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# Mitochondrial DNA supports the identification of two endangered river sharks (*Glyphis glyphis* and *Glyphis garricki*) across northern Australia

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**Abstract.** The river sharks (genus *Glyphis*) are a small group of poorly known sharks occurring in tropical rivers and estuarine waters across northern Australia, south-east Asia and the subcontinent. The taxonomy of the genus has long been unclear due to very few individuals having been caught and examined, resulting in a paucity of data regarding their distribution, biology and ecology. Only recently has attention focussed on the two Australian species, *G. glyphis* and *G. garricki*. This study is a result of a rare opportunity to collate the few samples that have been collected from these species and the bull shark *Carcharhinus leucas*, which shares an overlapping range. These samples were analysed using the DNA barcoding approach (*cox1* mitochondrial gene), compared with six other species of carcharhinids and evaluated in light of the current taxonomic classification. Nine species-specific nucleotide differences were found between *G. glyphis* and *G. garricki* and no intra-specific variation provides strong support for the separation into distinct species. Significant differences were also observed at the inter-generic level, with *Glyphis* forming a distinct clade from *Carcharhinus*. This study provides the basis for future molecular studies required to better address conservation issues confronting *G. glyphis* and *G. garricki* in Australia.

**Additional keywords:** cytochrome oxidase, northern river shark, speartooth shark.

## Introduction

Global concerns regarding the status of shark species and populations have been raised in response to reports of declining numbers, increased fishing pressure and habitat degradation (Fowler *et al*. 2005; Aires-da-Silva and Gallucci 2007). These reports are in light of the typical life histories of sharks that are long-lived, have a late age-at-first-breeding, and have long gestation periods and low fecundities (Fowler *et al*. 2005; Powter and Gladstone 2008). Conservation efforts are often hampered by a lack of base-line information, especially for a group whose marine and/or estuarine distributions make them difficult to study. Molecular genetic markers such as mitochondrial DNA (mtDNA) have been widely used to study marine organisms and are useful for stock/population identification crucial for conservation management programs (Ovenden 1990). The Barcode of Life Database (BOLD; www.boldsystems.org, verified 2 May 2009) is a repository of short standardised DNA sequences that aid in species identification for a wide range of taxa. The DNA barcoding technique is adopted in this study to confirm the presence of two endangered species of river shark in northern Australia and investigate the relationship between them.

The river sharks from the genus *Glyphis* are a small group of poorly known sharks with an apparent fragmented distribution across northern Australia, south-east Asia and the Asian subcontinent. The river sharks are distinctive in that they can occur in coastal waters, in the estuarine waters of tropical river mouths and in river systems well inland from the coast (Thorburn *et al*. 2003; Peverell *et al*. 2006). However, beyond the few individuals captured from the numerous sites sampled throughout the region, limited information is available pertaining to the distribution, abundance, morphology, reproduction and ecology of all species within the group. Indeed, until 2008, formal descriptions were

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only available for half the species recorded, with uncertainty as to the exact number of species in the genus, and what species name should be assigned to each population. This lack of information is in part due to the limited number of sites where these sharks have been found and the difficulty in catching individuals in remote areas. As a consequence, there has been a paucity of specimens, with some being lost, which had made further taxonomic evaluation and research difficult (Fowler 1997; Compagno and Niem 1998; Compagno *et al*. 2005; Fowler *et al*. 2005; Martin 2005).

Six possible species had been identified: *Glyphis glyphis* (speartooth shark from Papua New Guinea and possibly the Bay of Bengal), *G. gangeticus* (Ganges River shark from India and possibly Pakistan), *G. siamensis* (from Myanmar), *G.* sp. A (Bizant River shark from northern Australia), *G.* sp. B (Borneo River shark from Borneo) and *G.* sp. C (the northern river shark from northern Australia and New Guinea). Compagno *et al*. (2005) noted that the level of similarity between *G. glyphis* and *G.* sp. A were such that they were possibly conspecific. Recently, this proposition was confirmed when specimens of *G.* sp. A from New Guinea and northern Australia were compared with the holotype for *G. glyphis* (Compagno *et al*. 2008). Compagno *et al*. (2008) provided the first formal description of *G.* sp. C, describing the species as *G. garricki*. The present study focuses on the two species reported from Australia (*G. glyphis* and *G. garricki*) and uses the species names given by Compagno *et al*. (2008).

The distribution of *G. glyphis* in Australia has been reported to extend from the Bizant and Wenlock Rivers (east and west coasts of Cape York, Queensland) and the East Alligator, South Alligator and Adelaide Rivers in the Northern Territory (Peverell *et al*. 2006; Ward and Larson 2006*a*; Compagno *et al*. 2008). Recent photographs of *G. glyphis* in the Ord River, Western Australia, suggest that its distribution may extend further west (Thorburn 2006). *Glyphis garricki* has been reported from waters of the Northern Territory (East Alligator, South Alligator and Adelaide Rivers and tentatively from the Daly River) and Western Australia (Doctors Creek near Derby, King Sound and Cambridge Gulf) (Thorburn and Morgan 2004; Ward and Larson 2006*b*; Compagno *et al*. 2008). The accurate distributional limits of each species within Australia are not known, but the 'Top End' of the Northern Territory appears to be where the two species overlap. Furthermore, the Wenlock River population of *G. glyphis* (from the west coast of Cape York) differs in pectoral fin colour from the Northern Territory specimens (H. Larson and S. Peverell, unpubl. data), but the significance of this is unknown. Both species have also been reported from rivers in New Guinea (Compagno *et al*. 2008).

Although both species appear to be relatively uncommon, juveniles can be locally abundant in estuarine creeks (Thorburn and Morgan 2004; Peverell *et al*. 2006; Field *et al*. 2008; Museum and Art Gallery of the Northern Territory records). There are reports of adult *Glyphis* (2–3 m total length (TL)) being found along the coast away from rivers, from Finke Bay (Van Diemen Gulf) and off the Wessel Islands (J. D. Stevens and D. Lindner, pers. comm.). Adult *Glyphis* (2 m TL or greater) are known from the Northern Shark Fishery in the Northern Territory, but their numbers and species are unknown due to confusion with the more abundant bull shark *Carcharhinus leucas* (R. Buckworth and C. Tarca, pers. comm.).

The concern regarding the conservation status of *Glyphis* in Australia is due to their apparently restricted habitats as indicated by the limited number of sites from where they have been reported, low population sizes, whether these are increasing or decreasing, and pressure from line and net fishing (Pogonoski *et al*. 2002; Ward and Larson 2006*a*, 2006*b*; Field *et al*. 2008). The 2007 IUCN Red List recognised four species, with *G. glyphis* listed as Endangered, and *G. gangeticus*, *G.* sp. A and *G.* sp. C (*G. garricki*) listed as Critically Endangered (IUCN 2007). *Glyphis* sp. B is not listed, probably because it is currently known from only one specimen collected from Borneo more than 100 years ago (Compagno and Niem 1998; Pogonoski *et al*. 2002; Fowler *et al*. 2005). These listings are likely to be revised upon recognition of *G. glyphis* and *G.* sp. A as conspecific. In Australia, the *Environmental Protection and Biodiversity Conservation* (EPBC) *Act* 1999 lists *G.* sp. A (*G. glyphis*) as Critically Endangered and *G.* sp. C (*G. garricki*) as Endangered.

While the listing of *Glyphis* under the EPBC Act 1999 means that these species cannot be targeted for commercial exploitation in Commonwealth waters, capture and retention by commercial fishers working in state waters (nearshore and rivers), such as those associated with the *C. leucas* fishery or for the purpose of crab-pot bait, is covered by an exemption (S. Matthews, pers. comm.). A review of their level of protection in State waters (where they come under more than one Act), subsequent increase in awareness of the morphological differences between *Glyphis* and *C. leucas*, along with clarification of species identity and geographical distribution would be highly beneficial for the conservation of these species.

The aim of this study was to identify genetic differences that may occur between Australian populations of *G. glyphis* and *G. garricki*, and to determine whether such differences support the current separation of these species based on morphological characters alone. 'DNA barcoding' is a technique based on mitochondrial cytochrome oxidase I gene (*cox1*) sequences and was employed in this study because it is highly discriminating in the identification of marine fauna (Ward and Holmes 2007; Moura *et al*. 2008; Ward *et al*. 2008). We also aimed to explore the level of intra-specific mtDNA diversity to gain a preliminary insight into possible population substructure across the wide geographic range of each species. Such information may assist in providing a basis upon which species-specific management plans can be drafted and implemented.

## Materials and methods

Tissue samples from 29 specimens of *G. glyphis*, 13 *G. garricki*, 7 *C. leucas* and 3 'unknown' specimens (tentatively identified as *Glyphis*), all held in separate collections throughout northern Australia (Northern Territory Department of Primary Industries and Fisheries Management, the Museum and Art Gallery of the Northern Territory, the Queensland Department of Primary Industries and Fisheries, the Queensland Museum, the Western Australian Museum or Centre for Fish & Fisheries Research and Murdoch University), were utilised for the current study. Samples were either preserved in 95% or 100% ethanol, 20% salt-saturated DMSO or were frozen. They included 30 voucher specimens (Table 1, Fig. 1) and were collected from individuals identified in the field using morphological characters to

**Table 1. Details of carcharhinid samples used in this study**
Specimens retained as vouchers in museum collections are indicated by the catalogue number (NTM, Museum and Art Gallery of the Northern Territory; WAM, Western Australian Museum; QM, Queensland Museum)

| Species | State | River system | No. samples | Sample label | Catalogue no. |
|---|---|---|---|---|---|
| *Glyphis glyphis* | NT | Murganella Creek | 1 | GL1 | NTMS.15122–001 |
| | NT | West Alligator | 3 | GL2, GL46, GL47 | NTMS.15299–001 |
| | NT | East Alligator | 2 | GL3, GL6 | NTMS.15301–001, NTMS.15303–001 |
| | NT | Adelaide | 6 | GL5, GL7, GL8, GL10, GL11, GL12 | NTMS.15305–001, NTMS.15304–001, NTMS.15351–001, NTMS.16255–001, NTMS.16262–001, NTMS.16258–001 |
| | Qld | Wenlock | 16 | GL9, GL23–36, GL38 | NTMS.16217–001, QMI.36880-QMI.36885 |
| | Qld | Ducie | 1 | GL37 | |
| *Glyphis garricki* | NT | East Alligator | 1 | GL4 | NTMS.15302–001 |
| | NT | West Alligator | 2 | GL42–43 | |
| | WA | King Sound | 10 | GL13–18, GL22, GL39–41 | WAM03001–03006, WAM02001 |
| | WA | Unknown | 2 | GL794-GL795[A] | BW-A3734, BW-A3733 (Ward *et al*. 2008) |
| 'Unknown' *Glyphis* | NT | West Alligator | 1 | GL44 | |
| | NT | South Alligator | 1 | GL45 | |
| | NT | Unknown – frozen specimen | 1 | GL48 | |
| *Carcharhinus leucas* | NT | East Alligator | 1 | CL1 | NTMS.16284–001 |
| | NT | South Alligator | 1 | CL2 | NTMS.15161–006 |
| | NT | Daly | 2 | CL3–4 | NTMS.16165–002 |
| | WA | Unknown | 3 | CL19–21 | |

[A]Sequences obtained from GenBank (EU398794 and EU398795).

distinguish each species (Table 2; Thorburn and Morgan 2004; Compagno *et al*. 2008).

Total genomic DNA was extracted from a small section of macerated tissue from these samples using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for 'Animal Tissue'. DNA quality was assessed following electrophoresis through 1% agarose stained with ethidium bromide and visualised under UV light.

The cytochrome oxidase subunit I gene (*cox1*) was amplified using a combination of the primers FishF1, FishF2, FishR1 and FishR2 as described in Ward *et al*. (2005). The PCR reaction mix was 50 μL, containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 U Taq polymerase (Genesearch, Arundel, Qld, Australia), 0.1 mM of each dNTP (Astral Scientific, Sydney, NSW, Australia) and 0.4 μM each of the forward and reverse primers. PCR conditions were 95°C for 2 min, 35 cycles of (94°C for 30 s, 54°C for 30 s, 72°C for 1 min), 72°C for 10 min, 4°C hold. PCR products were separated by electrophoresis through 1% agarose at 100 V for 30 min. The major band at ~650 base pairs was excised with a scalpel blade and purified using a QIAquick Gel Extraction kit (Qiagen). The quantity of purified PCR product was estimated by gel electrophoresis (80 V for 60 min) and comparison with a Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA). Sequencing reactions were conducted with the BigDye Terminator Cycle Sequencing system (Applied Biosystems, Foster City, CA, USA) using the FishF1 and FishR1 primers. Sequencing conditions were 94°C for 5 min, 30 cycles of (96°C for 10 s, 50°C for 5 s, 60°C for 4 min), and the reactions were separated on a 3130 Genetic Analyser (Applied Biosystems) following manufacturer's instructions.

Raw data were analysed using ABI Prism GeneScan Analysis Software Version 3.1 (Applied Biosystems), and evaluated and aligned using Clustal W (Thompson *et al*. 1994) within MacVector with Assembler (2007). Sequence and phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al*. 2004). Two *G. garricki* sequences from Ward *et al*. (2008) were included for analysis (GenBank accession, label used in analysis: EU398794, GL794 and EU398795, GL795), as were sequences from additional taxa including: *Carcharhinus dussumieri* (DQ108301, 305 – CD1&2), *C. leucas* (EF609311 – CL5), *Carcharhinus obscurus* (DQ108291, 306 – CO1&2), *Carcharhinus sorrah* (DQ108292, 295 – CS1&2), *Carcharhinus tilstoni* (DQ108283, 298 – CT1&2), *Negaprion acutidens* (DQ108284 – NA1) and *Prionace glauca* (DQ108285, 288 – PG1&2) (Ward *et al*. 2005; Ward and Holmes 2007).

The control region (CR) was amplified from a subset of the *G. glyphis* and *G. garricki*. The PCR reaction and sequencing conditions were as described above, but the primers used were a combination of GWF, GWR and 470R2 (Pardini *et al*. 2001).

## Results

### *Cytochrome oxidase 1 (*cox1*)*

DNA was successfully extracted and sequence data obtained from 29 *G. glyphis*, 13 *G. garricki*, 3 *Glyphis* sp. 'unknown' and 7 *C. leucas* samples. Raw sequences of slightly different lengths were aligned, and the ends cropped resulting in 602 base pairs available for analysis. These sequences were aligned with additional GenBank sequences from *G. garricki*, four additional

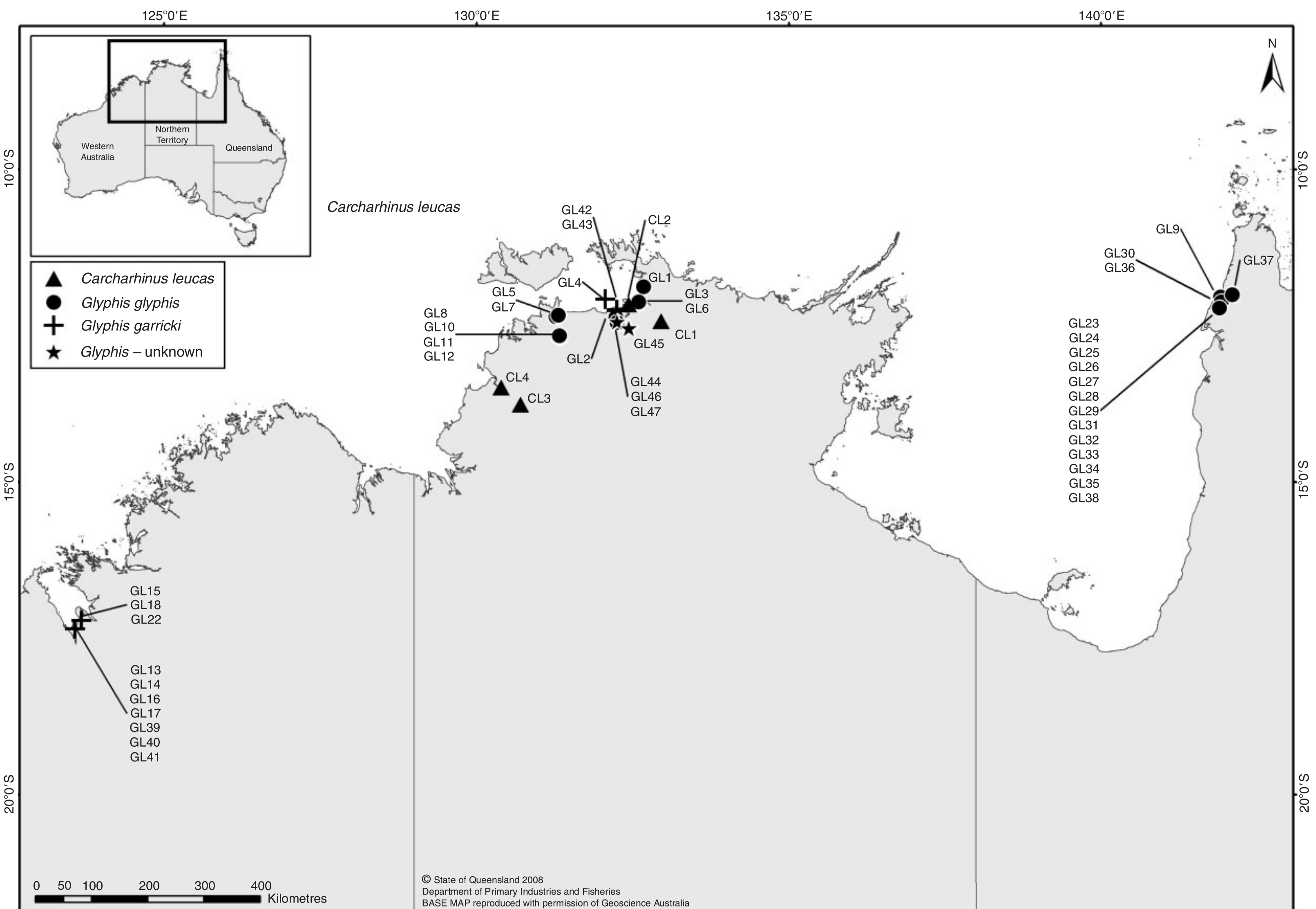


**Fig. 1.** Map of northern Australia showing distribution of samples used in this study.

**Table 2. The distinguishing features of the three carcharhinid species used to differentiate individuals in the field for this study**

| Character | *Carcharhinus leucas* (bull shark) | *Glyphis glyphis* (speartooth shark) | *Glyphis garricki* (northern river shark) |
|---|---|---|---|
| Position of anteriormost edge of upper lip | Anteriormost edge of upper lip below centre of eye | Anteriormost edge of upper lip over or just forward of anterior margin of eye | Anteriormost edge of upper lip well (about an eye diameter) forward of anterior margin of eye |
| Snout shape in lateral view | Snout short, robust and rounded | Snout somewhat long, slightly flattened | Snout short, somewhat fleshy |
| Pectoral fin markings | Fin with blackish tip in juveniles, adults with dusky grey to faintly dusky tip on underside of fin; other fins may have dark tips | Pectoral fin not distinctly darker at ventral tip; other fins without dark tips | Pectoral-fin tip black ventrally; other fins usually without dark tips |
| Lower jaw tooth shape | Teeth stout, triangular, serrated | Teeth slender, smooth, upright | Teeth slender, smooth, upright |
| Closest distance from mouth to nostril | ~2 times width between nostrils | 1.8–2.1 times width between nostrils | ~1.5 times width between nostrils |
| Dorsal/ventral markings bordering eye | Grey dorsal colour running through bottom of eye | Grey dorsal colour bordering bottom of eye | Grey dorsal colour extending more than eye diameter below eye |

**Table 3. Pairwise differences between taxa, with the number of differences below divide and the p-distance above (expressed as a percentage)**

GLA, *Glyphis glyphis*; GLC, *Glyphis garricki*; CL, *Carcharhinus leucas*; CO, *Carcharhinus obscurus*; CS, *Carcharhinus sorrah*; CT, *Carcharhinus tilstoni*; CD, *Carcharhinus dussumieri*; PG, *Prionace glauca*; NA, *Negaprion acutidens*

| | GLA | GLC | CL | CO | CS | CT[A] | CD | PG | NA[A] |
|---|---|---|---|---|---|---|---|---|---|
| GLA | – | 1.5 | 6.8 | 7.1 | 8.1 | 7.6 | 8.6 | 8.1 | 8.7 |
| GLC | 9 | – | 7.6 | 7.5 | 8.8 | 7.9 | 8.8 | 8.5 | 9.2 |
| CL | 41 | 46 | – | 4.0 | 5.8 | 4.7 | 4.8 | 6.1 | 7.4 |
| CO | 43 | 45 | 24 | – | 4.8 | 5.2 | 3.8 | 3.8 | 7.4 |
| CS | 49 | 53 | 35 | 29 | – | 5.6 | 6.0 | 6.5 | 8.4 |
| CT | 45.5 | 47.5 | 28.5 | 31.5 | 33.5 | – | 6.6 | 5.7 | 8.0 |
| CD | 52 | 53 | 29 | 23 | 36 | 39.5 | – | 5.8 | 7.9 |
| PG | 49 | 51 | 37 | 23 | 39 | 34.5 | 35 | – | 7.9 |
| NA | 52.5 | 55.5 | 44.5 | 44.5 | 50.5 | 48 | 47.5 | 47.5 | – |

[A] A single mutation was observed between the two sequences in these groups, while no intra-specific variation was detected in all other species.

*Carcharhinus* species, *N. acutidens* and *P. glauca*, bringing the total number of sequences analysed to 66. Overall, there were 499 conserved sites, 103 variable sites and 79 parsimony informative sites.

Nine species-specific nucleotide differences were observed between *G. glyphis* and *G. garricki*, and a significant number was found between *C. leucas* and these species (41 and 46, respectively) (Table 3). No sequence variation was observed within each of these three species. The sequences included from GenBank for *G. garricki* and *C. leucas* were found to be the same as those obtained from these species in this study (Fig. 2). Representative sequences from each of the species obtained from this study have been submitted to GenBank (accession EU818708–710). Due to the invariant nature of this gene at the intra-specific level, it was found to be unsuitable for the investigation of potential genetic differentiation between populations of *Glyphis*.

The utility of the DNA barcoding approach was highlighted with the inclusion of the three 'unknown' specimens from the Northern Territory that were tentatively identified as *Glyphis* but had not been identified further (Table 1). The individual from the West Alligator River was identified as *G. glyphis* (GL44), while the individual from the South Alligator River was identified as *G. garricki* (GL45) (Fig. 2). The sequence from the third sample of unknown origin (GL48) showed 52 and 55 nucleotide differences between those of *G. glyphis* and *G. garricki*, respectively. A search using BLAST (www.ncbi.nlm.nih.gov/blast, verified 2 May 2009) showed that the closest sequence was that of *N. acutidens* (lemon shark) with only one nucleotide difference between them (Fig. 2).

Overall, there were significant nucleotide differences observed between all taxa included in analysis, with little or no intra-taxon variation (Table 3). The intra-genus difference observed for *Glyphis* was far less than that observed for *Carcharhinus* (Fig. 2).

### Control region (CR)

Two regions of the CR were amplified and sequenced, with the larger fragment using primers GWF and GWR (~1000 base

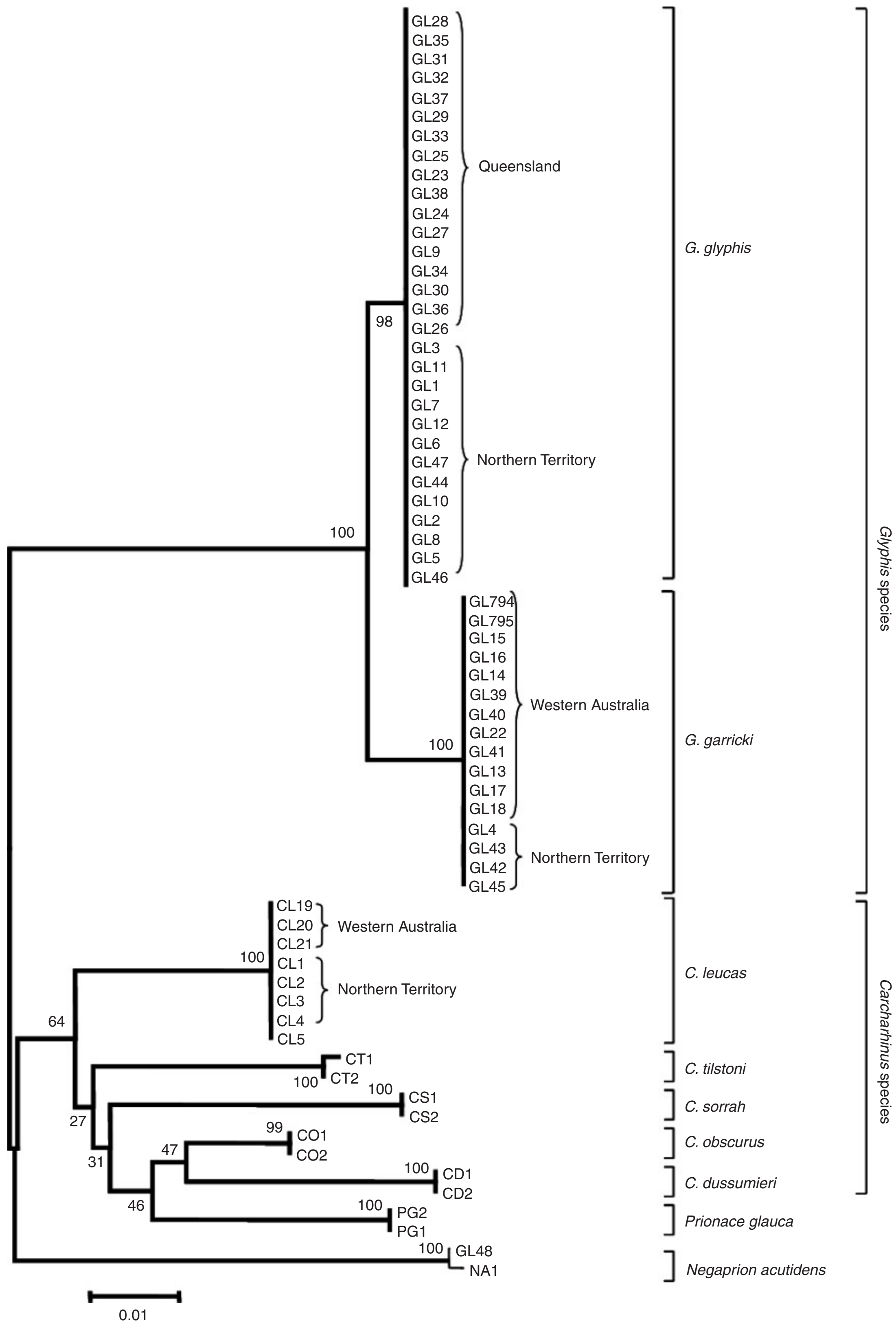


**Fig. 2.** Neighbour joining tree using Kimura 2-parameter distance, 10 000 bootstrap replications.

pairs) and the smaller fragment using GWF and the internal reverse primer 470R2 (~500 base pairs). Four samples were chosen to trial the primers and determine whether the 500-base pair fragment would be sufficient to detect genetic variation within the *Glyphis* species. The four samples represented the extremes of the geographic range of both species: *G. glyphis* (GL5 from the Northern Territory and GL25 from Queensland) and *G. garricki* (GL4 from the Northern Territory and GL13 from Western Australia).

The larger fragment did not amplify in GL4 and GL13, possibly as a result of a mutation in the binding site for the GWR primer for *G. garricki*. The larger fragments from GL5 and GL25 were sequenced, giving a truncated sequence length of 934 base pairs. No variable sites were detected.

The smaller fragment amplified in all four samples, and these were sequenced. The truncated aligned sequence was 421 base pairs, and 12 of these were variable between *G. glyphis* and *G. garricki*. There was no intra-specific variation observed for either species. The CR sequences obtained from each species have been submitted to GenBank (accession numbers FJ460518 and FJ460519).

## Discussion

The DNA barcoding gene employed in this study showed strong support for the separation of *Glyphis glyphis* and *G. garricki* as distinct species. The nine species-specific nucleotide differences detected across 602 base pairs of *cox1* were far fewer than were detected between any of the *Carcharhinus* species examined in this study. This suggests a much closer relationship within the *Glyphis* genus than seen within *Carcharhinus*. Such clear differences between species allowed unambiguous identification of three unknown specimens included in the study. Two of the unknown *Glyphis* specimens were identified to species, and the third unknown was identified as an unrelated species. Examination of this specimen by one of the authors (H. Larson) confirmed the original misidentification, as its morphological characters agreed with that of a lemon shark (*N. acutidens*). The *cox1* gene was therefore found to be an excellent marker for distinguishing *Glyphis* species and can be effectively used to support identifications made in the field, especially where confusion with *C. leucas*, *N. acutidens* or other species may occur.

The genetic differences observed between *G. glyphis* and *G. garricki* confirm the reports of their overlapping distribution, with both detected in the East and West Alligator Rivers in this study. Indeed, one *G. glyphis* individual was caught within metres of two *G. garricki* individuals in the same trip indicating that these species not only occur within the same river systems, but may also have overlapping niches. This also raises ecological questions about potential competition and possible hybridisation. While the data obtained in this study do not support introgression with the absence of shared haplotypes between the *Glyphis* species, the inclusion of nuclear markers alongside a more thorough sampling of these river systems would assist in addressing this issue.

To strengthen the findings of this study regarding the taxonomy of the Australian *Glyphis* species, it would be beneficial to incorporate samples of these species collected from populations identified from New Guinea and the Bay of Bengal. Cryptic species have been identified in at least one shark species using molecular techniques (Quattro *et al*. 2006; Ward *et al*. 2008) and sampling across the geographic ranges of both species would be useful to explore this possibility as the large distances between river systems may foster segregation to such an extent as to result in speciation. The addition of samples from other *Glyphis* species would also serve to reinforce the taxonomic findings to date. These would include samples from *G. gangeticus* from India, *G. siamensis* from Myanmar and *G.* sp. B from Borneo. While the logistics of obtaining such samples may be prohibitive, specimens identified in the course of any field research in these regions should be sampled and stored for future molecular-based studies.

A striking feature of the *cox1* sequence data was that no variation was observed between individuals within each of the species investigated. While this enhances the ability to identify species, this molecular marker appears unsuitable for addressing questions at the intra-specific level. A preliminary investigation into the utility of the mitochondrial CR for exploring population subdivision within *G. garricki* and *G. glyphis* was undertaken using several individuals from the extremes of the sampled range. The CR has often been demonstrated to be hyper-variable and has been used in a range of diverse marine and riverine taxa to address population genetic questions (e.g. Zane *et al*. 2006; Castro *et al*. 2007; Mabuchi *et al*. 2008). In this study, however, the intra-specific sequences were found to be invariant, and this was not pursued further at this time. While the CR has shown significant variation within several shark species (e.g. Pardini *et al*. 2001; Keeney *et al*. 2003; Quattro *et al*. 2006), only five polymorphisms identifying six haplotypes were detected over 1085 base pairs in the basking shark (Hoelzel *et al*. 2006). As such, while only a handful of *Glyphis* samples were sequenced albeit from the extremes of their range in Australia, to detect possible variation within the CR requires the entire region to be sequenced incorporating more samples from both species.

An absence of genetic variation was also noted between the populations of *G. glyphis*, which showed morphological differences in the Wenlock River population compared with individuals found in the Northern Territory. The significance of marked differences in pectoral fin colouration was not known; however, such differences were not reflected in the genetic data. Similarly, Thorburn and Morgan (2004) reported that the presence of fused vertebrae and spinal deformation was not uncommon in a Western Australian population of *G. garricki* and suggested that this might be indicative of a small gene pool. The present genetic data do not reflect any differentiation between geographically distant populations of this species, and to explore whether such morphological differences are reflected as genetic variation would require the screening of additional, more variable markers.

The absence of intra-specific variation may be a result of panmixia. Conversely, significant population subdivision may well exist across the geographical range, but was simply not detected using the *cox1* and CR markers. Such a scenario is possible if insufficient time has lapsed in relation to the mutation rate of the marker. Given the paucity of data pertaining to the reproduction and ecology of both *Glyphis* species, it is difficult to address either hypothesis adequately. Based on limited data and

comparison to similar species, *G. garricki* and *G. glyphis* are thought to be viviparous, with females giving birth to live young (Stevens *et al*. 2005). The local abundance of *Glyphis* juveniles reported in rivers and the absence of adults suggests that it is possible that these species adopt a similar strategy to *C. leucas*, whereby juveniles utilise riverine or nearshore waters before migration to marine waters to mature and breed (Thorburn 2006). If female sharks show philopatry to particular river systems, then population divergence might be expected to occur and could be reflected in mitochondrial genes. However, if the females show little or no philopatry then the migration to, and breeding in, marine waters where greater mixing of adults might occur, could act to reduce or negate population subdivision. To address whether panmixia is possible across such broad geographical distances, more information regarding adult movements is required (e.g. through tagging studies) before any conclusions can be drawn.

The lack of intra-specific variation was also observed for *C. leucas* across a similar geographic range to *G. garricki*, as samples from both Western Australia and the Northern Territory were included. The invariant nature of the *cox1* gene for the three species of interest in this study suggests that the marker is uninformative rather than being indicative of an absence of population subdivision *per se*. While mtDNA has been found to be variable within several shark species, including the Australian gummy shark (*Mustelus antarcticus*), sandbar shark (*C. plumbeus*), shortfin mako shark (*Isurus oxyrinchus*), sharpnose shark (*Rhizoprionodon terraenovae*) and deepwater sharks (*Centrophorus* and *Centroscymnus* species; Heist *et al*. 1995, 1996*a*, 1996*b*; Gardner and Ward 1998; Moura *et al*. 2008), evidence for population subdivision was lacking in all but the gummy shark. This was not the case for hammerhead shark (Genus *Sphyrna*) populations where *cox1* and CR markers showed evidence of cryptic speciation (Quattro *et al*. 2006).

This study provided the first investigation into the distribution of *Glyphis* species across northern Australia and confirms the identity of the two species reported to occur here. Future studies will require more variable markers (e.g. alternative mitochondrial genes and nuclear genes, such as ribosomal ITS regions and/or microsatellite loci) to better explore the genetic variation within these species. An increase in the sample sizes of populations and broader sampling across the geographic ranges will serve as a solid basis to better address the future conservation issues confronting *G. glyphis* and *G. garricki* in Australia.

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## References

Aires-da-Silva, A. M., and Gallucci, V. F. (2007). Demographic and risk analyses applied to management and conservation of the blue shark (*Prionace glauca*) in the North Atlantic Ocean. *Marine and Freshwater Research* **58**, 570–580. doi:10.1071/MF06156

Castro, A. L. F., Stewart, B. S., Wilson, S. G., Hueter, R. E., Meekan, M. G., *et al.* (2007). Population genetic structure of Earth's largest fish, the whale shark (*Rhincodon typus*). *Molecular Ecology* **16**, 5183–5192.

Compagno, L. J. V., and Niem, V. H. (1998). Family Carcharhinidae. In 'The Living Marine Resources of the Western Central Pacific FAO Species Identification Guide for Fisheries Purposes Volume 2'. (Eds K. E. Carpenter and V. H. Niem.) pp. 1212–1360. (FAO: Rome.)

Compagno, L. G. V., Dando, M., and Fowler, S. (2005). 'A Field Guide to the Sharks of the World.' (Harper Collins Publishing Ltd: London.)

Compagno, L. J. V., White, W. T., and Last, P. R. (2008). *Glyphis garricki* sp. nov., a new species of river shark (Carcharhiniformes: Carcharhinidae) from northern Australia and Papua New Guinea, with a redescription of *Glyphis glyphis* (Müller & Henle, 1839). In 'Descriptions of New Australian Chondrichthyans'. (Eds P. R. Last, W. T. White and J. J. Pogonoski.) pp. 203–225. (CSIRO Marine and Atmospheric Research: Canberra.)

Field, I. C., Charters, R., Buckworth, R. C., Meekan, M. G., and Bradshaw, C. J. A. (2008). Distribution and Abundance of *Glyphis* and Sawfishes in Northern Australia and their Potential Interactions with Commercial Fisheries. Department of Environment and Heritage, Canberra.

Fowler, S. (1997). River shark discovered in Sabah. *Shark News: Newsletter of the IUCN Shark Specialist Group* **9**, 11.

Fowler, S. L., Cavanagh, R. D., Camhi, M., Burgess, G. H., Cailliet, G. M., *et al.* (2005). 'Sharks, Rays and Chimaeras: The Status of the Chondrichthyan Fishes.' (IUCN Species Survival Commission Shark Specialist Group, IUCN: Gland and Cambridge.)

IUCN (2007). '2007 IUCN Red List of Threatened Species.' Available at http://www.iucnredlist.org [Accessed 24 March 2008].

Gardner, M. G., and Ward, R. D. (1998). Population structure of the Australian gummy shark (*Mustelus antarcticus* Günther) inferred from allozymes, mitochondrial DNA and vertebra counts. *Marine and Freshwater Research* **49**, 733–745. doi:10.1071/MF98009

Heist, E. J., Graves, J. E., and Musick, J. A. (1995). Population genetics of the sandbar shark (*Carcharhinus plumbeus*) in the Gulf of Mexico and mid-Atlantic Bight. *Copeia* **1995**, 555–562. doi:10.2307/1446752

Heist, E. J., Musick, J. A., and Graves, J. E. (1996*a*). Genetic population structure of the shortfin mako (*Isurus oxyrinchus*) inferred from restriction fragment length polymorphism analysis of mitochondrial DNA. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 583–588. doi:10.1139/CJFAS-53-3-583

Heist, E. J., Musick, J. A., and Graves, J. E. (1996*b*). Mitochondrial DNA diversity and divergence among sharpnose sharks, *Rhizoprionodon terraenovae*, from the Gulf of Mexico and mid-Atlantic Bight. *Fishery Bulletin* **94**, 664–668.

Hoelzel, A. R., Shivji, M. A., Magnussen, J., and Francis, M. P. (2006). Low worldwide genetic diversity in the basking shark (*Cetorhinus maximus*). *Biology Letters* **2**, 639–642. doi:10.1098/RSBL.2006.0513

Keeney, D. B., Heupel, M., Heuter, R. E., and Heist, E. J. (2003). Genetic heterogeneity among blacktip shark, *Carcharhinus limbatus*, continental nurseries along the U.S. Atlantic and Gulf of Mexico. *Marine Biology* **143**, 1039–1046. doi:10.1007/S00227-003-1166-9

Kumar, S., Tamura, K., and Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**, 150–163. doi:10.1093/BIB/5.2.150

Mabuchi, K., Senou, H., and Nishida, M. (2008). Mitochondrial DNA analysis reveals cryptic large-scale invasion of non-native genotypes of common carp (*Cyprinus carpio*) in Japan. *Molecular Ecology* **17**, 796–809.

Martin, R. A. (2005). Conservation of freshwater and euryhaline elasmobranchs: a review. *Journal of the Marine Biological Association of the United Kingdom* **85**, 1049–1073. doi:10.1017/S0025315405012105

Moura, T., Silva, M. C., Figueiredo, I., Neves, A., Munoz, P. D., *et al.* (2008). Molecular barcoding of north-east Atlantic deep-water sharks: species

identification and application to fisheries management and conservation. *Marine and Freshwater Research* **59**, 214–223. doi:10.1071/MF07192

Ovenden, J. R. (1990). Mitochondrial DNA and marine stock assessment: a review. *Australian Journal of Marine and Freshwater Research* **41**, 835–853. doi:10.1071/MF9900835

Pardini, A. T., Jones, C. S., Noble, L. R., Kreiser, B., Malcolm, B., *et al.* (2001). Sex-biased dispersal of great white sharks. *Nature* **412**, 139–140. doi:10.1038/35084125

Peverell, S. C., McPherson, G. R., Garrett, R. N., and Gribble, N. A. (2006). New records of the river shark *Glyphis* (Carcharhinidae) reported from Cape York Peninsula, northern Australia. *Zootaxa* **1233**, 53–68.

Pogonoski, J. J., Pollard, D. A., and Paxton, J. R. (2002). Conservation Overview and Action Plan for Australian Threatened and Potentially Threatened Marine and Estuarine Fishes. Environment Australia, Canberra.

Powter, D. M., and Gladstone, W. (2008). Demographic analysis of the Port Jackson shark *Heterodontus portusjacksoni* in the coastal waters of eastern Australia. *Marine and Freshwater Research* **59**, 444–455. doi:10.1071/MF07096

Quattro, J. M., Stoner, D. S., Driggers, W. B., Anderson, C. A., Priede, K. A., *et al.* (2006). Genetic evidence of cryptic speciation within hammerhead sharks (Genus *Sphyrna*). *Marine Biology* **148**, 1143–1155. doi:10.1007/S00227-005-0151-X

Stevens, J. D., and Pillans, R. D., and Salini, J. P. (2005). Conservation Assessment of *Glyphis* sp. A (speartooth shark), *Glyphis* sp. C (northern river shark), *Pristis microdon* (freshwater sawfish) and *Pristis zijsron* (green sawfish). Department of Environment and Heritage, Canberra.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680. doi:10.1093/NAR/22.22.4673

Thorburn, D. C. (2006). Biology, ecology and trophic interactions of elasmobranchs and other fishes in riverine waters of Northern Australia. PhD Thesis, Murdoch University.

Thorburn, D. C., and Morgan, D. L. (2004). The northern river shark *Glyphis* sp. C (Carcharhinae) discovered in Western Australia. *Zootaxa* **685**, 1–8.

Thorburn, D. C., Peverell, S., Stevens, J. D., Last, P. R., and Rowland, A. J. (2003). Status of Freshwater and Estuarine Elasmobranchs in Northern Australia. Natural Heritage Trust, Canberra.

Ward, R. D., and Holmes, B. H. (2007). An analysis of nucleotide and amino acid variability in the barcode region of cytochrome oxidase I (*cox1*) in fishes. *Molecular Ecology Notes* **7**, 899–907. doi:10.1111/J.1471-8286.2007.01886.X

Ward, S., and Larson, H. K. (2006*a*). Threatened Species of the Northern Territory: Northern River Shark, *Glyphis* sp. C. Threatened Species Information Sheet. Northern Territory Government, Darwin.

Ward, S., and Larson, H. K. (2006*b*). Threatened Species of the Northern Territory: Speartooth Shark, Bizant River Shark, *Glyphis* sp. A. Threatened Species Information Sheet. Northern Territory Government, Darwin.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., and Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B* **360**, 1847–1857. doi:10.1098/RSTB.2005.1716

Ward, R. D., Holmes, B. H., White, W. T., and Last, P. R. (2008). DNA barcoding Australian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research* **59**, 57–71. doi:10.1071/MF07148

Zane, L., Marcato, S., Bargelloni, L., Bortolotto, E., Papetti, C., *et al.* (2006). Demographic history and population structure of the Antarctic silverfish *Pleuragramma antarcticum. Molecular Ecology* **15**, 4499–4511. doi:10.1111/J.1365-294X.2006.03105.X

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