Vol. 533: 237–244, 2015
doi: 10.3354/meps11354

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published August 6

Whole mitogenome sequencing refines population structure of the Critically Endangered sawfish *Pristis pristis*

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ABSTRACT: The largetooth sawfish *Pristis pristis* (Linnaeus, 1758) is a highly threatened euryhaline elasmobranch that in recent times has undergone a significant range contraction. It now only remains in a few areas, with northern Australia being the main stronghold. Previous work using a single mitochondrial gene approach suggested the existence of regional barriers to gene flow in northern Australia. In this study, whole mitochondrial sequences of 92 *P. pristis* from 7 river drainages across northern Australia were used to refine the population structure. This approach revealed barriers to gene flow at a scale as fine as between adjacent river drainages. Except for those flowing into the Gulf of Carpentaria, all river drainages appeared to host a genetically distinct population. The apparent genetic homogeneity in the Gulf is probably due to freshwater connectivity between river drainages, either during the last glaciation event when the Gulf was a freshwater lake or through contemporary wet season flooding. These results suggest that each river drainage across the species' range should be considered a discrete management unit unless there is evidence of freshwater connectivity. More broadly, the improved resolution of population structure obtained with whole mitogenome analysis compared to single mitochondrial gene approaches suggests that female reproductive philopatry may have been overlooked in previous studies of some elasmobranch species.

KEY WORDS: Population genetics · Elasmobranch · Philopatry · Dispersal · Control region · D-loop

INTRODUCTION

The largetooth (or freshwater) sawfish *Pristis pristis* (Linnaeus, 1758) was once widespread, but is now listed as Critically Endangered on the IUCN Red List of Threatened Species, due to population declines and extirpation from large parts of its former distribution (Kyne et al. 2013). Significant range contractions have occurred in the tropical Eastern Atlantic, Western Atlantic, Eastern Pacific and Indo-West Pacific Oceans, and the species is now only found in a limited number of core population areas (Peverell 2005,

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Publisher: Inter-Research · www.int-res.com

Morgan et al. 2011, Kyne et al. 2013, Dulvy et al. 2014, Moore 2014). The distribution of *P. pristis* overlaps with areas of intense human activity in coastal zones, and the species has suffered from both unregulated and regulated fishing, and habitat modification and loss (with threats persisting across its range) (Peverell 2005, Morgan et al. 2011, Kyne et al. 2013). *P. pristis* reaches at least 6.5 m total length (TL) (Compagno & Last 1999), has a late age at maturity (8–10 yr) (Thorson 1982, Peverell 2008), is estimated to live to 44 yr (Tanaka 1991) and have 1–13 young every 1 or 2 yr (Thorson 1976, Peverell 2008). These life history traits limit productivity and restrict the ability of this species to recover from depletion (Dulvy et al. 2014).

International trade in *P. pristis* is regulated through the species' listing on the Convention on International Trade in Endangered Species (CITES) Appendix I. *P. pristis* is also protected in many countries (Dulvy et al. 2014), including across its Australian range, where it is listed as Vulnerable in Australia's Environment Protection and Biodiversity Conservation Act. Northern Australia is a key remaining population centre of global significance for *P. pristis* (Peverell 2005, Morgan et al. 2011, Kyne et al. 2013, Dulvy et al. 2014). Much of northern Australia remains remote and sparsely inhabited, although pressure is mounting to develop the region (The Parliament of the Commonwealth of Australia 2014), which has the potential to degrade natural systems, including key *P. pristis* habitats (Chin et al. 2012).

P. pristis is a euryhaline elasmobranch, with life-cycle stages that encompass freshwater, estuarine and marine waters; juveniles are born in estuaries and move into river systems, including upstream reaches and floodplain waterholes, where they are believed to spend some 4–5 yr before moving back to the sea (Thorburn et al. 2007, Peverell 2008, Whitty et al. 2009, Kyne et al. 2013). Adults are elusive and their rarity has rendered them difficult to sample for scientific research. However, samples of juveniles are more easily collected in river drainages, and genetic analyses from different river drainages (nurseries) can inform population structure and management strategies if admixture is limited.

Mitochondrial DNA (mtDNA) markers are maternally inherited and thus reflect female behaviour, which is particularly useful to detect female reproductive philopatry, as is often exhibited by elasmobranch species (Hueter et al. 2005). Different phylogeographic signals can be observed using nuclear DNA instead of mtDNA if males display a reproductive behaviour which is in contrast to females. In a recent study, Phillips et al. (2011) provided the first population genetic analyses of each of the Indo-West Pacific *Pristis* species and found evidence of female reproductive philopatry in *P. pristis*. They analysed a portion of the mitochondrial control region (CR) or d-loop across 149 individuals from northern Australia and demonstrated the existence of several barriers to gene flow at a broad scale. The *P. pristis* CR study revealed a low genetic diversity, with >80% of the individuals harbouring 1 of the 2 main haplotypes (Phillips et al. 2011).

The power to detect population structure directly depends on the variability of the genetic marker(s) analysed (Ryman et al. 2006), as the typical way to test for population differentiation is to assay 1 or more loci and to test the null hypothesis of identical allele frequencies. Thus, finer-scale resolution of population structuring can be missed if an appropriate marker (i.e. one exhibiting enough variability) is not analysed (Feutry et al. 2014). Shamblin et al. (2012) and Feutry et al. (2014) demonstrated that the use of whole mitogenome sequencing can greatly increase the resolution of matrilineal genetic structure patterns compared to single gene approaches, especially in groups where mitochondrial gene evolutionary rates are low, such as elasmobranchs (Martin et al. 1992).

In the present study, we apply whole mitogenome sequencing to investigate patterns of matrilineal gene flow in *P. pristis* amongst northern Australian rivers. Phillips et al. (2011) noted limitations in both sample size and the number of locations sampled, which hindered the examination of fine-scale patterns of variation (e.g. that study had only 8 individuals sampled from the Northern Territory [NT]). The main objective is to further refine the population boundaries discovered by Phillips et al. (2011) for this Critically Endangered species, which is crucial information for conservation and recovery planning.

MATERIALS AND METHODS

Tissue samples from 92 juvenile *Pristis pristis* were collected between 2009 and 2013 from 7 major river drainages across their northern Australian range, from the Kimberley region of Western Australia (WA) to the eastern Gulf of Carpentaria in Queensland (QLD). From west to east, samples were obtained from the Fitzroy River (n = 20) in WA; the Daly River (n = 21), Adelaide River (n = 13) and South Alligator River (n = 5) in the NT; and the Norman River (n = 11), Gilbert River (n = 11) and Mitchell River (n = 11)

in QLD (Fig. 1). DNA was extracted from fin clips or biopsies using DNeasy Blood & Tissue kits (Qiagen).

The *P. pristis* mitogenomes were amplified in 2 overlapping fragments using 2 new primer pairs: PM6405F: 5'-TAG ACG TAG ACA CTC GAG CC-3' and PM15026R: 5'-TGA GTA GGG AGG TTA GGA CAA G-3'; and PM14570F: 5'-TGG CTG ACT CAT CCG AAA C-3' and PM6829R: 5'-TAG GGT GTA GGC GTC TGG-3'. To design the primers, we first aligned the mitogenomes of narrow sawfish *Anoxypristis cuspidata* (Chen et al. in press) and dwarf sawfish *Pristis clavata* (Feutry et al. 2015). The primers were then designed to sit in conserved regions of the resulting alignment. PCR mix was 1 unit of Takara LA *Taq*, 2 µl of 10× LA PCR Buffer II (Mg^{2+}-free), 2 µl of 25 mM $MgCl_2$, 3.2 µl dNTP (2.5 mM each), 0.5 µl of each primer at 10 mM, and 10.6 µl of sterilised distilled water. PCR conditions followed the manufacturer's recommendations: initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 35 s and 68°C for 10 min, with a final extension step at 72°C for 10 min. After being purified with Agencourt AMPure XP magnetic beads (Beckman Coulter) and quantified with the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific), the PCR products for each individual were pooled at equimolar concentrations. Individual libraries were then prepared with Nextera XT DNA Sample Preparation kits (Illumina) and sequenced on a Miseq (Illumina) following the manufacturer's protocol.

Read trimming and filtering and mitogenome assemblies were conducted in GENEIOUS PRO (v. 7.0.6) (Biomatters). For each read, 5 and 3 ends, as well as regions with >5% chance of an error were trimmed. Sequences shorter than 150 bp after trimming were discarded as well as sequences with >15 low-quality bases. Remaining reads for each individual were mapped onto a reference sequence (P. Feutry et al. unpubl. data) using the custom sensitivity tool with the following parameters: Maximum gaps per read = 10; Word length = 24; Ignore word repeated more than 8 times; Maximum mismatches per read = 5%; Maximum gap size = 3; Minimum overlap identity = 95%; Index word length = 14; and, Map multiple best matches randomly. After removal of PCR duplicates using the rmdup SAMTools toolkit (v. 1.0.0) (Li et al. 2009), the majority rule consensus (>50% of reads for any single nucleotide polymorphism, insertion, deletion) for each fish was calculated. Consensus sequences were then aligned using the MUSCLE alignment tool with default parameters in GENEIOUS PRO. A 106–318 bp section starting at position 15 632 was removed from alignment and subsequent analyses due to the presence of a repetitive region resulting in alignment uncertainties.

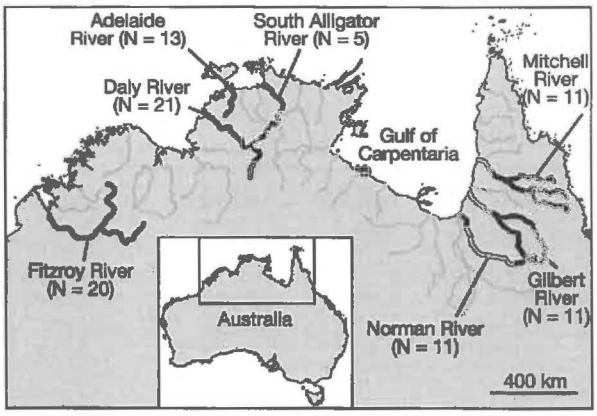

Fig. 1. Locations and number of samples of *Pristis pristis* obtained from northern Australian river drainages

Seven genetic diversity indices were calculated for northern Australia (i.e. all 7 river drainages combined) and for each river separately using DNASP (v. 5.10.01) (Librado & Rozas 2009). These were the nucleotide diversity index π (Nei 1987), number of variable sites (Watterson 1975), number of singleton sites, number of haplotypes and the haplotype diversity index H_d (Nei 1987). Tajima's D (Tajima 1983) and Fu's F_s (Fu 1997) statistics were also calculated to infer the demographic history of *P. pristis*.

Analyses of molecular variance (AMOVA) were conducted with ARLEQUIN to test for the existence of structure among animals from different river drainages, and the pairwise fixation index (Φ_{ST}) was calculated to investigate the genetic differentiation between the 7 river drainages (Fig. 1). The model of nucleotide evolution used for the AMOVA and to calculate Φ_{ST} values was Tamura-Nei (Tamura & Nei 1993). AMOVA and Φ_{ST} values were calculated for the whole mitogenome sequences and also on the same 352 bp portion of the CR used by Phillips et al. (2011) for comparative purposes.

A Mantel's test (Mantel 1967) was conducted to test for isolation by distance (IBD). The approximate shortest distances through marine waters between the mouth of each of the 7 rivers were estimated using Google Earth (v. 7.1.2.2041) and tested against the pairwise Φ_{ST} values. The Mantel's test was performed using the online version of GENEPOP (v. 4.2) (Raymond & Rousset 1995) with 1000 permutations. Prior to the test, Φ_{ST} were converted to $\Phi_{ST}/(1 - \Phi_{ST})$, and geographic distances were log-transformed (Rousset 1997).

RESULTS

All mitogenomes were successfully assembled, with an average sequencing coverage ranging from 105× to over 7500×, ensuring highly reliable sequences (Sequence Read Archive accession nos. SRR1732112 to SRR1732203). A total of 106 variable sites (positions can be found in Table S1 in the Supplement at www.int-res.com/articles/suppl/m533p237_supp.xls) and 2 insertions were observed across the 92 *Pristis pristis* mitogenomes, defining 22 haplotypes. The number of haplotypes per river drainage ranged from 2 to 7, and the overall nucleotide diversity was 0.0012. The Daly River exhibited the highest nucleotide diversity (π = 0.0065), whereas the Fitzroy, Adelaide, Norman, Gilbert and Mitchell Rivers all had nucleotide diversity indices below 0.0005 (Table 1).

The AMOVA uncovered a high level of genetic differentiation among the different populations (AMOVA Φ_{ST} = 0.71099, $p < 0.00001$). Pairwise fixation indexes indicated the presence of barriers to gene flow between all drainages in the NT as well as barriers to gene flow between samples from WA, NT and QLD, but not between drainages within the Gulf of Carpentaria (Table 2). The differentiation between populations was due to both differences in haplotype frequencies and the presence of population-specific haplotypes (Fig. 2). The same analysis carried out on the 352 bp portion of the CR used by Phillips et al. (2011) also indicated a high level of genetic differentiation among sites (AMOVA Φ_{ST} = 0.68752, $p < 0.00001$). The pairwise fixation indexes based on this portion of the CR revealed fewer and/or weaker barriers to gene flow than using the whole mitogenome sequences (Table 2). The genetic differentiation found using this portion of the CR was only due to differences in haplotype frequencies, as there were no population-specific haplotypes. In fact, in our samples, this portion of the CR only exhibited 2 different haplotypes: the main 2 found by Phillips et al. (2011).

There was no evidence of IBD; the correlation between Φ_{ST} and geographic distance estimates was not significant for *P. pristis* across the 7 river drainages considered (R^2 = 0.13728, p = 0.22700).

Table 1. Genetic diversity indices for northern Australian *Pristis pristis* across all sites and for each of the 7 river drainages sampled. π: nucleotide diversity index, H_d: haplotype diversity index

Diversity index	All sites (N = 92)	Fitzroy River (N = 20)	Daly River (N = 21)	Adelaide River (N = 13)	South Alligator River (N = 5)	Norman River (N = 11)	Gilbert River (N = 11)	Mitchell River (N = 11)
π	0.0011	0.0002	0.0065	0.0003	0.0015	0.0001	0.0003	0.0001
Variable sites	104	15	33	36	43	4	27	5
Singleton sites	15	3	4	36	3	3	2	2
Haplotypes	22	7	4	2	3	5	6	6
H_d	0.919	0.579	0.652	0.154	0.700	0.709	0.891	0.855
Tajima's *D*	−0.2534	−0.7549	0.7813	−2.3120	1.6634	−0.8342	−1.7006	−0.4036
Significance	p > 0.10	p > 0.10	p > 0.10	p < 0.001	p > 0.10	p > 0.10	p > 0.05	p > 0.10
Fu's F_s	0.3168	0.1516	1.0749	−3.2378	1.7993	−1.2580	−2.1895	−0.1841
Significance	p > 0.10	p > 0.10	p > 0.10	p < 0.02	p > 0.05	p > 0.10	p > 0.05	p > 0.10

Table 2. Pairwise fixation index (Φ_{ST}) for northern Australian *Pristis pristis* population comparisons using whole mitogenome (upper matrix) and 352 bp control region portion (lower matrix). ns: not significant (p > 0.05), *p < 0.05, **p < 0.01, ***p < 0.001 after false discovery rate (FDR) adjustments (Benjamini & Hochberg 1995)

Population	Fitzroy River (N = 20)	Daly River (N = 21)	Adelaide River (N = 13)	South Alligator River (N = 5)	Norman River (N = 11)	Gilbert River (N = 11)	Mitchell River (N = 11)
Fitzroy River		0.654***	0.873***	0.755***	0.738***	0.620***	0.727***
Daly River	−0.037 (ns)		0.753***	0.464**	0.621***	0.532***	0.617***
Adelaide River	0.723***	0.798***		0.729***	0.894***	0.833***	0.888***
South Alligator River	0.332 (ns)	0.471*	0.203 (ns)		0.721***	0.620***	0.712***
Norman River	0.802***	0.870***	−0.014 (ns)	0.441 (ns)		0.010 (ns)	−0.034 (ns)
Gilbert River	0.701***	0.782***	−0.090 (ns)	0.146 (ns)	0.000 (ns)		−0.022 (ns)
Mitchell River	0.802***	0.870***	−0.014 (ns)	0.441 (ns)	0.000 (ns)	0.000 (ns)	

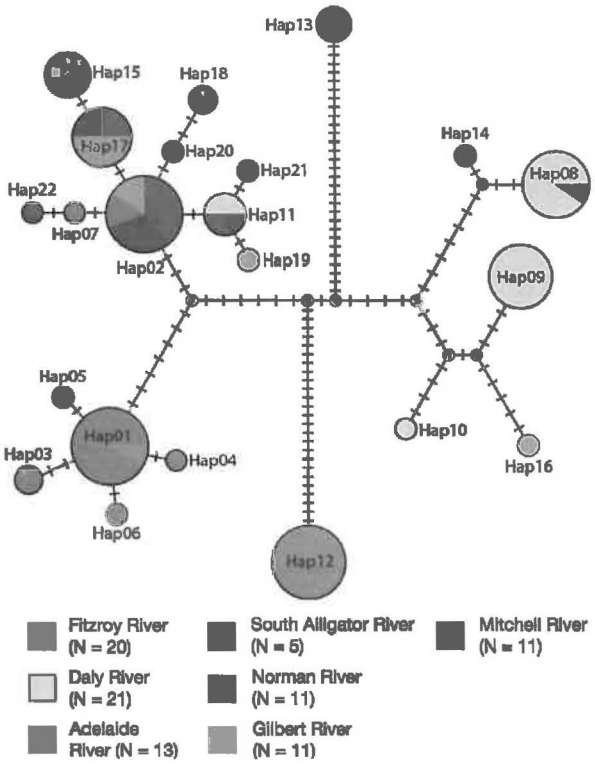

Fig. 2. Median-joining haplotype network inferred from whole mitochondrial sequences of northern Australian *Pristis pristis*. A circle depicts each haplotype, with size proportional to the number of copies found in this study. Each hash line represents 1 variable site between haplotypes

DISCUSSION

While Phillips et al. (2011) demonstrated the existence of population structure among *Pristis pristis* at a regional scale, with samples from the west coast (WA) appearing to belong to a different population than samples from the north coast and the Gulf of Carpentaria (NT/QLD), the present study showed that genetic heterogeneity can occur at a scale as fine as between 2 adjacent river drainages. The mitogenomic study of *P. pristis* thus uncovered significant levels of population structure across northern Australia, which was previously unrevealed. This was made possible by the combined use of new samples from rivers in the NT and the analysis of whole mitochondrial sequences instead of only a portion of the CR. While the use of the same CR portion as Phillips et al. (2011) along with additional samples from the NT uncovered some new barriers to gene flow (e.g. between the Daly, or the Fitzroy, and the Adelaide Rivers), it was the use of the whole mitogenome data which was necessary to uncover all barriers reported in this study (Table 2).

The phylogeographic history of *P. pristis* in northern Australia revealed in this study presents some similarities to those of freshwater teleost species. Indeed, Huey et al. (2014) showed that within a variety of teleost species from this region, genetic differentiation tended to be low between river drainages within the Gulf of Carpentaria compared to genetic differentiation between river drainages outside of the Gulf. In northern Australian freshwater fishes, biogeographic history is believed to have played a major role in shaping current genetic diversity. Briefly (see Huey et al. 2014 for more details), depending on sea level, the Gulf of Carpentaria alternated between being a brackish gulf and a freshwater lake (Lake Carpentaria). Given the life history of *P. pristis* (adults are marine and juveniles use river systems as nursery areas), populations from rivers in this region may be extirpated each time the Gulf turns into a freshwater lake. Following the last inundation by salt water ~10 000 yr ago, females moving across northern Australia may have colonised river drainages flowing into the Gulf of Carpentaria, thereby starting a new population. Alternatively, a population of *P. pristis* may have persisted in Lake Carpentaria and connected the different rivers flowing into the lake. This hypothesis is plausible, as according to Astorqui (1967) and Thorson (1982), *P. pristis* appears to be able to complete its entire life cycle in the freshwater Lake Nicaragua. Unfortunately, verification of this hypothesis can not be tested, as the Lake Nicaragua population was fished to near extinction (McDavitt 2002). It is also supported by the absence of evidence of recent population expansion in those rivers as revealed by Tajima's and Fu's indices.

Another possible explanation to the absence of any apparent barriers to matrilineal gene flow between the 3 river drainages sampled in the Gulf of Carpentaria is topography. In the southeastern Gulf of Carpentaria where these rivers are located, the coast is flat, and during the wet season, the area is a large continuous floodplain (Turnadge et al. 2013). In contrast, the sampled river drainages of WA and NT are separated by non-inundated woodland and rocky escarpment country, preventing admixture of these populations during the wet season. In the southeastern Gulf of Carpentaria, Peverell (2008) demonstrated a >200 km movement of a sub-adult *P. pristis* (247 cm TL) between different river drainages, highlighting the extent to which individuals are capable of moving. Additional samples from WA, the NT and within the northern and western Gulf of Carpentaria are needed to fully comprehend gene flow in the northern Australian range.

IBD is the consequence of limited dispersal across space (i.e. pairs of populations close to each other are more genetically similar than populations farther away because individuals are less likely to travel longer distances and not because of any selective pressures acting on those populations). The absence of IBD patterns in *P. pristis* is not surprising, as Φ_{ST} are either very low between river drainages in the Gulf of Carpentaria or high between all other river drainages, even adjacent ones. The data from south-eastern Gulf of Carpentaria rivers is consistent with freshwater teleost fish from across the entire Gulf (Huey et al. 2014), suggesting that the mitochondrial genetic diversity observed in *P. pristis* across northern Australia was shaped by the biogeographic history of the region rather than very limited (reproductive) dispersal of females as suggested by Phillips et al. (2011).

This study is the first to use whole mitogenome sequencing to investigate genetic structure in batoids and only the second in elasmobranchs (sharks and batoids) (Feutry et al. 2014). In both studies, this method greatly improved the resolution of matrilineal population structure and showed a very high fidelity for reproductive sites in females (Feutry et al. 2014). As noted by Phillips et al. (2011), when only the CR is considered, levels of genetic structure in *P. pristis* are not higher than in other elasmobranch species. In the speartooth shark *Glyphis glyphis*, the analysis of the CR only provided no evidence of population structure (Feutry et al. 2014), and yet, in both this species and *P. pristis*, very fine-scale levels of population structure were revealed through whole mitogenome sequencing. This raises the question: have previous studies underestimated the degree of female reproductive site fidelity in elasmobranchs and overestimated the size of management units using single gene approaches?

P. pristis and *G. glyphis* have unique life histories compared to most elasmobranchs, given their use of fresh or brackish waters as nursery areas (Thorburn et al. 2007, Pillans et al. 2010, Whitty et al. 2009). Although this could explain the higher degree of genetic structure found in those species compared to fully marine species (Phillips et al. 2011), parentage analysis recently provided direct evidence of female lemon shark *Negaprion brevirostris* returning to the same marine site to pup year after year (Feldheim et al. 2014). Also, a number of shark-tagging and long-term recapture studies have showed females returning to the same marine nursery areas to pup (Pratt & Carrier 2001, Sims et al. 2001, Feldheim et al. 2002). These elements suggest that the resolution of matrilineal population structure could probably be improved in many other elasmobranch species using whole mitogenome sequencing and that females may return to the exact same site to reproduce in more species than suggested by previous genetic studies using single genes.

For species of conservation concern and for which the current understanding of the boundaries of management units are based on single gene studies, we strongly recommend a reassessment of population boundaries using whole mitogenome sequencing. For example, in a study of dusky shark *Carcharhinus obscurus*, spottail shark *Carcharhinus sorrah*, blue shark *Prionace glauca* and scalloped hammerhead *Sphyrna lewini* using neutral genetic markers (CR sequences and allelic variation at co-dominant microsatellite loci), population subdivision between northern Australia and Indonesia was only found in *C. sorrah* (Ovenden et al. 2009). The use of whole mitogenome sequencing may help to further refine population structure resolution between countries that potentially harvest the same stock or at even finer scales. A previous study of the bull shark *Carcharhinus leucas*, a euryhaline species which uses rivers as nursery areas, using single gene sequencing also uncovered the existence of barriers to gene flow at a broad scale in northern Australia (Tillett et al. 2012). Whole mitogenome sequencing might reveal matrilineal population structure boundaries occurring at the river drainage scale as seen in other euryhaline elasmobranch species in this region (*P. pristis*: the present study; *G. glyphis*: Feutry et al. 2014). Sampling should be undertaken in nursery areas, ideally with neonate catches to ensure they were born in this area, or on females just after parturition to ensure genetic data reflect differences among nursery areas and not adult movements (Keeney et al. 2003). However, the genetic composition of samples of juveniles caught close to their presumed birthplace could be biased by the occurrence of siblings. Without nuclear markers to test for kinship, the possibility of such sampling bias cannot be rejected. In the present study, a sampling regime spanning several years should have limited this bias. Furthermore, the haplotype diversity could also be biased by the relatively small sample size (Sinclair & Hobbs 2009) inherent to a threatened and rare species and the results presented here should be considered tentative.

Northern Australia represents one of the few remaining population centres for *P. pristis* from a once widespread occurrence (Kyne et al. 2013, Dulvy et al. 2014). Population structure analysis has revealed that each river drainage, which acts as critical juvenile

habitat, should be considered as a separate management unit throughout the species' range, unless there is evidence of freshwater connectivity between river drainages. Furthermore, the likelihood of population recovery as a result of emigration is low due to the high degree of female reproductive philopatry that would limit recruitment from alternative sources. In geographic regions where *P. pristis* is likely to be extinct (large parts of its former range, e.g. West Africa; see Dulvy et al. 2014), re-establishment of viable populations would therefore be nearly impossible. Further research should sample the entire range of *P. pristis* to investigate links with northern Australia. Additionally, future genetic studies on this species should include the analysis of nuclear markers in order to test for kinship among samples and estimate patterns of gene flow in males.

Acknowledgements. Thanks to Stirling Peverell, Nicole Phillips and Jenny Ovenden for access to samples. We also thank Nicole Phillips for providing details on the sequencing strategy used in a previous study of *Pristis pristis*, Gareth Elvidge for advice on the use of the Illumina Miseq and Rasanthi Gunasekera for help in the laboratory. We appreciate the support of Murdoch University's Freshwater Fish Group, Team Sawfish, in particular Jeff Whitty, and the Nyikina-Mangala Rangers (WA) and the Malak Malak Rangers (NT), in particular Rob Lindsay. Thanks to the Traditional Owners and staff of Kakadu National Park, especially Anne O'Dea and Khan Spokes, to Northern Territory Fisheries, in particular Grant Johnson, Mark Grubert and Thor Saunders, and to Kate Buckley (Charles Darwin University) and the Territory Wildlife Park for field and sample collection assistance. This study was supported by the Marine Biodiversity Hub, a collaborative partnership supported through funding from the Australian Government's National Environmental Research Program (NERP). P.F. was partly supported by the North Australia Marine Research Alliance (NAMRA). P.M.K. was partly supported by the NERP Northern Australia Hub. NT samples were collected under NT Fisheries Special Permit S17/3252, Kakadu National Park Research Permit RK805, and Charles Darwin University Animal Ethics Committee A11041.

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Editorial responsibility: Philippe Borsa, Montpellier, France

Submitted: January 5, 2015; Accepted: May 15, 2015
Proofs received from author(s): July 26, 2015