 h and 6.7 h, respectively, with a total distance covered of 78 km, 89 km, 84 km and 92 km, respectively (Fig. 1). Surveys were conducted to the west of Ascension Island, i.e. in the approximate zone that turtles approaching directly from Brazil might be expected to arrive at the island. Ascension Island was always visible to the human eye during surveys.

Results

Population genetic structure

Table 1 summarizes the general characteristics of the loci. No significant deviations from Hardy–Weinberg equilibrium were found for any locus, each locus had 10–16 alleles, and expected heterozygosities ranged from 0.469 to 0.871. The distribution of genotypes was not identical across nesting populations for the female data set ($\chi^2 = 23.226$, d.f. = 10, $P = 0.010$), and allele distribution was nearly significantly different across populations ($\chi^2 = 17.837$, d.f. = 10, $P = 0.058$). Regardless, F_{ST} analyses did not detect significant population structure for this data set (GENEPOP global $F_{ST} = 0.001$; SPAGEDI global $F_{ST} = 0.001$, $P = 0.731$). For the male data set, alleles and genotypes were distributed identically across beach locations with no significant population structure (GENEPOP global $F_{ST} = -0.007$; SPAGEDI global $F_{ST} = -0.016$, $P = 0.262$).

The reconstructed male data set

Possible paternal solutions for 17 of 18 nests were found with DADSHARE and GERUD2.0. One progeny array had such a large number of possible fathers that paternal genotypes could not be resolved by either method. This nest was excluded from further analyses. We then examined statistics that could indicate confidence for each of the remaining paternal genotypes, summarized in Table 2. Of the 22 genotypes reconstructed with GERUD2.0 that had at least six representative offspring, 12 were exact

solutions. Of these, only one (TT06.F2) had fewer than 90% iterations where the correct male genotype was recovered in simulation tests. Since the possible error rate for this genotype was more than 10%, it was eliminated from further analyses. Interestingly this genotype also had the fewest representative offspring in the list (six), confirming that it is not beneficial to consider paternal genotypes with fewer than six offspring. Within the other 10 progeny arrays with multiple rather than exact solutions, all genotypes except for four were consistent with those reconstructed with DADSHARE. According to simulation tests, GERUD2.0 had a good chance of finding the correct genotype for these four (the correct genotype was found in > 90% of iterations; Table 2). However, if we look across the possible multiple solutions for these arrays, each of these four genotypes occurred in fewer than 65% of the GERUD2.0 solutions (Table 2) — so it was not surprising that DADSHARE estimated alternative genotypes. Given the uncertainty, these four were not used in further analyses. The final male reconstructed data set included only the 17 paternal genotypes in which we had sufficient confidence (Table 2).

There was no indication of null alleles, either in the inferred male data set or the female data set. MICROCHECKER also did not detect any other problems such as short allele dominance (large allele dropout) or scoring errors due to stuttering.

Analyses of assignment probabilities

The power to assign any individual to only one of the nesting populations was low. If the assignment criterion is to assign the individual to the population with the highest probability value, only 53% of the females were assigned back to the nesting population they had been sampled in (LB: 62%, NB: 49%, SB: 15%). If instead, the assignment criterion is by exclusion at $P < 0.01$ (Cornuet *et al.* 1999), there were only 10 instances where any of the three possible nesting populations could be excluded as the source population ($P < 0.01$); among these only three females could be assigned to one population with certainty, and of these only two were assigned to the population they had been sampled in. Similarly for male

Locus	Reference	Sample size	Number of alleles	H_O	H_E	HWE probability test
CC7	FitzSimmons (1998)	140	13	0.779	0.818	0.932
CC117	FitzSimmons <i>et al.</i> (1995)	141	13	0.823	0.871	0.300
CM3	FitzSimmons <i>et al.</i> (1995)	140	10	0.500	0.469	0.493
CM58	FitzSimmons <i>et al.</i> (1995)	134	14	0.873	0.818	0.216
CM84	FitzSimmons <i>et al.</i> (1995)	139	16	0.813	0.832	0.840

Table 1 Summary statistics for five microsatellite loci from adult Ascension green turtles used in this study, with source references for each locus

Table 2 Support for paternal genotypes with at least six offspring assigned to them. If the solution (i.e. the set of paternal genotypes consistent with the genotypes of the progeny array and known mother) is not exact, the solution with the best probability score is accepted. The frequency of each particular genotype across all probable solutions, and the frequencies of iterations in simulations (GERUDSIM 2.0) for which the correct paternal genotypes were found are also presented

Nest code	Number of sampled offspring per nest	Father code	Number of offspring assigned to the father†	Probability scores	Frequency in solutions %	% iterations correct
LB:						
TP36	30	TP36.F1	30	Exact	NA	100
TP39	26	TP39.F1	26	Exact	NA	100
TT01	56	TT01.F1*	23	1.06E-44	64	99.5‡
		TT01.F2*	17		26	98‡
		TT01.F2*	14		12	98‡
TT02	14	TT02.F1	14	Exact	NA	100
TT04	12	TT04.F1	10	Exact	NA	93
TT05	14	TT05.F1	14	Exact	NA	100
TT06	19	TT06.F1	13	Exact	NA	97
		TT06.F2	6	Exact		83‡
NB:						
TP44	44	TP44.F1	44	Exact	NA	100
TP51	29	TP51.F1	17	1.45E-37	100	99
		TP51.F2	11		82	99
TP53	55	TP53.F1	51	8.14E-40	100	99
TT08	33	TT08.F1	33	Exact	NA	100
TT09	46	TT09.F1	39	Exact	NA	100
TT10	40	TT10.F1	25	1.32E-43	100	99
		TT10.F2*	10		50	94‡
TT11	14	TT11.F1	12	Exact	NA	91
TT13	16	TT13.F1	13	3.79E-25	100	98
TT14	11	TT14.F1	11	Exact	NA	97
SB:						
TP48	23	TP48.F1	21	Exact	NA	98

*Genotypes that were different from those found with DADSHARE.

†Note that if some offspring are compatible with more than one father, then the sum of the number of offspring assigned to all fathers will be more than the total number of offspring in the nest. (Adam G. Jones, personal communication)

‡Genotypes excluded from downstream analyses.

genotypes, there was only one instance where one of the nesting populations could be excluded. All other genotypes had assignment probabilities of > 0.01 for any of the three nesting populations.

However, on average, the assignment probabilities for females were significantly higher for nesting populations where these had been sampled from compared with their assignment probabilities for other populations (Fig. 2a; Mann–Whitney U -test: $z = -3.718$, $P < 0.001$). When the data were examined for each nesting population, this was also observed for LB and NB, but not for SB (Fig. 2b; Mann–Whitney U -tests: LB, $N = 79$, $z = -4.016$, $P < 0.001$; NB, $N = 47$, $z = -1.972$, $P = 0.049$; SB, $N = 13$, $z = -1.192$, $P = 0.233$).

Assignment probabilities of males were not significantly different for populations from which the male data had

been sampled compared with their assignment probabilities for other populations (Fig. 2c–d; Mann–Whitney U -test: $z = -0.070$, $P = 0.944$). A possible confounding factor for the male data is the relatively small sample size. There were only 17 reconstructed male genotypes compared with the much larger set of 139 adult female genotypes. To gauge if this could lead to a nonsignificant result, a reduced female dataset was tested consisting of only mothers for each of the offspring families used in reconstructing the paternal data ($N = 17$), and indeed found to be nonsignificant (Mann–Whitney U -test: $z = -1.379$; $P = 0.168$).

F_{ST} and vAI_c tests for sex-biased gene flow were nonsignificant ($P = 0.464$ and $P = 0.855$, respectively). However, the mAI_c test provided a significant result for the one-tailed test of females being more philopatric than males ($P < 0.001$).

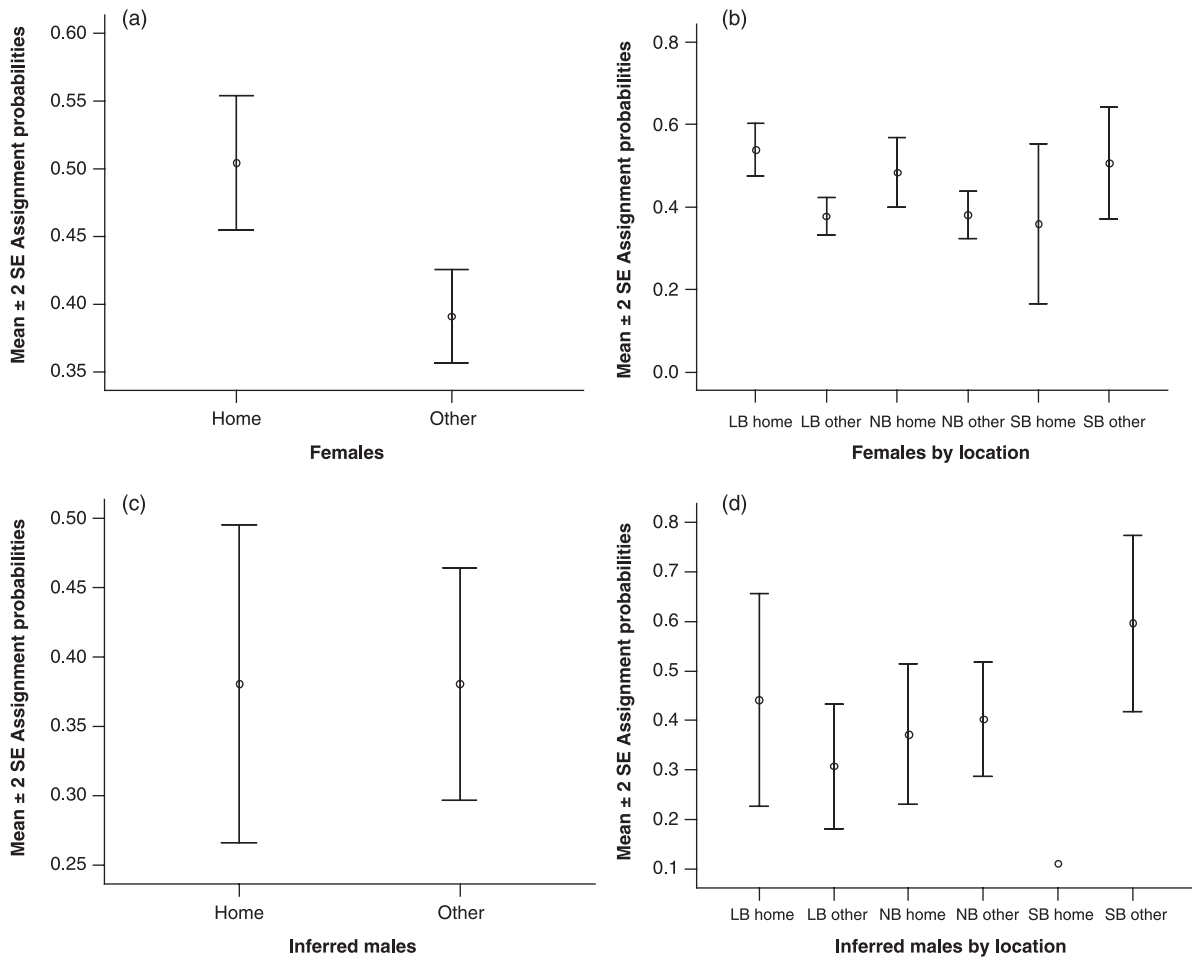


Fig. 2 Average assignment probabilities (\pm SE) of green turtle genotypes for the beach location in which they had been sampled from ('Home') or for other locations ('Other'). Overall average assignment probabilities of all locations (\pm SE) for females (a) and males (c), and average assignment probabilities (\pm SE) for each beach for females (b) and (d) males are shown.

Boat surveys

We observed 53 turtles at least 2 km from the land. As the seabed shelves rapidly around Ascension, these were observed in very deep water (water depth 100 s or 1000 s of metres). Turtles were sighted in all sectors covered (Fig. 1). We observed three mating aggregations totalling 12 turtles (23% of all turtles seen in deep water) (Fig. 1). At 11:08 on 13 January, we observed a mating pair 9.5 km NW of Ascension Island along with two attendant males. At 13:30 on the same day, we observed a mating pair with three attendant males 14.5 km west of the island. At 12:34 on 15 January, we observed a mating pair 6 km west of the island, with one attendant male.

Discussion

Precise natal site fidelity raises many questions about sea turtle homing and nesting behaviour, but there are also

further ramifications for the sustainable management of populations. It has been argued that precise natal site fidelity is an 'evolutionary handicap' for sea turtles, the major implication being that local extinction of nesting populations would lead to irretrievable loss of genetic variation for the species (Schroth *et al.* 1996). Thus an important goal for sea turtle research is to establish the degree to which natal philopatry occurs. In this study, although individual assignment probabilities to nesting populations of origin were generally low, when all assignment values for a nesting location were considered for females sampled from that location, these were nevertheless significantly higher than probabilities of the same individuals for other locations. A possible explanation is that females nesting in the same location have a greater genetic similarity with each other than with females nesting in other locations. That this pattern is consistent for two of the three nesting locations examined (LB and NB) is evidence for female precise natal philopatry for at least

these two locations. The third, SB, represented by a relatively small number of samples (13 females only; LB and NB had 79 and 47 samples, respectively), did not provide any evidence for precise natal philopatry. For the LB and NB nesting populations, it is reasonable to suggest that the results, based on relatively large sample sizes of > 45 and using the conservative leave-one-out control, are consistent with females nesting in the same location having a greater genetic similarity with each other than with females nesting elsewhere. This corresponds with a scenario of females returning to their natal site to nest. Previously, tagging studies have shown strong nest site fidelity by female green turtles of Ascension (Mortimer & Carr 1987), but this provides the first genetic evidence supporting the theory that some are returning to natal beaches.

Significant genetic structure among the female green turtles of Ascension Island could not be detected using F_{ST} . Given this, the low power for assigning individuals to a source population was not surprising. Simulation studies have shown that assignments will improve as the degree of F_{ST} between populations increases (Cornuet *et al.* 1999). For the green turtles of Ascension, either female natal site fidelity is 'leaky' (females occasionally straying to nest on other beaches) or male-mediated gene flow between populations is high enough that nesting groups within Ascension are not strongly genetically differentiated. A tagging study found that although it was rare for a female to be observed emerging at three different beach locations (0.3%), a small proportion (15%) was recorded as nesting on two beach sites (Mortimer & Carr 1987). These observations suggest that female nesting behaviour can contribute towards genetic exchange between nesting populations, but male contribution to this gene flow has been previously unexplored.

Our field observations did show that males could attempt to mate females in areas other than where most matings take place. Matings usually happen close to the shores of nesting beaches, but the boat surveys demonstrated that, while relatively rare, they could also occur in much deeper waters further offshore (Fig. 1). These may involve individuals in their last stages of migration from Brazil to Ascension Island, or those moving offshore after completing their migration. One possibility is that males are trading-off a lower female encounter rate for reduced competition with other males in the less densely populated areas further offshore. Even if males had a tendency to remain adjacent to their own natal beaches while in these deeper waters, the females encountered in such far offshore areas could still include those heading for different beaches to nest (Carr *et al.* 1974; Mortimer & Portier 1989). Mating with females long before they reach the nesting areas is a viable strategy since females can store sperm (Pearse & Avise 2001). A female is likely to have been

mated on several occasions by the time she is ready to lay eggs, and multiple paternity of clutches has been documented for the Ascension rookery (Lee & Hays 2004). Although it is not known for sea turtles, first male precedence in offspring fertilization occurs for other reptiles (Olsson & Madsen 1998) and may be a factor here. Thus, there appears to be opportunity as well as possible explanations for males to mate with any female of the rookery, regardless of her choice of nesting location, and it stands to reason that male-mediated gene flow would be a good explanation for the lack of strong genetic differentiation between nesting populations.

To assess this, we attempted to reconstruct male paternal genotypes from their offspring but only a small number of genotypes could be obtained despite having data for over 400 offspring. Similar to the female data, males could not be assigned with certainty to single source populations. Unlike the female data, the assignment probabilities of male genotypes for nesting populations where their offspring had been sampled were not significantly different from their assignment probabilities for other nesting populations. However, this may not be conclusive evidence that males are less likely than females to have offspring born in natal sites, because the sample size for males was small. On the other hand, analyses of AI_c values (mAI_c test) revealed strong evidence for a sex-bias in gene flow between nesting populations, with females being the more philopatric sex. There is no doubt that there is considerable gene flow between the nesting populations of Ascension, and although some of this would be female-mediated, the significant mAI_c result implies that males did indeed contribute more towards this genetic exchange.

Comparison with other sea turtle studies

For sea turtles, precise natal philopatry was shown for loggerhead turtles on the east Mediterranean coast (Schroth *et al.* 1996) and in the Japanese Archipelago (Hatase *et al.* 2002), but not for those in Florida (Moore & Ball 2002). Studies of olive ridley turtles nesting in east India (Shanker *et al.* 2004), and those for leatherback turtles (Dutton *et al.* 1999) found no significant population genetic structure within breeding regions. Other studies of green turtles have presented mixed results: there was significant correlation between genetic similarity and nesting site distances in Tortuguero (Costa Rica) but not in Melbourne (Florida) (Pearse & Parker 1996), and genetic analyses also did not discern significant population structure along the Michoacan (Mexico) coast (Chassin-Noria *et al.* 2004).

Perhaps precise natal beach fidelity is not a general characteristic for sea turtles, but varies between and within the species. On the other hand, perhaps some instances of the lack of evidence can be explained with markers or techniques that do not provide the required level of resolution,

particularly if there is male-mediated gene flow. Lack of evidence for population genetic substructuring may not always signify panmixia. Mitochondrial DNA, for example, has been a popular marker for sea turtle research because its extensive use in phylogeography studies (Bowen *et al.* 1992; Bowen *et al.* 1994; Avise 1998; Encalada *et al.* 1998) provides well-characterized haplotypes, but its slow evolutionary rate in turtles (Avise *et al.* 1992; Bowen *et al.* 1993) means it is less likely to be useful over short geographical distances. For example, Shanker *et al.* (2004) did not find any significant differences in the frequencies of eight mtDNA haplotypes for olive ridley turtles nesting over a distance of about 500 km of the Orissa coast of India, or even between Orissa & Madras, 2000 km away. For leatherback turtles, phylogeographical structure at the global scale could be detected with 11 mtDNA haplotypes, but proximal nesting populations were not distinguishable (Dutton *et al.* 1999). Chassin-Noria *et al.* (2004) concluded there was no evidence for genetic structure among different green turtle nesting sites within 45 km of the Michoacan coast in Mexico, from either mtDNA or microsatellite data. Schroth *et al.* (1996) however, detected genetic differences in between adjacent loggerhead turtle nesting sites across 800 km of the Turkish coast, but only from the distribution of a single mtDNA haplotype — they needed additional evidence from DNA fingerprinting. Over a similar distance, Hatase *et al.* (2002) also demonstrated significant genetic differences between Japanese nesting sites for loggerhead turtles by detecting variation in the distribution of only three mtDNA haplotypes. Although these latter studies discovered mtDNA variation over such short distances, of the three studies that have demonstrated genetic differentiation between sea turtle nesting sites at the local scale, two also used multilocus DNA fingerprinting methods (Peare & Parker 1996; Schroth *et al.* 1996). Such markers are far more variable than those based on mitochondrial DNA, and hence more likely to reveal fine-scale genetic structure for turtles (Freedberg *et al.* 2005). These multilocus methods are now considered less reliable or less informative than markers such as microsatellites, which is the current method of choice for many population studies (Jarne & Lagoda 1996).

However, studies using microsatellite markers, considered 'hypervariable', have so far not demonstrated precise natal site fidelity in sea turtles. Microsatellites are biparentally inherited markers and only a half-dozen or fewer loci are scored in many ecological studies (including the current one), compared with > 100 loci for markers such as the random amplified polymorphic DNAs (RAPDs) used by Schroth *et al.* (1996). Also, studies using microsatellites often rely on *F*-statistics (or similar) to describe the genetic data. The results in this study may be interpreted as an indication of male contribution towards genetic exchange between nesting populations. Male-mediated

gene flow, together with the low number of loci typically used in resource-limited studies, could explain the general lack of detecting fine-scale genetic structure in sea turtles. Interestingly, in the two DNA fingerprinting studies with evidence for precise natal site fidelity, neither F_{ST} nor microsatellites were used. Peare & Parker (1996) showed a significant correlation between genetic similarity (band sharing scores) of minisatellite DNA fingerprints for pairs of nesting green turtles and the geographical distances between nest sites within Tortuguero Beach in Costa Rica — an impressive result given the extremely short distances (turtles were sampled approximately every 0.2 km within just 8 km). However, a more recent study on green turtles using microsatellite data did not find significant fine-scale genetic structure: Chassin-Noria *et al.* (2004) supplemented mtDNA data with data from three microsatellite loci but did not find evidence for significant genetic structure (F_{ST}) among different green turtle nesting sites within 45 km of the Michoacan coast in Mexico. For loggerhead turtles, Schroth *et al.* (1996) collected data on 152 RAPD markers for five adjacent nesting sites along the Turkish coast over a distance of about 800 km. Analysis of molecular variance indicated that a large proportion of the genetic variance could be explained by differences among nesting sites, but further evidence was a significant correlation between genetic distances (calculated as dissimilarity coefficients) and geographical distances. In contrast, Moore & Ball (2002) collected data for four microsatellite loci from two loggerhead turtle nesting locations within Melbourne Beach in Florida (8 km apart) but there was no significant population structure as calculated by F_{ST} .

Genetic analyses with assignment tests

Recently the usefulness of F_{ST} and similar methods for analysing the genetic data of natural populations have been criticized (Whitlock & McCauley 1999; Pearse & Crandall 2004). These reviews highlight that F_{ST} , which is derived from a set of idealized and highly unrealistic assumptions must be interpreted with caution when it comes to natural populations, particularly those of conservation concern where such assumptions are likely to be violated. To calculate F_{ST} the allele frequencies of predefined groups of individuals are used to compute expected heterozygosities that are then compared among groups, thereby losing individual information. In contrast, the assignment test developed by Paetkau *et al.* (1995) calculates the likelihood of drawing a single multilocus genotype from several groups of genotypes that were potential sources, based on the observed allele frequencies at each locus at each source. With current methods, an assignment probability of each individual for each potential source population is estimated, and the similarity of individual multilocus genotypes provides a means of

clustering individuals into populations as well as excluding a population as the origin of an individual (Cornuet *et al.* 1999). The individual-based approach provides potentially greater power than F_{ST} in discerning fine-scale genetic patterns (Waser & Strobeck 1998), particularly when used with highly variable loci (Campbell *et al.* 2003). Indeed Paetkau *et al.* (1995) originally developed the method to quantify subtle degrees of genetic differentiation among polar bear populations that had previously proved difficult to study because of low variation in markers such as allozymes and mtDNA. Since this first use, the tests have been predominantly applied to the assignment of individuals or samples to source populations. These include genetic analyses of fisheries stocks (Nielsen *et al.* 1997; Engbrecht *et al.* 2002; Hansen 2002), assignment of migrating or dispersed organisms to breeding populations (Haig *et al.* 1997; Palsboll *et al.* 1997; Berry *et al.* 2004) and analysis of admixed populations (Roques *et al.* 1999; Pritchard *et al.* 2000). Further applications are species identification (Reed *et al.* 1997; Roques *et al.* 1999), real-time estimation of migration rate (Paetkau *et al.* 2004) and various others (see reviews by Waser & Strobeck 1998; Davies *et al.* 1999; Campbell *et al.* 2003; Evanno *et al.* 2005; Manel *et al.* 2005). Many applications, including Paetkau *et al.* (1995)'s original work, required exact assignment of individuals to source populations. In this study, this could not be achieved because virtually all individuals had high probabilities for more than one nesting population. Instead, we compared the overall average assignment probabilities of individuals for the nesting populations they had been sampled from with their probabilities for other nesting populations, and detected significantly higher values for at least two of the three Ascension nesting populations. Judging from the lack of significant genetic structure and the low power for individual assignment, the apparent genetic cohesion of females in nesting populations is most certainly weak, but the critical point is that it would seem to have been detected with analysis of assignment probabilities.

Another important application for assignment tests has been in detecting sex-biased gene flow (Favre *et al.* 1997; Goudet *et al.* 2002; Prugnolle & de Meeus 2002). F_{ST} approaches can also be used to detect sex-biased gene flow (Fontanillas *et al.* 2004), but AI_c statistics are considered more advantageous because it is more versatile, less sensitive to the pattern of isolation by distance for the populations under investigation, can be applied to a single population, and measures instantaneous dispersal rates rather than long-term effective gene flow (Goudet *et al.* 2002). In this study, the mAI_c test effectively demonstrated sex-biased gene flow, whereas both F_{ST} and vAI_c tests did not do so. Simulations show that these tests differ in their sensitivity to various parameters (Goudet *et al.* 2002). For example, F_{ST} and vAI_c are best used at high (> 10%) and low (< 10%)

dispersal rates, respectively, whereas mAI_c is intermediate between these tests. In recent studies analyses of AI_c values have been successfully used to demonstrate sex-biased dispersal in many animals including terrestrial mammals (van Hooft *et al.* 2003; Devillard *et al.* 2004; Hammond *et al.* 2006), birds (Dallimer *et al.* 2002), reptiles (Berry *et al.* 2005), amphibians (Lampert *et al.* 2003; Palo *et al.* 2004), dolphins (Moller & Beheregaray 2004; Cassens *et al.* 2005), fish (Fraser *et al.* 2004) and invertebrates (de Meeus *et al.* 2002; Sundstrom *et al.* 2003). The situation for sea turtles is somewhat more complex than the dispersal behaviour studied in these other examples. Both male and female sea turtles migrate between feeding and breeding grounds (FitzSimmons *et al.* 1997), but there could be sex-biased gene flow between populations without actual dispersal taking place (Bowen *et al.* 2005). Here, we were looking for a genetic pattern resulting from females preferring to lay eggs on natal beaches but having been mated by any male of the rookery — hence, we were interested in sex-biased gene flow rather than 'dispersal' between natal beaches. Nevertheless, the AI_c approach is still applicable because it tracks the movement of genes and not individuals.

We conclude that assignment probabilities provided some evidence for females to precisely nest in natal sites. Out of three tests for sex-biased gene flow, a single test (mAI_c test) appeared to indicate that gene flow between nesting populations was mediated more by males than by females. One can view the latter result as corroborative evidence for female natal site fidelity because it would be consistent with relatively fewer females than males involved in genetic exchange between nesting populations. However, this study also highlighted the necessity for adequate sample sizes for assignment tests, a point also suggested by simulation tests (Goudet *et al.* 2002). We acknowledge that larger sample sizes and also more microsatellite loci could have yielded stronger evidence. Nevertheless, we propose that where large numbers of individuals can be sampled, either directly or by reconstruction from offspring genotypes, assignment-based approaches should always be considered for future studies of similar problems, as an alternative or at least an addition to the more traditional methods of analysing population genetic structure.

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