Biological Conservation 171 (2014) 200–208

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

High rates of hybridisation reveal fragile reproductive barriers between endangered Australian sea snakes

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ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 22 December 2013
Accepted 8 January 2014

Keywords:
Endangered species
Hybridisation
Reverse speciation
Microsatellites
Sea snakes

ABSTRACT

The viviparous sea snakes include 62 ecologically diverse species, many of which are of very recent evolutionary origin and have overlapping distributions. Peak sea snake diversity and endemism is recorded from the isolated emergent reefs of the Timor Sea in Northwest Australia. However, nine species have disappeared from Ashmore, the largest of these reefs, over the last 15 years, including two critically endangered *Aipysurus* species that have also disappeared from neighbouring Hibernia Reef. A third Timor Sea endemic, *Aipysurus fuscus*, is now known only from Scott and Hibernia reefs, where it coexists with closely related and locally abundant *Aipysurus laevis*. We analysed microsatellite markers for *A. fuscus* and *A. laevis* sampled across four Timor Sea reefs to assess evidence for recent inter-specific gene flow and historical introgression. Our data fit an Isolation–Migration model, which showed significant and asymmetrical levels of gene flow following species divergence, and highest rates of introgression from the large *A. laevis* population into the much smaller *A. fuscus* population. Population assignment analyses recovered two ancestral clusters that broadly corresponded to morphological species designations, but revealed high frequencies of hybrids on all four reefs and individuals of pure *A. fuscus* ancestry only at Scott and (historically) Ashmore. Most unexpectedly, 95% of snakes sampled at Hibernia were hybrids that resembled *A. laevis* in phenotype, revealing a collapse of reproductive barriers ('reverse speciation') at this reef. These results have dire implications for the conservation status of *A. fuscus*, and highlight the fragility of reproductive barriers in a recent marine radiation.

1. Introduction

Hybridisation between individuals of genetically distinct species is a major theme in both evolutionary and conservation biology. Inter-specific mating can maintain biodiversity by reinforcing pre-existing reproductive barriers (Dobzhansky, 1937; Servedio and Noor, 2003), or might result in novel gene combinations and new hybrid species (Anderson and Stebbins, 1954; Abbott et al., 2013). Alternatively, hybridization can threaten biodiversity when rare species are assimilated with introduced populations (Laikre et al., 2010), or via 'reverse speciation' of previously distinct species when ecological conditions change to favour the formation and viability of hybrids (Taylor et al., 2006; Seehausen et al., 2008). Such introgressive hybridisation not only results in the genomic extinction of parental species, but can cause marked fitness declines in outbreeding populations, particularly where parental species are highly divergent and/or adapted to contrasting environments (Allendorf et al., 2010). Evidence from molecular studies suggests that hybridisation occurs much more frequently in natural systems than was previously recognised (Mallet, 2005). However, anthropogenic species translocations and habitat modifications have caused a dramatic increase in rates of hybridisation worldwide, contributing to the extinction of numerous species and populations (Rhymer and Simberloff, 1996; Allendorf et al., 2001). Understanding the dynamics and consequences of natural and anthropogenic hybridisation in endangered species is therefore an important priority for biodiversity conservation (Allendorf et al., 2010).

Viviparous sea snakes (Hydrophiinae: Hydrophiini) are the only fully aquatic and by far the most speciose marine reptiles (Rasmussen et al., 2011). The group includes more than 60 species that are ecologically very diverse and are distributed throughout the tropical and sub-tropical Indo-West Pacific (Rasmussen et al., 2011; Elfes et al., 2013). However, the majority of these species are of very recent evolutionary origin, having diversified within

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http://dx.doi.org/10.1016/j.biocon.2014.01.013
0006-3207/

only the last approximately 3.5 million years (Sanders et al., 2013). The highest endemism and sympatric diversity of sea snakes is recorded from Northwest Australia, where five emergent reefs in the Timor Sea support 14 species including three regional endemics (Minton and Heatwole, 1975; Guinea and Whiting, 2005; Elfes et al., 2013). These isolated reefs have also suffered the most severe population declines (Guinea 1993, 2006, 2007, 2012a,b; Guinea and Whiting, 2005; Lukoschek et al., 2013). Nine sea snake species were previously resident at the Ashmore Reef Marine Reserve, with high population densities in coral, sea grass and lagoon habitats; however, following unexplained declines over the last ~15 years, all of these species have now disappeared (Guinea, 2012a,b; Lukoschek et al., 2013). The critically endangered and endemic short-nosed and leaf-scaled sea snakes, *Aipysurus apraefrontalis* and *Aipysurus foliosquama*, were previously known only from Ashmore and Hibernia Reefs, but have not been sighted at either reef since 1998 despite intensive survey efforts (Guinea, 2006, 2007, 2012a,b; Guinea and Whiting, 2005; Lukoschek et al., 2013). A third Timor Sea endemic, the dusky sea snake (*Aipysurus fuscus*: Tschudi, 1837), is listed as endangered and is only known from Scott, Hibernia and Ashmore Reefs (IUCN, 2012; Lukoschek et al., 2013). Following the extinction of *A. fuscus* at Ashmore, this species is now restricted to a maximum range area of 262 km^2, and the Hibernia population is isolated from conspecifics at Scott Reef by 260 km of unsuitable (>200 m deep water) habitat.

Dusky sea snakes co-occur with olive sea snakes (*Aipysurus laevis*: Lacepede, 1804) throughout their range. These species were strongly resolved as closest relatives based on multilocus analysis of a broad phylogenetic sampling of *Aipysurus*, and were estimated to share a common ancestor dated at approximately 500,000 years ago (Sanders et al., 2013). While *A. fuscus* has a tiny distribution, *A. laevis* is widely distributed across northern Australia to the south coast of New Guinea and New Caledonia (see online kmz file for species distributions), and is often the most abundant sea snake on coral reefs throughout this range (Guinea and Whiting, 2005; Lukoschek et al., 2007; IUCN, 2012). Both species have large heads with similar scalation and musculature and long tooth rows, but they are readily distinguished by body size, body scale number and colour pattern (Fig. 1) (Smith, 1926; McDowell, 1972; Rasmussen, 2000). *A. laevis* is heavy bodied and reaches almost 2 m in total length, whereas *A. fuscus* is moderately built and rarely exceeds ~90 cm (Smith, 1926; Cogger, 1975; Rasmussen, 2000). *A. laevis* has a variable colour pattern but in the Timor Sea is usually tan to dark brown dorsally and pale ventrally; *A. fuscus* are a uniform dark brown or purplish brown, sometimes with faint cross-bands (Smith, 1926; McDowell, 1972; Cogger, 1975). Both species are diurnally active on shallow reef flats and edges (Cogger, 1975) and prey on a variety of fishes by probing holes and crevices in the reef matrix (McCosker, 1975; Voris and Voris, 1983).

As for many closely related sympatric species (Seehausen et al., 2008), it is likely that adaptive divergence (probably of body size, e.g. Shine, 2005) facilitates the ecological and genetic coexistence of *A. laevis* and *A. fuscus* in the Timor Sea. However, the strength of contemporary fertility barriers and history of gene flow between these species have not previously been investigated. In the present paper, our aim was to better understand the conservation status of the remaining *A. fuscus* populations by examining their past and present levels of hybridisation with the more abundant *A. laevis*. Microsatellite analyses uncovered high levels of recent and historical hybridisation between the species across four Timor Sea reefs, with much higher rates of introgression from *A. laevis* into *A. fuscus* than in the opposite direction. Unexpectedly, however, almost all snakes sampled at Hibernia were hybrids that closely resembled *A. laevis* in phenotype. The near-complete collapse of reproductive barriers at one of only two reefs that support *A. fuscus* has critical implications for the conservation of this endangered species.

2. Materials and methods

2.1. Sampling sites and strategy

Sampling was carried out by the authors at Scott, Seringapatam and Hibernia Reefs in Northwest Australia, primarily in February 2012 and March 2013. These are emergent reefs located in the Timor Sea ~800 km west of Darwin. Ashmore and Hibernia are situated ~30 km apart on the edge of the Sahul continental shelf ~180 km northeast of Scott and Seringapatam, which are separated by ~24 km; each of these reefs is isolated by waters of at least 200 m to more than 1 km deep (Skewes et al., 1999). The type locality of *A. fuscus* is given as Sulawesi (Indonesia) but this provenance is doubtful and the species has not been reported from any other (unsampled) reefs in the region (Minton and Heatwole, 1975; Guinea, 1993, 2006, 2007, 2012a,b).

Sea snakes were caught in nets while snorkelling and at night from a boat using spot lights and dip nets. Global positioning system coordinates were recorded at each sampling area. Preliminary morphological species identifications were made using colour pattern characters and scale counts (*A. laevis* typically has 21 scale rows at mid body versus 19 in *A. fuscus*: Smith, 1926; Cogger, 1975; Rasmussen, 2000). A narrow strip (approximately 1 × 3 mm) of tail tissue was collected and stored in saturated salt solution for DNA preservation; snakes were then released at the collection locality. Muscle and skin tissues were also obtained from nine specimens collected at Ashmore by ML Guinea between 1998 and 2001. The locations of the four reefs sampled in this study are shown in the online kmz file.

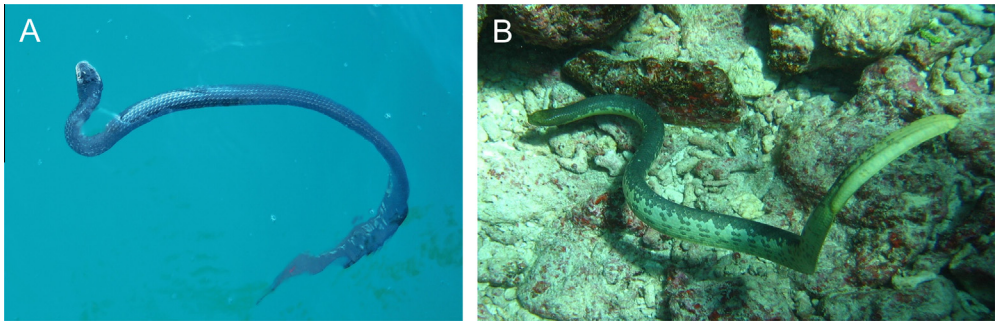

Fig. 1. Photographs of (A) *Aipysurus fuscus* and (B) *A. laevis* from the Timor Sea showing representative differences in colour pattern. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Microsatellite data collection

We used 11 microsatellite markers previously developed for a closely related sea snake genus, *Emydocephalus*, and shown to cross-amplify in *Aipysurus* species (Lukoshek and Avise, 2012). Primers were labelled with four different coloured fluorescent dyes: 6-FAM, VIC, NED or PET. For all markers, PCR amplification used an annealing temperature of 55 °C and 35 cycles. The markers were then pooled and run on an Applied Biosystems 3730 DNA analyser platform for capillary separation. Genotyping data were scored for alleles using Applied Biosystems GeneMapper software.

2.3. Summary statistics and population structure

Deviations from Hardy–Weinberg (H–W) expectations within loci, linkage disequilibrium (LD) between loci, mean number of alleles per locus, and observed and H–W expected heterozygosities were analysed using Arlequin (Excoffier and Lischer, 2010). MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) was used to further check for large allele dropout and stuttering errors. Analyses were carried out using only individuals identified as genetically-pure species at Scott and Seringapatam ($Q > 0.9$) based on population assignment analyses (see Section 2.4 below), and separately using all individuals sampled at each reef including hybrids.

Using only genetically-pure individuals of each species, F_{ST} values were calculated using the Weir and Cockerham (1984) method in Arlequin. F_{ST} values were calculated for *A. laevis* and *A. fuscus* at Scott Reef and *A. laevis* at Scott and Seringapatam; comparisons involving Hibernia and Ashmore were excluded due to low sample sizes of non-hybrid individuals. Significance was tested using 100 random permutations of the data for each value.

2.4. Admixture analysis and genetic assignment

To assign the species or hybrid origin of each sampled snake and calculate the frequency and admixture proportions of hybrids at each reef, we analysed genotype data from all 11 loci for all 80 snakes using the Bayesian program STRUCTURE (Pritchard et al., 2000). STRUCTURE estimates the probability that each individual belongs to an ancestral cluster (K), recording posterior probability distributions for admixture proportion (Q) in each cluster. We ran the program 10 times for each of $K = 1$ to $K = 5$ under the admixture model. We assumed independent allele frequencies because the species are quite divergent (estimated split is ~500,000 years ago) so we expect them to have reasonably different allele frequencies. Each run used a burn-in step of 1,000,000 followed by 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. The best supported K was determined by examining posterior probability distributions and using log-likelihood ratio tests (Pritchard et al., 2000) as implemented in STRUCTURE Harvester (http://taylor0.biology.ucla.edu/structureHarvester/). Runs for the optimum K were then summarised using CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) and Q values were plotted using Distruct 1.1 (Rosenberg, 2004).

To exclude the possibility that our results for *A. laevis* and *A. fuscus* are influenced by gene flow with other congeners, we performed a separate STRUCTURE analysis including three additional *Aipysurus* species that co-occur with *A. laevis* and *A. fuscus* in the Timor Sea reefs. In addition to the 80 *A. laevis* and *A. fuscus* samples, this analysis used four individuals of *A. apraefrontalis* (from Ashmore and Exmouth), five *A. foliosquama* (Ashmore), and 12 *Aipysurus duboisii* (Scott and Seringapatam).

2.5. Analysis of simulated genotype data

To test the power to distinguish between hybrid and non-hybrid individuals in our study system, we generated simulated data sets using only individuals that had high probabilities ($Q > 0.90$) of belonging to either of the pure bred clusters identified in the STRUCTURE analysis. The genotypes of these 12 *A. fuscus* and 20 *A. laevis* were then used to generate simulated data sets using the program Hybridlab v 1.0 (Nielsen et al., 2006). We simulated 120 genotypes for *A. fuscus*, 200 genotypes for *A. laevis*, and 80 genotypes for each of four hybrid classes: F1, F2, *A. fuscus* first generation backcross (F1 × *A. fuscus*), and *A. laevis* first generation backcross (F1 × *A. laevis*). These proportions reflect the percentage of genetically-pure individuals and hybrids in the real data (see Section 3). Simulated data were analysed with STRUCTURE for five runs at $K = 2$ using the same settings and parameters as for the real data. The results were used to determine appropriate thresholds for separating genetically-pure and hybrid individuals, and calculate the efficiency (proportion of pure or hybrid individuals correctly assigned) and accuracy (proportion F1 hybrids correctly assigned to that category) of our chosen assignment method following Vähä and Primmer (2006).

2.6. Demographic analysis

Demographic migration, divergence time and population size parameters were estimated by fitting coalescent Isolation with Migration (IM) models as implemented in the program IMa (Hey and Nielsen, 2004, 2007). The full IM model estimates the splitting time (t) of an ancestral population with an effective population size (qA) into two sister populations that can have different sizes (q_1 and q_2) and asymmetrical migration (m_1 and m_2). This full demographic model was run using the Markov chain Monte Carlo (MCMC) approach and the Stepwise Mutation Model (SMM) with alleles converted into numbers of repeats. Three loci with imperfect repeats or containing repeat motifs of varying length were excluded from the analyses, so that the model was fit using the remaining eight loci.

Two different datasets were analysed using only individuals with admixture coefficients (Q) of >0.75 in the STRUCTURE outputs to avoid errors in assignments of hybrids to parental species clusters. In the first analysis, we used all sampled *A. laevis* ($n = 23$) and *A. fuscus* ($n = 15$) with no missing data and $Q > 0.75$. We then repeated the analysis using only individuals sampled at Scott Reef and randomly discarded *A. laevis* individuals so that equal numbers of individuals were sampled for the two species ($n = 9$). This second analysis was performed to exclude the effects of unequal sample sizes and geographic population structure among reefs (although the IM method has been shown to be relatively robust to these assumptions: Strasburg and Rieseberg, 2010).

Appropriate prior distributions for demographic parameters were set based on posterior distributions from five preliminary runs: the maximum m_1 and m_2 were set to 5; and the maximum q_1 and t were set to 10. Each analysis was then run 5 times in M-mode for 1 million MCMC steps (with the default sampling of every 100 steps) after a burn-in period of 1 million steps, using different random number seeds to check for convergence of the chain. Log-likelihood ratio (2LLR) tests as implemented in the program were performed on the 16 nested models: population, migration and divergence time parameter estimates variously to set to zero, fixed as equal to other parameters, or free to vary. Model fitting was used to assess changes in effective population sizes and the level and directionality of migration in the speciation history of these snakes. We did not convert the divergence time parameter to an

absolute time estimate because a reliable mutation rate is not currently available for our microsatellite markers.

3. Results

3.1. Summary statistics and population structure

Genetically-pure individuals ($Q > 0.9$) of each species sampled at Scott and Seringapatam showed no evidence of deviations from HWE expectations at any locus or linkage disequilibrium between loci. Tests using all sampled individuals at each reef (hybrids included) showed two locus pairs in significant linkage disequilibrium at Scott Reef (Table 1), and HWE deviations for three loci at Scott Reef, and one locus each of Ashmore and Seringapatam. MICRO-CHECKER did not find evidence of null alleles consistent with the findings of Lukoschek and Avise (2012). Based on pure individuals ($Q > 0.9$) of each species, F_{ST} values were 0.102 between *A. laevis* at Scott Reef ($n = 11$) and *A. fuscus* at Scott Reef ($n = 8$); and 0.014 between *A. laevis* at Scott and Seringapatam ($n = 7$); both values were significant based on 100 permutations.

3.2. Admixture analysis and genetic assignment

Based on the Evanno method, STRUCTURE supported 2 genetic clusters (K) with admixture proportions shown in Fig. 2. Here each species represents an ancestral cluster; hybrids result from

Table 1
Summary statistics and STRUCTURE results from all 11 sampled microsatellite loci. *N* is the sample size; # alleles is the mean number of alleles per locus; Obs. Het. and Exp. Het. are the mean observed and Hardy–Weinberg (H–W) expected heterozygosities; H–W is the number of loci that deviate from H–W expectations; LD is the number of locus pairs in significant linkage disequilibrium after Bonferroni correction (adjusted $p = 0.0009091$). STRUCTURE results show the number of sampled individuals of pure *A. laevis* ancestry, *A. fuscus* ancestry, and hybrids, at each reef using a threshold *Q* value for pure ancestry of >0.90.

	Reef area (km^2)	*N*	# Alleles	Obs. Het.	Exp. Het.	H–W	LD	STRUCTURE		
								'Pure' *laevis*	'Pure' *fuscus*	Hybrid (%)
Scott	250.13	42	10.4	0.772	0.808	3	2	11	8	23 (54.8%)
Seringapatam	55.19	12	7.6	0.800	0.784	1	0	7	0	5 (41.7%)
Ashmore	226.97	7	5.4	0.615	0.730	1	0	0	4	3 (42.8%)
Hibernia	11.47	19	9	0.784	0.780	0	0	1	0	18 (94.7%)
Total	543.76	80	8.1	0.743	0.775	4	2	19	12	49 (61.2%)

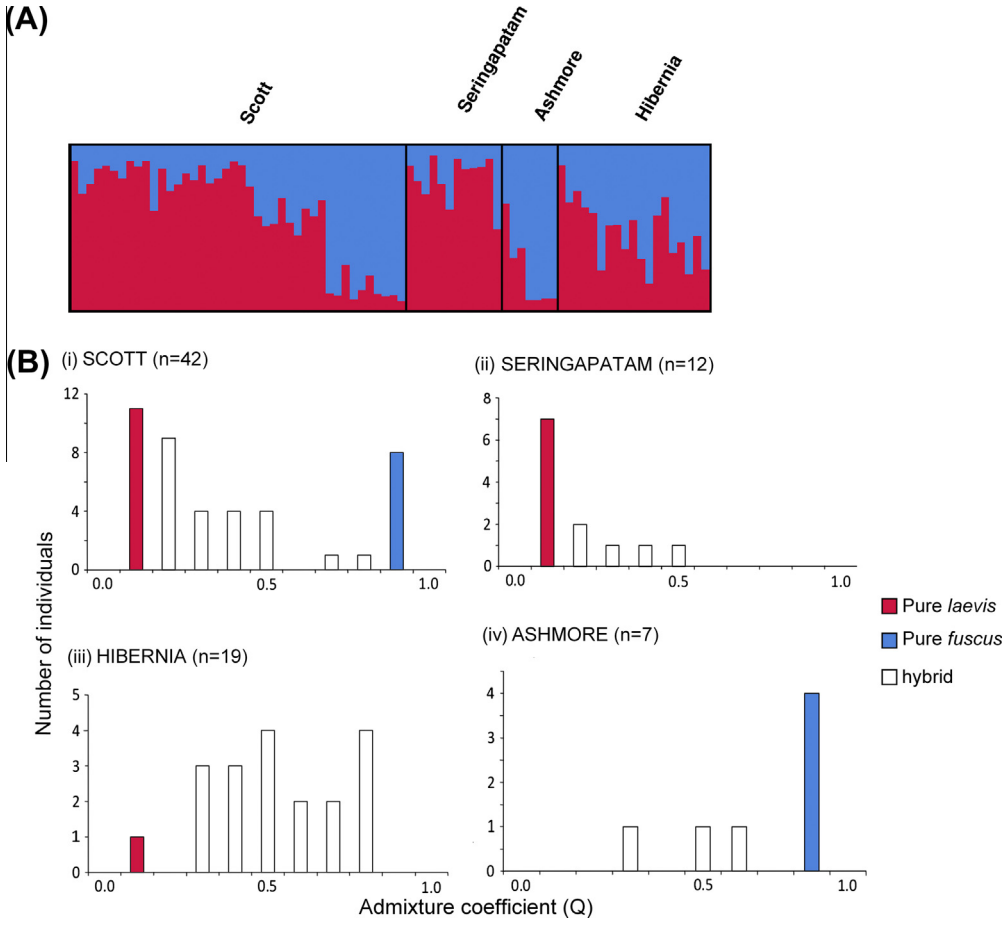

Fig. 2. STRUCTURE results for all sampled individuals showing (A) admixture proportion in *A. laevis* (red) and *A. fuscus* (blue), and (B) hybrid index plots of admixture for individuals sampled from (i) Scott Reef, (ii) Seringapatam Reef, (iii) Hibernia Reef, and (iv) Ashmore Reef. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

admixture between these clusters and make up a large proportion of sampled individuals on all four reefs at a threshold admixture coefficient (Q) of <0.9 (Table 1; Fig. 3).

Scott Reef had a bimodal admixture distribution with most individuals showing $Q > 0.80$ in either *A. fuscus* or *A. laevis* clusters, however 54.8% of individuals were assigned as hybrids at $Q > 0.90$. At this reef, morphological data were collected for 8 of the 9 individuals with $Q > 0.80$ in *A. fuscus*, all had *A. fuscus* phenotypes (dark uniform colour, small body size and 17 or 19 mid body scale rows). One individual had 19 mid body scale rows but the *A. laevis* colour pattern and pure *A. laevis* ancestry. All remaining snakes for which morphological data were available (21 of 32 individuals) had *A. laevis* phenotypes (21 or 22 mid body scale rows and light–dark contrasting colour patterns), including eight pure *A. laevis* and 13 snakes with hybrid genotypes (seven of which had $Q > 0.80$ in *A. laevis*). At Seringapatam, all 12 sampled individuals had *A. laevis* phenotypes. Of these, seven were pure *A. laevis* and five (41.7%) were hybrids, all showing $Q > 0.50$ in the *A. laevis* cluster consistent with the observation that *A. fuscus* has not previously been recorded from Seringapatam. Of the seven samples (that yielded sufficient DNA) from Ashmore, four were pure *A. fuscus* and three (42.8%) were hybrids. Hibernia showed by far the highest proportion of hybrids, with only one pure *A. laevis*, no pure *A. fuscus*, and 94.7% of individuals assigned as hybrids. At this reef, morphological data were recorded for 15 of 19 snakes; all had 21 or 23 scale rows at mid body (corresponding to *A. laevis*) but one individual with hybrid ancestry had a uniform dark colour pattern and was provisionally designated as *A. fuscus*.

3.3. Analysis of simulated genotype data

Structure analysis of simulated genotypes showed that 100% of pure *A. fuscus* and 98% of pure *A. laevis* individuals could be identified using a threshold Q value of >0.90. This justifies our classification of real individuals with $Q > 0.90$ as genetically-pure species and individuals with $Q < 0.90$ as hybrids. All F1 and F2 genotypes were correctly identified as hybrids using the threshold Q-value of >0.90, although their Q value distributions overlapped (0.22–0.64 and 0.14–0.89 for F1s and F2s, respectively). Of first generation backcrosses, 6% were wrongly assigned to a genetically-pure species category, and in all cases these were assigned to the parental species category to which the hybrid was backcrossed. Frequency distributions of admixture coefficients for simulated genotypes of parental species and F1 and F2 hybrids are shown in Fig. 3.

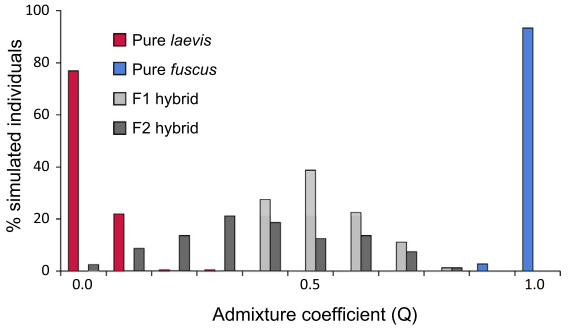

Fig. 3. Frequency distribution of admixture coefficients for simulated genotypes for parental species and first and second generation hybrids estimated using STRUCTURE.

3.4. Demographic analysis

Independent IMa runs yielded ESS (effective sample size) values above 50 and broadly similar unimodal posterior density distributions for the six model parameters in the analyses of both the reduced (9 individuals per species with $Q > 0.75$ from Scott Reef) and full (all 38 individuals with $Q > 0.75$) data sets, suggesting good mixing and adequate convergence of the Markov chains. Parameter estimates differed between the two data sets, but all five runs of each data set indicated much higher effective population size for *A. laevis* compared to *A. fuscus*, and for the ancestral population compared to the two extant species (Table A1; Fig. 4a). All runs for both data sets also consistently indicated much higher migration rates (movement of alleles) from *A. laevis* into *A. fuscus*, than in the opposite direction (Table A1; Fig. 4b). Divergence time estimates were the most variable parameters, but all runs yielded unimodal distributions with lower 90% HPD estimates that excluded zero (upper 90% HPD estimates exceeded the prior maximum regardless of the prior set).

Likelihood ratio tests for the genealogies sampled under the full data set strongly rejected all models in which migration rates were set to zero (2LLR > 880, $p < 0.001$), or fixed to zero from *A. laevis* into *A. fuscus* but not in the opposite direction (2LLR > 50, $p = < 0.001$). We were also able to reject models in which migration rates were equal in both directions and effective population sizes were equal for two out of the three populations (2LLR 5-12, $p < 0.05$). The model with the highest likelihood allowed a different effective population size for *A. laevis* than for *A. fuscus* and the ancestral population, and different but non-zero migration rates in both directions. For the genealogies sampled under the reduced data set, all models in which migration rates were set to zero were again strongly rejected (2LLR > 12-144, $p < 0.01$). We were also able to reject models in which migration rates were fixed to be equal in both directions and with equal effective population sizes for the three populations (2LLR 6-9, $p = < 0.05$). The model with the highest likelihood had different effective population sizes for

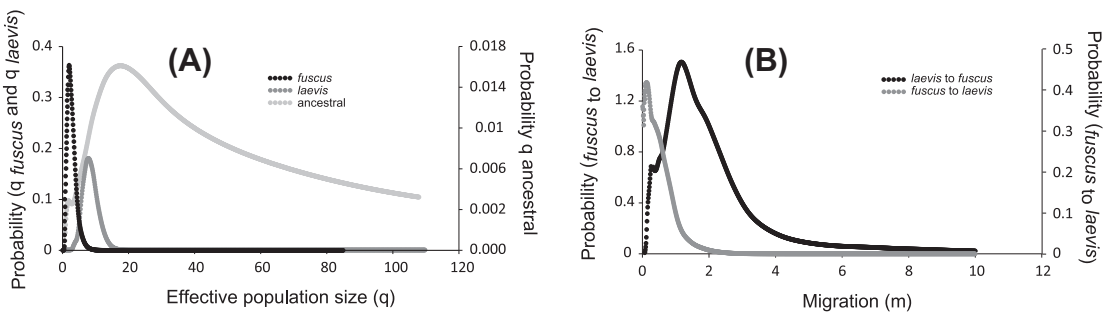

Fig. 4. Posterior density distributions of (A) effective population size and (B) migration rate parameters estimated from a single run fitting an Isolation with Migration model to 8 microsatellite loci for all 38 sampled individuals with $Q > 0.75$.

each deme, and migration from *A. laevis* into *A. fuscus* but not in the opposite direction.

4. Discussion

Our microsatellite analyses demonstrate that endangered dusky sea snakes (*A. fuscus*) hybridise very frequently with olive sea snakes (*A. laevis*) throughout their small range in the Timor Sea. The species formed two ancestral clusters based on population assignment analyses (Fig. 2a), but hybrids were common on all four reefs. Most unexpectedly, almost all (95% of) snakes sampled at Hibernia were hybrids that mostly resembled *A. laevis* in phenotype. Despite such high levels of contemporary gene flow, our data fit an IM model of historical divergence that yielded unimodal divergence time parameters with credibility intervals that excluded zero (Table A1). IM analyses further indicated significant levels of gene flow following divergence, with much higher rates of introgression from the larger *A. laevis* population into *A. fuscus* than in the opposite direction (Fig. 4). Together, these results suggest 'reverse speciation' of *A. laevis* and *A. fuscus* at Hibernia Reef.

Our findings follow several previous studies that have found evidence of significant gene flow following speciation in other squamate reptiles and marine vertebrates (e.g. Rabosky et al., 2009; Prada and Hellberg, 2013; Leaché et al., 2013). Whether the initial divergence of *A. laevis* and *A. fuscus* occurred as a result of parapatric ecological speciation, or involved periodic isolation and introgression, is unknown. However, opportunities for gene flow may have been enhanced by dynamic range changes in response to late Pleistocene sea level fluctuations, which dramatically altered the distribution and community structure of coral reefs in the Timor Sea (Wilson, 2013).

In the following, we discuss the implications of our results for understanding the dynamics and conservation impacts of hybridisation in these sea snakes.

4.1. Hybrid frequency: Collapse of species boundaries at Hibernia?

Hybrid frequencies and admixture proportions differed considerably among the four reefs (Fig. 2a and b). At Scott, genetically pure individuals of both parental species were present, but 55% of individuals were assigned as hybrids and most of these had highest admixture proportions in the *A. laevis* cluster. Seringapatam and Ashmore were less densely sampled, but hybrids were also common (~42%) at these reefs, albeit with different admixture proportions: *A. fuscus* has never been recorded from Seringapatam, where all hybrids showed $Q > 0.5$ in *A. laevis*. Most unexpectedly, 95% of individuals at Hibernia were hybrids, and no pure *A. fuscus* and only one pure *A. laevis* were identified at this reef. With a few exceptions, most hybrids on all reefs closely resembled *A. laevis* in phenotype (21–23 scale rows at mid body and light–dark contrasting colour pattern). In contrast, *A. fuscus* phenotypes (17–19 scale rows at mid body and dark uniform colour pattern) were only found in individuals with close to pure *A. fuscus* ancestry.

Accurate estimation of hybrid frequency using population assignment requires that all species connected by gene flow be included in the analysis. Three other *Aipysurus* species (*A. duboisii*, *A. foliosquama* and *A. apraefrontalis*) are found at Hibernia (and formally at Ashmore), and one of these (*A. duboisii*) also occurs at Scott and Seringapatam. A separate population assignment analysis that included all five species showed negligible admixture ($Q < 0.05$) between the three additional species and either *A. laevis* or *A. fuscus* (not shown), indicating that our results were not significantly influenced by gene flow with these other species. Estimates of hybrid frequencies can also be biased by sampling strategy. We collected snakes opportunistically, targeting reef edge, flat and lagoon habitats at each locality (Guinea, 2012b), so it is unlikely that our sampling was biased towards encountering more hybrids at Hibernia compared to the other reefs. Larger *A. laevis* may have been more conspicuous, but *A. fuscus* were collected preferentially because they occurred at lower densities (Guinea, 2012b). Given these observations, our results are most consistent with generally high rates of hybridisation at Scott and Ashmore, but an almost complete breakdown of reproductive barriers ('reverse speciation') at Hibernia.

Proportions of first versus later generation hybrids can provide insights into the nature and strength of pre- and post-mating barriers (Barton and Hewitt, 1989). Unfortunately, we were not able to probabilistically assign individuals to hybrid class. However, distributions of hybrid admixture coefficients (Fig. 2b) can provide some insight into their genetic composition compared to parental species. If hybridisation was restricted to the first generation, we would expect most hybrids to have admixture coefficients close to 0.5 (Fig. 3; see also Jiggins and Mallet, 2000). However, in the present study, the majority of hybrids at each reef had Q values between 0.6 and 0.8. These results suggest that hybridisation also involves later generation crossings (F2 hybrids and backcrosses) indicating that, regardless of their fitness relative to parental species, hybrids must be viable beyond the first generation. At Hibernia, an absence of intrinsic hybrid dysfunction is further suggested by the lack of violations of Hardy–Weinberg and linkage equilibrium at all 11 loci (Table 1). In contrast, several loci show Hardy–Weinberg and linkage disequilibrium at Scott Reef, which together with a bimodal admixture distribution (Fig. 2b) indicates some level of non-random mating at this reef (Jiggins and Mallet, 2000). Behavioural isolation (e.g. size-assortative mating, different breeding season) might be the most plausible pre-mating barrier in these species given that they overlap in habitat use.

4.2. Asymmetry of introgression

IM estimates of migration rates following divergence were consistently higher from *A. laevis* into *A. fuscus* than in the opposite direction (Table A1; Fig. 4a), and the best fitting IM models had unequal but non-zero migration rates (full data set) or migration from *A. laevis* into *A. fuscus* but not from *A. fuscus* into *A. laevis* (reduced data set). Such asymmetrical hybridisation has been documented in numerous taxa, and is often thought to reflect differential fitness of hybrids or unequal ability to backcross with parental species (e.g. Broyles, 2002). Future studies comparing the ecology and reproductive behaviour of parental species and hybrids would be useful to understand the influence of selection on hybridisation dynamics in these sea snakes.

At present, the best explanation for asymmetrical introgression into *A. fuscus* from *A. laevis* following their divergence is the proportionally higher abundance of the latter species. In species that lack strong reproductive barriers, hybridisation rates are expected to increase when species abundances are unbalanced, with unequal introgression of genes into the rarer species (Hubbs, 1955; Borge et al., 2005; Lepais et al., 2009). This is because mate-searching males of the rarer species are more likely to encounter heterospecific females (in the context of the present study taxa), and F1 hybrids are likewise more likely to mate with the more abundant species, producing backcrossed individuals that will be genetically closer to that species. Surveys of sea snake abundance in the Timor Sea between 1992 and 2013 reported much higher numbers of *A. laevis* compared to *A. fuscus* at all surveyed reefs (Guinea, 1993, 2007, 2012b; Lukoschek et al., 2013). Consistent with these field studies, our IM effective population size parameters were much higher for *A. laevis* than *A. fuscus* (Table A1; Fig. 4b). Such unequal abundance of parental species probably explains admixture proportions at Scott Reef, where most (17 of 23) hybrids have more

than 50% admixture in the *A. laevis* cluster, indicating that this species mates more frequently with hybrids creating an unequal distribution of *A. laevis* backcrosses.

4.3. Natural or anthropogenic hybridisation?

Collapse of reproductive barriers in natural populations is usually associated with habitat modification and/or reduced population densities, especially where these disrupt assortative mating and weaken selection against hybrids (reviewed in Seehausen et al., 2008). In the present study, an extremely high frequency of hybrids was found just at Hibernia, and this reef is situated only ~30 km from Ashmore where sea snakes have declined so dramatically that all nine previously resident species have disappeared within the last ~15 years (Guinea, 2012a,b; Lukoschek et al., 2013). This might implicate recent environmental or demographic change in driving a breakdown of reproductive barriers at Hibernia. However, coral and other habitats at the Ashmore Marine Reserve have remained intact and the reef is not subjected to intensive fishing (Skewes et al., 1999; Guinea, 2012b; Lukoschek et al., 2013). Hibernia, although not a marine protected area, has also maintained high coral cover and diversity (Skewes et al., 1999) but has lost at least two *Aipysurus* species (*A. apraefrontalis* and *A. foliosquama*) over the same timeframe. In contrast, the southern Timor Sea reefs (Scott and Seringapatam) have maintained stable sea snake populations (Guinea, 2012a,b; Lukoschek et al., 2013) despite a severe coral bleaching event in 1998 that caused >76% loss of coral cover (Skewes et al., 1999; Smith et al., 2008). Proximity to Ashmore might nevertheless be important if *A. fuscus* at Hibernia previously relied on external recruitment of migrants from that larger neighbouring reef; historical tissue samples would be useful to test this hypothesis.

Natural processes might also explain the collapse of species boundaries between *A. laevis* and *A. fuscus*. The small size of Hibernia (11.5 km^2) might simply not support high enough sea snake densities to maintain ecologically and genetically distinct populations of both species: *A. fuscus* (but not *A. laevis*) is absent on two other small reefs in the region, Cartier Islet (10.8 km^2) and Seringapatam (55.2 km^2). Alternatively, the two species might have only recently come into secondary contact at Hibernia so that there has been less time for them to establish isolating mechanisms (e.g. assortative mating) via reinforcement compared to at Scott or Ashmore reefs. However, recent contact at Hibernia seems unlikely in light of the directionality of introgression revealed by IM model fitting and hybrid admixture proportions. When a species invades an area already occupied by a close relative, the invading species is expected to be initially rare so that it experiences high rates of hybridisation with the local species; as the invading population grows it retains introgressed genes that then increase in frequency (Barton and Hewitt, 1989; Currat et al., 2008; but see also Scascitelli et al., 2010). *A. fuscus* is endemic to the Timor Sea reefs, so that widespread *A. laevis* is more likely to be the invading species in this scenario. Hence, if secondary contact explained our data, we would expect biased introgression of *A. fuscus* alleles into *A. laevis*. On the contrary, we find much higher introgression in the opposite direction, from *A. laevis* into *A. fuscus*, which is less consistent with a recent invasion of *A. laevis* at Hibernia.

5. Conclusions and conservation implications

Our results show that endangered dusky sea snakes (*A. fuscus*) frequently hybridise with closely related olive sea snakes (*A. laevis*) throughout their range in the Timor Sea. For the neutral nuclear markers sampled, introgression following species

Table A1
Peak estimates and 90% highest posterior distributions (90% HPD) of the six parameters in the full Isolation with Migration model fit to 8 microsatellite markers. Effective population sizes of *A. fuscus*, *A. laevis* and their common ancestor are q_1, q_2 and q_A, respectively. Migration rate (movement of genes) from *A. laevis* to *A. fuscus* is m_1, and from *A. fuscus* to *A. laevis* is m_2. The divergence time parameter is *ut*. These parameter estimates were not converted to actual demographic quantities due to uncertainty in mutation rates for the loci used.

Run	Parameter estimates						Reduced data (n = 18 with Q > 0.75 from Scott Reef only)				
	Full data (n = 38 with Q > 0.75 from all reefs)										
	q_1 (90% HPD)	q_2 (90% HPD)	q_A (90% HPD)	m_1 (90% HPD)	m_2 (90% HPD)	*ut* (90% HPD)	q_1 (90% HPD)	q_2 (90% HPD)	q_A (90% HPD)	m_1 (90% HPD)	m_2 (90% HPD)	*ut* (90% HPD)
1	2.34	6.64	21.78	1.10	0.125	7.495	0.76	7.60 (0.83–19.03)	14.59	1.46	0.015	0.062
	(0.72–5.14)	(3.67–11.13)	(3.78–95.50)	(0.065–3.77)	(0.005–1.075)	(3.015–9.99)	(0.28–3.38)		(0.83–59.42)	(0.025–8.515)	(0.005–1.94)	(0.017–4.99)
2	2.25	6.97	13.33	1.11	0.025	5.585	0.84	7.82 (0.72–20.69)	12.71	1.64	0.005	0.172
	(0.98–5.48)	(3.78–10.91)	(5.76–96.26)	(0.005–3.085)	(0.005–1.265)	(0.285–9.99)	(0.36–3.15)		(1.72–51.43)	(0.005–8.09)	(0.005–1.88)	(0.03–4.99)
3	2.42	6.86	15.19	1.23	0.005 (0.005–0.905)	3.83	0.84	6.71 (0.39–20.03)	15.03	0.86	0.005	0.067
	(0.98–4.63)	(4.00–11.35)	(0.16–90.89)	(0.005–3.725)		(1.24–9.96)	(0.28–3.23)		(2.38–43.11)	(0.005–7.57)	(0.005–2.89)	(0.007–4.99)
4	2.59	6.64	18.70	0.89	0.265	5.85	0.84	8.60 (3.38–17.92)	12.71	2.05	0.025	0.055
	(1.15–5.22)	(3.89–10.37)	(4.99–95.59)	(0.125–2.975)	(0.005–0.94)	(2.76–9.99)	(0.35–3.07)		(0.17–77.73)	(0.51–8.37)	(0.005–1.43)	(0.025–4.99)
5	1.99	7.08	17.39	1.06	0.035	3.82	0.92	7.38 (0.61–16.81)	13.82	1.24	0.005	0.047
	(0.81–4.80)	(4.00–11.57)	(4.11–93.30)	(0.075–3.975)	(0.005–0.945)	(2.02–9.99)	(0.28–3.55)		(2.78–50.99)	(0.005–7.57)	(0.005–2.28)	(0.012–4.99)
Mean	2.32	6.84	17.28	1.08	0.091	5.32	0.84	7.62	13.78	1.45	0.011	0.081

divergence is strongly asymmetrical from widespread and locally abundant *A. laevis* into endangered *A. fuscus*. Moreover, most hybrids closely resemble *A. laevis* in phenotype regardless of their admixture proportions, indicating that introgression also erodes the distinctiveness of the species. These results have important implications for the conservation status of *A. fuscus*. Particularly concerning is the apparent collapse of species boundaries at Hibernia, where ~95% of sampled snakes were hybrids and none had pure *A. fuscus* ancestry. Given that Hibernia is situated only ~30 km from Ashmore, it is tempting to link the dramatic extinction of all sea snakes from Ashmore to the failure of reproductive barriers between *A. fuscus* and *A. laevis* at Hibernia. However, it is unclear what environmental or demographic factors might have increased hybridisation rates at Hibernia, and whether these have anthropogenic or natural causes. Unfortunately a lack of an adequate temporal series of specimens or DNA tissues from Hibernia prevents analysis of historical changes in hybridisation dynamics. Nonetheless, future studies should aim to understand the mating preferences and selection pressures that balance inter-specific gene flow at Scott Reef. Detailed studies are also needed to examine morphological and ecological differences among the two parental species and the hybrid population at Hibernia. In addition to providing valuable information for species conservation, such studies would shed light on the evolutionary processes that have shaped a global hotspot of marine snake diversity.

Acknowledgements

We thank PTTEP Australasia for funding survey trips to the Timor Sea reefs in 2012 and 2013. We are also grateful to survey team members T. Schroeder, D. Wright, S. Oughton, J. Crowe-Riddell, N. Nath, C. Malam, D. Guinea, A. Raith and C. Giuliano; also skipper J. Ralston and the crew of the Kimberley Quest II. Additional funds for travel and laboratory work were provided by an Australian Research Council grant to K.L. Sanders and a School of Conservation Research Fund 2013 to A.R. Rasmussen. The Australian Genomics Research Facility in Adelaide provided all laboratory analyses. Finally, we thank the Department of Environment and Conservation, Western Australia, and DSEWPaC in Canberra for granting us permission to carry out field research on sea snakes.

Appendix A

See Table A1.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.01.013. These data include Google maps of the most important areas described in this article.

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