Conserv Genet
DOI 10.1007/s10592-011-0298-6

RESEARCH ARTICLE

Detection of interspecies hybridisation in Chondrichthyes: hybrids and hybrid offspring between Australian (*Carcharhinus tilstoni*) and common (*C. limbatus*) blacktip shark found in an Australian fishery

Jess A. T. Morgan · Alastair V. Harry · David J. Welch · Raewyn Street · Jimmy White · Pascal T. Geraghty · William G. Macbeth · Andrew Tobin · Colin A. Simpfendorfer · Jennifer R. Ovenden

Received: 20 February 2011 / Accepted: 15 November 2011

Abstract Interspecies hybridisation in nature is a well-studied phenomenon, but it has not been analysed using genetic markers in the class Chondrichthyes (sharks, rays and chimeras). Two black-tip whaler shark species (Australian, *Carcharhinus tilstoni*; Common, *C. limbatus*) have overlapping distributions in Australia, distinct mitochondrial DNA sequence (ND4, COI, control region) and distinct morphological features such as length at sexual maturity, length at birth and number of vertebrae. A mismatch was observed between species identification using mtDNA sequence and species identification using morphological characters. To test whether hybridisation between the two species was responsible, a nuclear gene with species-specific mutations was sequenced. Extensive interspecies hybridisation was found to be occurring. Hybrids were found from five locations on the eastern Australian coastline, spanning 2,000 km. If hybrid fitness is low and hybrids are common, then fisheries recruitment may be overestimated and the productivity of the black-tip shark fishery may be well below that required to support commercial exploitation. To guard against identification errors, the likelihood of hybridisation and subsequent introgression should be assessed prior to using mtDNA (e.g. barcoding) to identify shark species. The *C. limbatus*–*C. tilstoni* species complex provides a unique opportunity to investigate the ability of sharks to adapt to environmental change, in particular, the impact of hybridization on species distributions which favour *C. tilstoni* along the north and *C. limbatus* along the south eastern Australian coastline.

Keywords Introgression · Hybrid fitness · Chondrichthyes · Barcoding · Mitochondrial DNA · Nuclear DNA

J. A. T. Morgan
Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD 4069, Australia

J. A. T. Morgan · R. Street · J. R. Ovenden (✉)
Molecular Fisheries Laboratory, Department of Employment, Economic Development and Innovation, Queensland Government, P.O. Box 6097, St Lucia, QLD 4069, Australia
e-mail: Jennifer.Ovenden@deedi.qld.gov.au

A. V. Harry · D. J. Welch · J. White · A. Tobin · C. A. Simpfendorfer
Fishing and Fisheries Research Centre, James Cook University, Townsville, QLD 4811, Australia

P. T. Geraghty · W. G. Macbeth
Department of Industry and Investment New South Wales Government, Cronulla Fisheries Research Centre, P.O. Box 21, Cronulla, NSW 2230, Australia

Introduction

Interspecies hybrids in nature are studied in detail because of their potential to illuminate the processes of speciation and adaptive evolution. Hybridisation can occur when incipient species come into contact following range expansion and is indicative that reciprocal reproductive isolation is incomplete (e.g. Mayr 1942). Arnold and Martin (2010) and Seehausen (2004) emphasize the role that hybridisation plays in evolutionary diversification. Hybrids of high fitness may readily adapt to environmental novelty and lead to new evolutionary lineages. Allendorf et al. (2001) recognised that hybridisation can be natural or anthropogenic and that the management of the latter is an enormous challenge to the conservation of biodiversity.

Published online: 17 December 2011

Hybridisation has been observed in viral, eukaryotic and prokaryotic groups and appears to be more common in plants than animals (Arnold 2006). Its documentation in animal taxa may reflect an observer bias toward closely related species that have divergent phenotypes (e.g. birds, cichlid fish) where hybrids are readily identifiable by eye. Hybridisation has not been observed in the class Chondrichthyes (sharks, rays and chimeras), possibly because their reproductive strategy is different to bony fishes. Bony fishes, which represent up to 95% of the modern fish species, generally produce millions of small gametes that are released into the water column where fertilisation occurs. Gamete and larval mortality is high due to factors such as predation and unfavourable environmental conditions. Chondrichthyans have internal fertilisation and lower numbers of offspring, but survival rates are much higher. Internal fertilization includes mate choice as an additional pre-zygotic barrier to hybridization that is absent from animals that have external fertilization. Male Chondrichthyes have modified pelvic fins (claspers) that allow sperm to be directly transferred to the female (Last and Stevens 2009).

The Australian (*C. tilstoni*, Whitley) and the Common (*C. limbatus*, Müller and Henle) black tip shark are morphologically similar species with overlapping distributions in northern Australian waters (Compagno et al. 2005; Last and Stevens 2009). The Australian species (*C. tilstoni*) is largely restricted to northern and eastern Australian waters (Last and Stevens 2009; Boomer et al. 2010), while the other (*C. limbatus*) is distributed worldwide in tropical and warm temperate seas (Last and Stevens 2009). Both are found inshore over continental shelves in mid-water or near the surface (Last and Stevens 2009). Phylogenetic analyses based on allozyme loci (Lavery 1992) showed that the two species form a closely related group with the Graceful shark (*C. amblyrhynchoides*), which was later confirmed by mitochondrial DNA (mtDNA) sequence data (Ward et al. 2008; Ovenden et al. 2010). Keeney and Heist (2006) studied mtDNA sequence variation in a worldwide sample of *C. limbatus* and grouped the sampling locations into two lineages (eastern Atlantic and Indo-Pacific). Based on the relative sequence divergence between the two lineages and the worldwide morphological variability of *C. limbatus*, Keeney and Heist (2006) suggested a taxonomic revision of *C. limbatus*, including the relative position of *C. tilstoni*.

The species status of the two species (*C. limbatus* and *C. tilstoni*) is confirmed by their evolutionary and morphological distinctiveness. Lavery and Shaklee (1991) found nearly fixed allelic differences at two allozyme loci between the two entities and showed that one entity was identical to *C. limbatus* from the type locality of that species (West Indies) and to *C. limbatus* from South Africa. Two phylogenetic analyses based on one (COI, Ward et al. 2008) and three (COI, control and ND4, Ovenden et al. 2010) mtDNA gene regions demonstrated separate evolutionary lineages per species. The targeted regions of mtDNA sequence contained one species-diagnostic single nucleotide polymorphism (SNP) in the control region (375 bp), two diagnostic SNPs in the COI gene (654 bp) and 10 diagnostic SNPs in the ND4 gene (873 bp) (Ovenden et al. 2010). Stevens and Wiley (1986) compared the two species in northern Australia and found that male *C. tilstoni* reached sexual maturity at smaller sizes compared to *C. limbatus* and that *C. tilstoni* had fewer precaudal vertebrae than *C. limbatus*. This was confirmed and extended by Harry (2011) who measured the stretched total length (STL) at birth, STL at reproductive stage and STL compared to male clasper length in 961 samples from the eastern Australian coast. Additionally, pre-caudal vertebral (PCV) counts were made from 224 specimens. The two species had largely non-overlapping PCV counts and could be separated by the life history characters measured.

The black tip sharks *C. tilstoni* and *C. limbatus* are a significant component of the Australian northern and eastern coast inshore shark fishery. During the course of investigating genetic stock structure on the eastern coast, several sharks were identified with clear mismatches between genetic and morphometric species-diagnostic characters. One hypothesis was that mismatches were due to hybridisation. A nuclear genetic marker was identified and applied to the mismatched animals, confirming our expectation. In this article we present the evidence for inter-species hybridisation involving *C. limbatus* and *C. tilstoni*, the spatial nature of the hybrid zone on the Australian eastern coast and preliminary data on the characteristics of interbreeding between the two species.

Materials and methods

Sample collection and DNA extraction

Carcharhinus tilstoni and *C. limbatus* were sampled along the eastern coast of Australia, from far north Queensland to northern New South Wales (Fig. 1). All samples were taken from the landed catch of the shark fishing sector. Using standardized datasheets, biological information was linked to samples taken for genetics. Approximately 200 mg of muscle tissue was dissected and preserved in 1 ml of NaCl saturated solution with 20% dimethylsulphoxide. Total genomic DNA was extracted from ten to 50 mg of muscle tissue using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Doncaster Victoria) into a final elution volume of 200 µl.

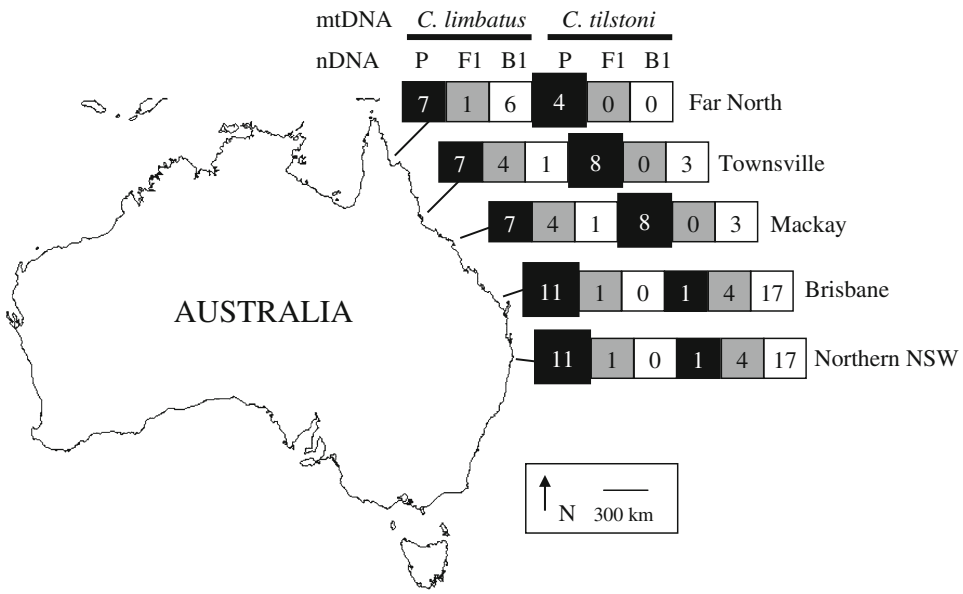

Fig. 1 Map showing the distribution of parental (P, *C. limbatus* or *C. tilstoni*) and different hybrid types (F1 first generation hybrid or B+ backcross hybrid, Table 3) along the east coast of Australia. The numbers do not represent the relative proportions of purebred or hybrid sharks at each location. *Enlarged boxes* indicate most abundant type based on ND4 mtDNA haplotype. Parental individuals had concordant nDNA, mtDNA ND4 and morphological characteristics (Table 2)

Mitochondrial DNA species identification

Species identifications were confirmed by a real-time high-resolution-melt PCR assay (Morgan et al. 2011). The assay targets species diagnostic mutations in the mtDNA NADH dehydrogenase subunit 4 (ND4) gene.

Species diagnostic nuclear DNA: development and screening

Diagnostic mutations in the flanking sequence of microsatellite locus CT06 (Ovenden et al. 2006) were identified by the direct sequencing of four homozygous individuals of each species. Once identified, 12 individuals per species with known PCV counts and matching ND4 genotypes were sequenced to validate species-diagnostic differences. Primers CT06F (CTGGCTGTCTCACTGAATGG) and CT06R (GGAAGGCCATATTCCAATCG) were used for amplification and sequencing (Ovenden et al. 2006). The flanking sequence of microsatellite locus CT06 was also obtained from 10 individuals of *C. amblyrhynchoides* (sourced from northern Australia and Indonesia) for comparison.

Amplification reactions were carried out in 10 µl volumes and contained 0.5 µM of each primer, combined with 5–50 ng of template DNA, 10× Taq buffer (containing 15 mM $MgCl_2$), 0.8 mM dNTP's, and 1.5 units of Taq DNA polymerase (Qiagen, Valencia, CA, USA). Thermal cycling conditions consisted of an initial denaturation (94°C for 1 min 30 s) followed by 35 cycles of 94°C for 25 s, 55°C for 25 s and 72°C for 1 min, with a final extension step of 72°C for 10 min. Cycling was performed in a PTC200 DNA Engine (MJ Research, USA). PCR products were viewed on a 1.5% agarose TAE gel stained with GelRed (Biotium, USA). PCR products were desalted prior to sequencing using Exosap-it® (USB Corporation distributed by GE Healthcare Bio-Sciences, Rydalmere NSW, Australia).

Sequencing reactions used 20 ng of DNA and ABI Big Dye Vers 1.1 technology, which is optimised for smaller amplicons where sequence is needed close to the primer (Applied Biosystems, California), and were run on an Applied Biosystems 3130xl Genetic Analyser. With the exception of the preliminary screen of the eight homozygous animals, only the forward primer (CT06F) was used as a sequencing primer due to frame shifts caused by heterozygous microsatellite alleles in the reverse primer reads. Sequence data was edited and aligned with Sequencher (Vers 4.8 Gene Codes Corporation, Ann Arbor, MI, USA).

Results

Mismatch between morphology and mtDNA for species identification

A set of 42 animals was found with discordant morphology and ND4 mtDNA species identifications. Pre-caudal vertebrae (PCV) counts for 24 animals ranged from 98 to 105, typing them as *C. limbatus* but the ND4 mtDNA assay identified them as *C. tilstoni*. Similarly, three animals had PCV counts from 83 to 88 indicating *C. tilstoni* morphology, but they possessed *C. limbatus* mtDNA ND4 haplotypes. An additional three animals had intermediate PCV counts of 93, with one identified as *C. limbatus* and the other two as *C. tilstoni* using the mtDNA ND4 marker.

A further 11 animals from northern NSW were genetically identified as *C. tilstoni*, but they had stretch total lengths well above (STL > 2,000 mm, but no PCV counts) the range recorded for this species. Finally, a neonate caught off Townsville was identified as a *C. limbatus* using ND4 mtDNA, but its length (STL = 651 mm) was well below that expected for *C. limbatus*. Neither morphological plasticity nor genotyping errors could readily explain the mismatches, thus a hybrid hypothesis was formulated that required a nuclear genetic marker for verification.

Nuclear marker

In the search for species-diagnostic mutations several nuclear markers were screened including ribosomal DNA ITS1 and ITS2 (primers designed by authors and available on request), RAG2 (Cooper et al. 2009) and flanking sequence for microsatellite loci CLI12 (Keeney and Heist 2003), CT06, CT07 and CS02 (Ovenden et al. 2006). Sequence for most of these markers were either identical within and between species (RAG2, CLI12, CT07, CS02) or showed only non-diagnostic polymorphisms (rDNA ITS1 and ITS2) (data not shown).

Species-diagnostic mutations were found in the 5 prime flanking sequence of nuclear microsatellite locus CT06 for *C. tilstoni, C. limbatus* and *C. amblyrhynchoides* (Table 1). Three diagnostic mutations were found at positions 111, 137 and 154. An allelic difference was identified within *C. limbatus* at position 138 with either a 'T' or a gap at this site. Heterozygote *C. limbatus* at position 138 were more difficult to score downstream (3 prime) due to the sequence frame-shift, however, the identity of position 154 was generally resolvable by eye as a 'G' or 'A' as the immediate flanking sequence both up- and down-stream was 'C'. Differences at positions 111 and 154 were fixed and diagnostic for *C. amblyrhynchoides*. In total, 46 *C. limbatus*, 23 *C. tilstoni* and 10 *C. amblyrhynchoides* were sequenced with the CT06 and ND4 mtDNA species-diagnostic markers. The flanking sequence of locus CT06 was concordant with the species identification of *C. limbatus* and *C. tilstoni* samples from a range of locations along the eastern Australian coastline based on mtDNA ND4 assay and morphological characteristics (Table 2).

Sequencing of the CT06 flanking region for 42 sharks that had previously been identified as having a mismatch between mtDNA ND4 sequence and one or more morphological characteristics confirmed the hybrid hypothesis; 40 animals returned a hybrid genotype (Table 3). Hybrids were identified because their CT06 genotypes contained DNA from both *C. tilstoni* and *C. limbatus*. Heterozygote genotypes were characterized by sequence chromatograms displaying both A and G peaks at position 111, however, they were difficult to score downstream of position 137 due

Table 1 Species diagnostic sites in 5 prime flanking sequence of nuclear DNA marker CT06 (GenBank AY545200.1, Ovenden et al. 2006) for *Carcharhinus tilstoni*, *C. limbatus* and *C. amblyrhynchoides*

Position	*C. tilstoni*	*C. limbatus*	*C. amblyrhynchoides*
109	C	C	C
110	T	T	T
111	G	A	A
112	T	T	T
113	G	G	G
114	T	T	T
115	C	C	C
116	A	A	A
117	T	T	T
118	C	C	C
119	C	C	C
120	T	T	T
121	A	A	A
122	A	A	A
123	G	G	G
124	A	A	A
125	C	C	C
126	A	A	A
127	G	G	G
128	A	A	A
129	T	T	T
130	T	T	T
131	T	T	T
132	T	T	T
133	T	T	T
134	T	T	T
135	T	T	T
136	T	T	T
137	–	T	T
138	–	T/–	–
139	G	G	G
140	A	A	A
141	C	C	C
142	A	A	A
143	G	G	G
144	C	C	C
145	T	T	T
146	A	A	A
147	C	C	C
148	A	A	A
149	G	G	G
150	A	A	A
151	T	T	T
152	T	T	T
153	C	C	C
154	G	A	G
155	C	C	C
156	C	C	C

Table 2 Collection location, sample size (N) and gender of *Carcharhinus limbatus* and *C. tilstoni* samples that had concordant species identification based on morphological characters (stretched total length, STL and pre-caudal vertebrae count, PCV) and nuclear (nDNA, locus CT06) and mitochondrial DNA (mtDNA ND4 region) genetic markers

Collection location	N	Gender	STL range (mm)	PCV range	mtDNA	nDNA
C. limbatus						
Far North, QLD	7	na	na	na	*C. limbatus*	*C. limbatus*
Cairns, QLD	5	5F	na	na	*C. limbatus*	*C. limbatus*
Townsville, QLD	7	5F, 2 M	676–1,380	93–101	*C. limbatus*	*C. limbatus*
Mackay, QLD	5	4F, 1 M	741–1,490	100	*C. limbatus*	*C. limbatus*
Brisbane, QLD	11	4F, 6 M, 1na	665–749	96–110	*C. limbatus*	*C. limbatus*
Northern NSW	11	6F, 5 M	740–2,670	na	*C. limbatus*	*C. limbatus*
Total	46	24F, 14 M	665–2,670	93–110		
C. tilstoni						
Far North, QLD	4	3F, 1 M	678–1,500	na	*C. tilstoni*	*C. tilstoni*
Townsville, QLD	8	3F, 5 M	611–856	83–93	*C. tilstoni*	*C. tilstoni*
Mackay, QLD	10	4F, 6 M	742–1,660	84–88	*C. tilstoni*	*C. tilstoni*
Brisbane, QLD	1	F	785	87	*C. tilstoni*	*C. tilstoni*
Total	23	11F, 12 M	611–1,660	83–93		

QLD Queensland; *NSW* New South Wales; *na* data not available

Table 3 Collection location, sample size (N) and gender of *Carcharhinus limbatus* and *C. tilstoni* hybrids that had discordant species identification based on morphological characters (stretched total length *STL* and pre-caudal vertebrae count *PCV*) and mitochondrial DNA (mtDNA ND4 region). First-cross (F1) hybrids were indicated by a heterozygous nuclear maker (nDNA, locus CT06) and back-crossed hybrids (B+) were indicated by a mismatch between mtDNA and nDNA markers

Collection location	N	Sex	STL range (mm)	PCV range	mtDNA	nDNA
F1 Hybrids						
Far North, QLD	1	na	na	na	*C. limbatus*	Heterozygote
Townsville, QLD	4	4F	651–1,000	98–101	*C. limbatus*	Heterozygote
Mackay, QLD	1	F	765	101[a]	*C. tilstoni*	Heterozygote
Brisbane, QLD	1	M	700	107	*C. limbatus*	Heterozygote
Brisbane, QLD	4	3 M, 1na	682–730	97–101[a]	*C. tilstoni*	Heterozygote
Northern NSW	1	F	730	na	*C. limbatus*	Heterozygote
Northern NSW	5	3 M, 2F	1,580–2,570[b]	na	*C. tilstoni*	Heterozygote
Total	17	7 M, 8F	651–2,570	97–107		
B + Hybrids						
Far North, QLD	6	3 M, 2F, 1na	840–1,620	na	*C. limbatus*	*C. tilstoni*
Townsville, QLD	1	M	678	101	*C. limbatus*	*C. tilstoni*
Townsville, QLD	3	1 M, 2F	1,130–1,415	93–99[a]	*C. tilstoni*	*C. limbatus*
Mackay, QLD	3	3F	631–996	83–88[c]	*C. limbatus*	*C. tilstoni*
Brisbane, QLD	17	9 M, 6F, 2na	650–904	96–105[a]	*C. tilstoni*	*C. limbatus*
Northern NSW	10	6 M, 4F	1,540–2,560[b]	na	*C. tilstoni*	*C. limbatus*
Total	40	20 M, 17F	631–2,560	83–105		

QLD Queensland; *NSW* New South Wales; *na* data not available

[a] MtDNA identification as *C. tilstoni*, but PCV count of *C. limbatus*

[b] MtDNA identification as *C. tilstoni*, but STL in range of *C. limbatus*

[c] MtDNA identification as *C. limbatus*, but PCV count of *C. tilstoni*

to the sequence frame-shift. It was possible to find both A and G peaks at position 154 (frame shifted by one or two bases) by visual editing. Two types of hybrids were identified, first- (F1) and back- (B+) crosses. The F1 genotype carried heterozygous CT06 alleles, one from *C. limbatus* and one from *C. tilstoni*, and may be a first or

subsequent generation hybrid. Both types of mtDNA were found among F1 hybrids indicating that hybridisation had occurred in both directions (e.g. female *C. tilstoni* with male *C. limbatus* and *visa versa*). B+ hybrids had homozygous alleles for the CT06 locus with conflicting mtDNA species identification. The B+ genotype could only result from a successful F1 cross, either as a backcross to a parental species, or by mating with another hybrid. For example, a hybrid with the mtDNA ND4 of *C. limbatus*, but the CT06 genotype of *C. tilstoni* was classified as a B+. The generation of B+ could not be determined from the markers used here. The presence of B+ indicates that the F1 animals are capable of producing offspring.

Three sharks were included in the mismatched group because they had PCV counts ($n = 93$) that were intermediate between that of *C. limbatus* and *C. tilstoni*. One of these typed as *C. tilstoni* at both the mtDNA and CT06 locus. The second animal typed as *C. limbatus* at both the mtDNA and CT06 locus. The third shark with PCV count of 93 was a B+ hybrid (*C. tilstoni* mtDNA and *C. limbatus* at locus CT06).

Screening of additional samples ($N = 60$) identified a further eight F1 and nine B+ hybrids. The hybrid animals were identified from every sampled population from the far north of Queensland to northern New South Wales (Fig. 1; Table 2) on the eastern Australian coast. The hybrids did not display any gender bias and both F1 and B+ animals were caught with STL over 2,500 mm suggesting they attain a large size. The relative proportions of *C. tilstoni*, *C. limbatus* and hybrids (F1 and B+) were not determined as sharks were not selected at random from populations. Animals more likely to be hybrids (i.e. the rare species in a population) were selected for genotyping.

Discussion

Hybridisation has not previously been reported for Chondrichthyan species, where fertilisation is internal. Observations in nature show that mating in shark and ray species can be preceded by behaviours (e.g. Powter and Gladstone 2008; Pierce et al. 2009; Marshall and Bennett 2010) that may facilitate the choice of a mate from the same species. Incorrect choices have presumably been made during reproductive activity in black tip sharks (*C. limbatus* and *C. tilstoni*) leading to F1 and B+ hybrids along the eastern Australian coastline. Nuclear DNA genotyping of black tip sharks (*C. limbatus* and *C. tilstoni*) identified 57 hybrid animals spanning 2,000 km of coastline, ranging from far north Queensland to northern New South Wales. Furthermore, *C. tilstoni* have been identified adjacent to Sydney (500 km to the south) based on mtDNA ND4 sequence (Boomer et al. 2010). These animals may also be hybrids, as some were thought too large to be purebred *C. tilstoni* (J. Boomer, pers. comm.). Hybrids may also be found on the extensive northern Australian coastline where the two species also co-occur. The widespread occurrence of hybrids suggests a 'hybrid swarm' model, which is common among native and introduced salmonid species (e.g. Muhlfeld et al. 2009), but is rarely reported for marine species. However, the apparent presence of pure *C. limbatus* and *C. tilstoni* does not support a hybrid swarm scenario.

Indeed, the co-occurrence of both hybrids and parental types is surprising. The hybrid swarm model predicts that due to extensive interbreeding and hybrid fertility all animals in the population are likely to have a hybrid ancestry within a few generations (Epifanio and Philipp 2000). Despite hybridisation, the two shark species appear to be maintaining species diagnostic morphometric and life history differences (Harry 2011). This agrees with genetic results where the nuclear marker identifies both parental types and hybrids. Relatively few genetically-confirmed hybrids had PCV counts intermediate to the parental range, which would be expected if PCV count was a continuous genetic trait. Of the 57 hybrids identified, only one animal had an intermediate PCV count of 93; the remaining hybrid animals had either less than 90, or more than 95, vertebrae. The range of PCV counts per species is large and, with the exception of two animals in this study, does not appear to overlap (Last and Stevens 2009; Ovenden et al. 2010). Although the mechanism of inheritance of PCV counts may be a factor, the absence of animals with intermediate PCV counts suggests that introgression has not merged the species.

The observed divergence in morphometric and life history characters between the two 'parental' types could also be maintained by low hybrid fitness. We have little concrete data on hybrid fitness, however, our data suggests F1 hybrids are reproductively viable due to the presence of presumably back-crossed individuals. Forty of the 57 hybrids identified could be the offspring of interbreeding between hybrids, or interbreeding between hybrids and purebred animals. We deduced this from a combination of nuclear DNA and mtDNA results; the nuclear DNA marker alone (locus CT06) could identify first or later generation hybrids (F1, heterozygous nuclear alleles) and second or later generation hybrids (B+, where homozygous nuclear alleles conflicted with mtDNA identity). A single nuclear gene marker, however, cannot distinguish all hybrids, nor can it distinguish recent from ancient hybridisation and thus, we may have underestimated the number of F1 and B+ hybrids. Increasing the number of nuclear diagnostic markers would assist in understanding the degree of introgression occurring between the two species, including a more robust test for the presence of purebred individuals.

Five microsatellite loci appeared to show allele frequency differences between *C. limbatus* and *C. tilstoni* (Ovenden et al. 2010) and may be potentially useful for hybrid analyses. The samples used for that study have not been genotyped with locus CT06 and the allele frequencies would need to be re-assessed if hybrids were present. The fitness of hybrids is highly likely to vary as a consequence of their mixed genetic background and interaction with heterogeneous environments (Arnold 2006). Shark hybrids were found across a large variety of environmental gradients, such as water temperature, turbidity and biotic communities, from north to south along approximately 2,000 km of the eastern Australian coastline.

Hybridisation between the shark species could be a natural phenomenon, occurring over evolutionary time-periods where the two species are sympatric. The occurrence of the hybrids over a wide spatial range supports this idea. Under this hypothesis, hybrid fitness would need to be low to maintain the observed amount of morphological divergence between parental phenotypes. Alternatively, if hybrid fitness was not low, the co-occurrence of both parental and hybrid types could be explained because hybridisation is a recent phenomenon. However, it is difficult to propose a mechanism that would lead to coincident, recent hybridisation on such a wide spatial scale. The key to testing these competing hypotheses in the future lies with the measurement of hybrid fitness and more extensive application of nuclear DNA markers to test the frequency of purebred compared to hybrid sharks.

Hybridisation between two commercially important shark species may affect fisheries resource sustainability under current management arrangements. If hybrids have reduced fitness and are common, then population productivity will be lower than predicted and harvesting at current levels could result in overfishing. Fishing techniques could unwittingly target one species over the other due to the size difference between of *C. tilstoni* and *C. limbatus*, which may be locally affecting the type of interspecies hybridization occurring in some populations. If hybrids have the same or higher fitness compared to the parental species, then over time the two species will merge into a single species and shark species biodiversity will be lost. The status of the third species in this lineage; *C. amblyrhynchoides*, is unknown. This species is reciprocally monophyletic with *C. limbatus* to the exclusion of *C. tilstoni* based on allozymes (Lavery 1992) and mtDNA sequence (Ovenden et al. 2010). However, the reverse is true morphologically; *C. amblyrhynchoides* has a divergent phenotype compared to the morphologically similar *C. limbatus* and *C. tilstoni* (Last and Stevens 2009). *Carcharhinus amblyrhynchoides* is not commonly caught on the east coast but may be interacting with the two other species in northern Australian waters. If the specific status of *C. tilstoni* and *C. limbatus* is in doubt due to hybridisation, then the species status of *C. amblyrhynchoides* should also be investigated.

Hybridisation may not be a threatening process as the presence of hybrids with varying fitness may facilitate the long-term adaptation of the species to environmental change. This scenario may explain the high incidence of *C. tilstoni* hybrids (*C. tilstoni* mtDNA, *C. limbatus* phenotype) in the temperate south of the range. Hybrid, large-bodied *C. tilstoni* may have an ecological advantage over pure *C. tilstoni* in cooler water. Ocean warming of southern, temperate waters may also be facilitating this process (Ridgway 2007). A similar scenario could be postulated for the northern Australian edge of the *C. tilstoni* range, where *C. limbatus* hybrids (*C. limbatus* mtDNA with smaller bodied *C. tilstoni* phenotype) could compete more efficiently with purebred *C. limbatus* in tropical waters. Indeed, the *Carcharhinus* genus is speciose (Compagno et al. 2005), which may have been facilitated by hybridization and adaptive evolution.

The potential for inter-species hybridisation should be accounted for when mtDNA is used for shark species identification (e.g. using 'barcoding'). Depending on the direction of the cross, hybrids will either be missed or will be genotyped as the 'wrong' type of mtDNA for a given phenotype (i.e. introgression). This is important in view of the forensic and regulatory implications of using 'barcoding' for species identification generally (Alacs et al. 2009; Wilson-Wilde et al. 2010) and specifically for species identification in the Chondrichthyes (Ward et al. 2008; Wong et al. 2009). As hybridization has the potential to cause species misidentification, we recommend that 'barcoding' should be used with caution for the identification of closely-related shark species that are co-distributed. Ideally barcoding should also include a nuclear marker. Note, however, that the nuclear DNA CT06 locus has been validated on *C. amblyrhynchoides*, *C. tilstoni* and *C. limbatus* from the east coast of Australia but may give confounding results against other members of the genus *Carcharhinus*. For black-tip shark species specifically, identification requires mtDNA 'barcoding' to maternal species prior to screening with the nuclear DNA CT06 assay to determine hybrid status. Other closely related species groups, with overlapping distributions and thus the potential to hybridize, include *C. melanopterus–C. cautus*, and *C. amblyrhynchos–C. albimarginatus* identified in an allozyme phylogeny (Lavery 1992). The mtDNA phylogeny of Ward et al. (2008) confirmed this, and reported another closely related species group (*C. plumbeus–C. altimus*).

Hybridisation can also bias phylogenetic inferences based on mtDNA sequences. For example, Keeney and Heist (2006) reported that eastern Atlantic and Indo-Pacific *C. limbatus* were paraphyletic with *C. tilstoni,* based on

mtDNA control region sequencing of five *C. tilstoni* (New South Wales, $n = 2$ and Western Australia, $n = 3$) and a worldwide sample of *C. limbatus*. Their observation of paraphyly could be explained by introgression of *C. limbatus* mtDNA into the *C. tilstoni* that they sampled as a result of current or past hybridization events. Ideally, the species identity of the five *C. tilstoni* samples included in the study by Keeney and Heist (2006) needs to be confirmed using pre-caudal vertebral counts, life-history traits, further mtDNA sequencing (e.g. ND4 region, Ovenden et al. 2010) and genotyping with the CT06 locus. The relationship between *C. tilstoni* and the widespread *C. limbatus* is more likely explained by allopatric speciation of *C. tilstoni* in Australian waters, followed by secondary contact and hybridization with *C. limbatus*, which may have been brought about by the subsequent colonization of Australian waters by the widespread species.

To find a wild hybrid animal is unusual. To find clear evidence of F1 and backcrossed (B+) hybrid sharks in nature is extraordinary. To find 57 hybrids along 2,000 km of coastline is unprecedented. The full spatial extent of the occurrence of hybridisation needs to be determined by testing samples from Western Australia, the Northern Territory, southern NSW and Indonesia. The frequency of F1 and B+ hybridisation needs to be determined across populations to test hypotheses about the origins and timing of the process. Likewise, hybrid fitness urgently needs to be investigated using an expanded range of nuclear DNA markers and reproductive and life-history characters. Now that hybrids can be identified, obtaining life history measurements to assess their capacity to reproduce, their susceptibility to parasites and general health compared to the parental species will assist in understanding whether the two species will be maintained or combined into one 'hybrid' species in the future.

Acknowledgments This study was made possible by the many fishers and scientists who collected tissue samples for this project, including those associated with the Marine and Tropical Science Research Facility project 4.8.4 (GBRWHA Inshore fisheries), James Cook University and the fisheries observer programs in Queensland and New South Wales. We thank three anonymous reviewers for their suggestions that improved the quality of the manuscript. The Australian Fisheries Research and Development Corporation supported this study (project 2007/035).

References

Alacs EA, Georges A, FitzSimmons NN, Robertson J (2009) DNA detective: a review of molecular approaches to wildlife forensics. Forensic Sci Med Pathol 6:180–194

Allendorf FW, Leary RF, Spruell P, Wenburg JK (2001) The problems with hybrids: setting conservation guidelines. Trends Ecol Evol 16:613–622

Arnold ML (2006) Evolution through genetic exchange. Oxford University Press, Oxford

Arnold ML, Martin NH (2010) Hybrid fitness across time and habitats. Trends Ecol Evol 25:530–536

Boomer JJ, Peddemors V, Stow AJ (2010) Genetic data show that *Carcharhinus tilstoni* is not confined to the tropics, highlighting the importance of a multifaceted approach to species identification. J Fish Biol 77:1165–1172

Compagno LJV, Dando M, Fowler S (2005) Sharks of the world, 1st edn. Princeton University Press, Princeton and Oxford

Cooper WJ, Smith LL, Westneat MW (2009) Exploring the radiation of a diverse reef fish family: phylogenetics of the damselfishes (Pomacentridae), with new classifications based on molecular analyses of all genera. Mol Phylogenet Evol 52:1–16

Epifanio J, Philipp D (2000) Simulating the extinction of parental lineages from introgressive hybridization: the effects of fitness, initial proportions of parental taxa, and mate choice. Rev Fish Biol Fish 10:339–354

Harry AV (2011) The life histories of commercially important tropical sharks from the Great Barrier Reef World Heritage Area. Doctor of Philosophy, James Cook University, Townsville, Queensland Australia

Keeney DB, Heist EJ (2003) Characterization of microsatellite loci isolated from the blacktip shark and their utility in requiem and hammerhead sharks. Mol Ecol Notes 3:501–504

Keeney DB, Heist EJ (2006) Worldwide phylogeography of the blacktip shark (*Carcharhinus limbatus*) inferred from mitochondrial DNA reveals isolation of western Atlantic populations coupled with recent Pacific dispersal. Mol Ecol 15:3669–3679

Last PR, Stevens JD (2009) Sharks and rays of Australia, 2nd edn. CSIRO Australia, Collingwood

Lavery S (1992) Electrophoretic analysis of phylogenetic relationships among Australian Carcharhinid sharks. Aust J Mar Freshw Res 43:97–108

Lavery S, Shaklee JB (1991) Genetic evidence for separation of two sharks *Carcharhinus limbatus* and *C. tilstoni* from northern Australia. Mar Biol 108:1–4

Marshall AD, Bennett MB (2010) Reproductive ecology of the reef manta ray *Manta alfredi* in southern Mozambique. J Fish Biol 77:169–190

Mayr E (1942) Systematics and the origin of species. Columbia University Press, New York

Morgan JAT, Welch D, Harry A, Street R, Broderick D, Ovenden JR (2011) Species-identification assay for Australian blacktip sharks, *Carcharhinus tilstoni*, *C. limbatus* and *C. amblyrhynchoides* using real-time PCR and high-resolution melt analysis. Mol Ecol Resour 11:813–819

Muhlfeld CC, Kalinowski ST, McMahon TE, Taper ML, Painter S, Leary RF, Allendorf FW (2009) Hybridization rapidly reduces fitness of a native trout in the wild. Biol Lett 5:328–331

Ovenden JR, Broderick D, Street R (2006) Microsatellite primers for two carcharinid sharks (*Carcharinus tilstoni* and *C. sorrah*) and their usefulness across a wide range of shark species. Mol Ecol Notes 6:415–418

Ovenden JR, Morgan J, Kashiwagi T, Broderick D, Salini J (2010) Towards better management of Australia's shark fishery: genetic analyses reveal unexpected ratios of cryptic blacktip species *Carcharhinus tilstoni* and *C. limbatus*. Mar Freshw Res 61:253–262

Pierce SJ, Pardo SA, Bennett MB (2009) Reproduction of the blue-spotted maskray *Neotrygon kuhlii* (Myliobatoidei: Dasyatidae) in south-east Queensland, Australia. J Fish Biol 74:1291–1308

Powter DM, Gladstone W (2008) The reproductive biology and ecology of the Port Jackson shark *Heterodontus portusjacksoni* in the coastal waters of eastern Australia. J Fish Biol 72: 2615–2633

Ridgway KR (2007) Long-term trend and decadal variability of the southward penetration of the East Australian Current. Geophys Res Lett 34:5

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Stevens J, Wiley P (1986) Biology of two commercially important carcharhinid sharks from northern Australia. Aust J Mar Freshw Res 37:671–688

Ward RD, Holmes BH, White WT, Last PR (2008) DNA barcoding in Australasian chondrichthyans: results and potential uses in conservation. Mar Freshw Res 59:57–71

Wilson-Wilde L, Norman J, Robertson J, Sarre S, Georges A (2010) Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (COI) gene. Forensic Sci Med Pathol 6:233–241

Wong EHK, Shivji MS, Hanner RH (2009) Identifying sharks with DNA barcodes: assessing the utility of a nucleotide diagnostic approach. Mol Ecol Resour 9:243–256