Genome-wide SNPs reveal complex patterns of introgressive hybridization between *Carcharhinus tilstoni* and *Carcharhinus limbatus* blacktip sharks

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Keywords: Population Genomics, Adaptation, Hybridisation, Fisheries, Simulations

This paper was presented as part of an invited session entitled 'Genomics for improved fisheries management and conservation: have the promises been fulfilled?' at the 7th World Fisheries Congress in Korea in 2016 which was sponsored by the OECD Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems, whose financial support made it possible for the invited speakers to participate in the Special Session.

Introduction

Overexploitation of apex predators raises great concern in terms of the ecological consequences that their removal may have over marine ecosystem function and stability [1]. Among these apex predators, sharks, particularly those associated to coastal habitats have been seriously overexploited, with some populations declining by over 99% [1,2]. Estimates of the total annual shark mortality due to fisheries are considered to be between 63 and 273 million sharks and include illegal unreported and unregulated (IUU) fishing driven by the high demand for shark fins in Asian markets [3] In Australia, species within the genus *Carcharhinus* make up a large component of local commercial fisheries [4-7]. However, as a result of the conserved morphology across several taxa, particularly Carcharhiniformes, misidentification in the field is common [8,5,9]. Despite stock assessments and observation programs on commercial fishing boats [4,7], the cryptic morphology of this taxa makes visual identification and reliability of catch records a challenge [5,6,9].

Recently, the first case of interspecies hybridization was reported between two cryptic blacktip sharks, the Australian blacktip (*Carcharhinus tilstoni*) and the common blacktip shark (*C. limbatus*) [10]. However, to understand the class of hybrids, the frequency of introgression, as well as the directionality and evolution of hybridization, a genome-wide approach is required [11]. Understanding the role that hybridization is playing in these two commercially and ecologically important species is imperative to develop appropriate conservation and management actions to either promote or protect this process or to minimize it. *C. tilstoni* and *C. limbatus* represent a significant component of the Australian northern and eastern coast inshore shark fishery [12]. They are morphologically indistinguishable externally, thus identification relies on pre-caudal vertebral count (PCV), but morphometric characteristics such as length-at-birth, length-at maturity and maximum body size can also facilitate identification [6].

The objectives of this study were (1) to develop novel genome-wide nuclear markers using a Reduced Representation Genotyping by Sequencing approach (RRGS) to evaluate the level of admixture between the sympatric populations of *C. tilstoni* and *C. limbatus* of the eastern coast of Australia; (2) to identify a minimum number of diagnostic single nucleotide polymorphisms (SNPs) with maximum power to delineate the hybrid classes present between the two blacktip species for application in fisheries enforcement and forensic cases; (3) to compare the power and resolution that diagnostic markers provide for hybrid class delineation with previous results that used mtDNA, microsatellite markers and pre-caudal vertebrae count discordance.

Materials and Methods

Sampling and DNA extraction

Given that all individuals analysed in this study proceeded from the study developed by Morgan et al. (2012), sample collection is essentially as described by that study. Briefly, tissue samples of *C. tilstoni* and *C. limbatus* were collected from the landed catch of the shark fishing sector from five different locations along the eastern coast of Australia, from far north Queensland to Northern New South Wales (Figure 1) Meristic and morphometric species diagnostic characters where recorded from all individuals for later comparison to genetic identification (Table 1).

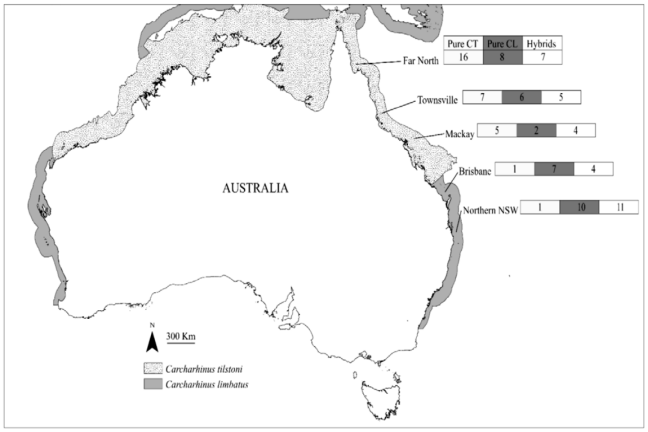

Figure 1. Sampling locations and distribution of *C. tilstoni* (CT) and *C. limbatus* (CL) along the coast of Australia. The boxes represent the number of individuals sampled per class in each location. Pure individuals had concordant nDNA, mtDNA ND4 and morphological characteristics [10].

SNP identification

Thirty pure *C. tilstoni*, 33 pure *C. limbatus* and 31 hybrids were preselected from 126 individuals as diagnosed by Morgan *et al.* [10] to ensure equal representation of each class (Figure 1; Table 1). Genomic DNA was extracted, selected samples where then used for DArT sequencing (Illumina HiSeq 2000 platform) and SNP discovery using methylation-sensitive restriction enzymes (RE). SNP quality control included the removal of non-informative loci with a call rate threshold less than 0.85 and loci deviating from Hardy-Weinberg equilibrium (HWE). Thus we adopted a conservative approach of using only markers with less than 15% missing value (i.e., call rate above 85%).

Location	Code	N	Gender	STL range	PCV
C. tilstoni					
Far North	FN	16	11F, 5M	795 – 1,517	83 – 89
Townsville	TSV	7	4F, 3M	611 – 1,740	85 – 88
Mackay	MKY	5	2F, 3M	789 – 1,660	85 – 88
Brisbane	BNE	1	1F	785	87
Northern NSW	NSW	1	Na	Na	na
Total		30			
C. limbatus					
Far North	FN	8	4F, 4M	1,019 – 1,959	96 – 102
Townsville	TSV	6	4F, 2M	676 – 1,380	93 – 101
Mackay	MKY	2	2M	748 – 1,490	100
Brisbane	BNE	7	3F, 3M,	700 – 749	99 – 110
Northern NSW	NSW	10	5F, 5M	2,160 – 2,670	na
Total		33			
Hybrids					
Far North	FN	7	5F, 2M	1,160 – 1,512	85 – 98
Townsville	TSV	5	4F, 1M	731 – 1,415	93 – 99
Mackay	MKY	4	4F	631 – 996	83 – 101
Brisbane	BNE	4	4M	690 – 730	97 – 107
Northern NSW	NSW	11	4F, 6M,	730 – 2,570	na
Total		31			
Total no.		94			

Table 1. Details of genetic samples for blacktip sharks including sampling locations, code, sample size (N), gender, stretch total length (STL) in mm, and pre-caudal vertebrae count (PCV) [10].

Clustering Analysis, species diagnostic SNPs and hybrids identification

To evaluate the degree of discreteness between *C. tilstoni* and *C. limbatus* and estimate the level of admixture of each individual, a Bayesian assignment analysis was conducted using STRUCTURE v2.3.4. [13], followed by the Bayesian statistical method NEWHYBRIDS v1.1 [14], to determine the posterior probability that each sampled individual belonged specifically to one the 10 pre-defined hybrid categories. Species-diagnostic SNPs were selected to design several lower density SNP assays, with high statistical power applicable in forensic/enforcement scenarios. A high-definition network visualization analysis pipeline, NETVIEW P v0.6 [15] was applied to each SNP data set to evaluate the genetic relatedness among individuals within and between both blacktip sharks species and identify individuals with mixed ancestry.

Results

Genome-wide SNP discovery and cluster analysis

DArT sequencing data from *C. tilstoni*, *C. limbatus* and hybrid individuals generated a total of 23,515 SNPs. Results from STRUCTURE identified two distinct clusters corresponding to the two blacktip shark species (Figure 2). For the 18,577 SNP data set, each individual was assigned to each cluster with an estimated membership (q) of 100%. These individuals where assumed pure parental species. However, in this same data set, 11 individuals presented various levels of admixed ancestry and where considered potential hybrids. Overall genetic differentiation between the two species was high (F_{ST} = 0.343, P value < 0.001), supporting the scenario of the two distinct cluster defined by STRUCTURE.

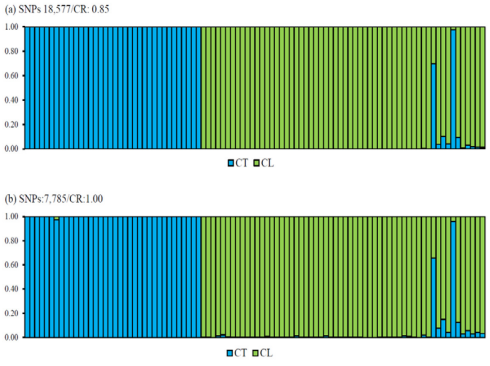

Figure 2. Admixture analysis in STRUCTURE assuming K=2. The blue and green clusters represent individuals assigned as *C. tilstoni* and *C. limbatus* respectively. Clustering based on a) 18,577 SNPs with a call rate (CR) of 0.85 and b) 7,785 SNPs with a CR of 1.0.

Species diagnostic SNPs

After the filtering criteria for selecting near diagnostic markers was applied to the genome-wide data set, three SNP assays where defined: (1) an assay of 301 SNPs with minimum call rate value of 0.85 (i.e., 15% missing value) and minimum pairwise F_{ST} values of 0.95; (2) an assay of 102 SNPs with a call rate of 1.00 (i.e., no missing values) and minimum pairwise F_{ST} values of 0.95 and; (3) an assay of 66 SNPs with a minimum call rate of 0.85 and pairwise F_{ST} values=1.00. Using the 301 SNP assay STRUCTURE classified 89 of the 94 individuals as pure parentals with a q value >0.99, while the remaining 11 individuals presented an admixed ancestry with a q value between 0.76 and 0.99 (Figure 3a-b). Based on the 102 SNP assay, only nine individuals presented admixture ancestry (Figure 3c-d). Contrary to the 301 SNP assay, individuals UBT11, UBT27 and UBT55 presented admixture proportions suggesting they were second-generation backcross with different degrees of *C. limbatus* ancestry. The remaining individuals where potentially third- or fourth-generation backcross. Contrary to the two large SNP assays, the 66 SNP assay classified 91 of the 94 individuals as pure parental with a q value >0.99, while the remaining individuals, also UBT11, UBT27 and UBT55 presented admixture proportions of second-generation backcross (Figure 3e-f).

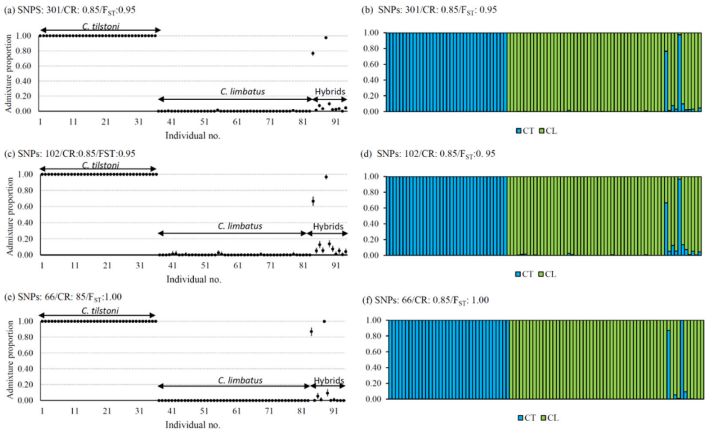

Figure 3. Admixture analysis in STRUCTURE, assuming the presence of two groups (K=2): *C. tilstoni* and *C. limbatus*. (a, c, e) Individual admixture proportions along with 95% credible intervals are shown for each individual. (b, d, e) Each vertical line represents one individual, partitioned into segments according to the proportion of *C. tilstoni*–CT (blue) and *C. limbatus*-CL (green).

To understand the relationship of each individual within and between the two species, five high- definition network visualizations including all 94 individuals where developed. Two networks were generated using the two large genome-wide data sets (Figure 4). The resulting topology clearly defined two clusters representing the two blacktip species, remarkably in accordance with the clustering analysis defined by STRUCTURE (Figure 3).

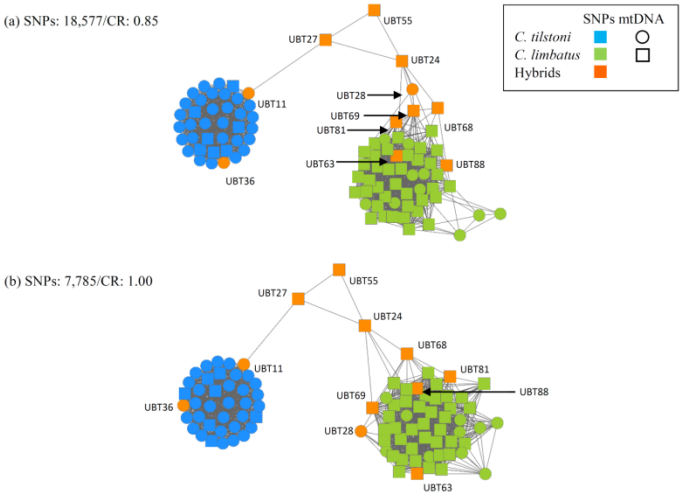

Figure 4. High definition organic network visualizations (NETVIEW) of *C. tilstoni* and *C. limbatus* with $k = 40$. (a) network based on 18,577 SNPs and; (b) network based on 7,787 SNPs. Each individual is represented by a node; the shape of the node represents genetic identity as denoted by mtDNA in Morgan *et al.* [10]; colours represent the ancestry defined by STRUCTURE analysis based on 18,577 SNPs, with *C. tilstoni*, *C. limbatus* and potential hybrids represented by the colours, green, blue and orange, respectively. Individuals considered real hybrids are found to be connecting the two main clusters in the network.

In the two networks designed by the genome-wide data sets each individual was assigned to their respective species with the exception of seven individuals in the 15,877 SNP generated network and four individuals in the 7,785 SNP generated network (Figure 4). Individuals within each cluster appeared highly interconnected, indicating close relatedness of conspecifics, despite discordance with mitochondrial genetic identification. The individuals not assigned to either cluster were by contrast connecting these clusters and were deemed hybrids.

Power of genome-wide SNPs vs mtDNA

The classification based on the 18,577 SNP panel and the low density 102 SNP assay which identified the higher number of hybrids was compared to the genetic identity previously defined on the basis of the mitochondrial ND4 gene and two microsatellite markers during the study of Morgan *et al.* [10]. Seven individuals classified with 100% accuracy as *C. tilstoni* by both STRUCTURE and NEWHYBRIDS had mtDNA from *C. limbatus* and 11 individuals classified as *C. limbatus* had mtDNA from *C. tilstoni* (Figure 4a). Ten individuals identified as hybrids by microsatellites were classified as pure by SNPs, whereas four individuals classified as pure species using mtDNA and microsatellite data, were identified as backcrosses using the SNP panels. Only three individuals considered hybrids due to incongruence between microsatellite and mtDNA were also classified as hybrids using the SNP genotyping classification (UBT27, UBT28, UBT69). All SNP genotyping classification was always in accordance with the PCV count of the species it was classified to. Individuals classified as *C. tilstoni*, all had a PCV count in the range of 80-91 and all individuals classified as *C. limbatus* had a PCV in the range of 94-101 (Table 1). However, this was not the case for two individuals classified as pure parental by SNPs, mtDNA and microsatellite markers (UBT52 and UBT56) and one individual with cytonuclear disequilibrium and hybrid classification on the basis of one microsatellite marker (UBT53). Individuals classified as hybrids on the basis of SNP genotyping classification had a PCV count in accordance to the species which provided the highest degree of ancestry.

Discussion

Morgan *et al.* [10] reported for the first time the presence of hybridization in an elasmobranch species complex. The phenomenon was discovered from a mismatch between mtDNA, microsatellite markers and meristic traits in the sympatric populations of *C. tilstoni* and *C. limbatus* along the eastern coast of Australia. Under this premise, DArTseq data was generated with the aim of enhancing our capacity to identify the level, frequency and directionality of hybridization and introgression between these two species. After the development of 23,515 genome-wide SNPs, a low density SNP assay of 102 markers with a call rate of 1.00 and with the highest F_{ST} values between both species showed the highest accuracy to reliably identify hybrids up to the third generation. As in previous studies [16,17], the approach of selecting highly differentiated SNPs prove to be successful in identifying hybridization and introgression in morphologically indistinguishable species and backcrosses.

Genome-wide levels of admixture between C. tilstoni and C. limbatus

After analysing 30 Australian blacktip sharks, 33 common blacktip sharks and 31 hybrids as defined by Morgan *et al.* [10], the genome-wide SNP classification reported 36 Australian blacktip and 47 common blacktip sharks and 11 individuals with admixed ancestry, irrespective of their mtDNA haplotype. The occurrence of eight individuals with mtDNA of *C. limbatus* in apparently pure *C. tilstoni* and eleven hybrids with mtDNA of *C. tilstoni* in seemingly pure *C. limbatus* was not detected at the genome-wide level, suggesting these individuals represent further backcrosses. The presence of seemingly pure individuals with haplotypes of the other species is suggesting that backcrossing and recombination potentially eroded any evidence of introgression at the nuclear level. However, among the eleven individuals with admixed ancestry, eight were not previously considered hybrids by Morgan *et al.* [10]. This indicates that genome-wide SNP can detect recent hybridization events with high accuracy.

Species diagnostic markers and patterns of hybridization

The power of diagnostic SNP markers to resolve hybrid status provided new insights into the level and nature of introgression between *C. tilstoni* and *C. limbatus*. A total of 35 hybrids were identified after taking into account the hybrids identified by SNP markers and the hybrids identified by Morgan *et al.* [10]. Backcrosses to *C. limbatus* were ubiquitous along sampled locations in the eastern coast of Australia. However, backcrosses to *C. tilstoni* were restricted to tropical waters (i.e., Far North Queensland, Townsville and Mackay), except for the second-generation backcross (bCLxCT) UBT11 found in New South Wales. This distribution suggests that the preference of warm tropical waters by *C. tilstoni* is to some extent inherited to hybrids that backcross to this species. An alternative interpretation is that introgression is increasing the hybrid's ability to adapt to more temperate water via adaptive introgression [18]. However, these assumptions remain to be tested.

Conservation implications of hybridization

Hybrids between *C. tilstoni* and *C. limbatus* suggest that earlier studies analysing abundance and distribution of these two species are potentially biased. For example, Ovenden *et al.* [5] found that the ratio of occurrence between both species was ~1:1. Although that study used mtDNA and microsatellite markers to identify both species, the presence of admixture may have gone unnoticed. Additionally, Boomer *et al.* [19] reported the presence of five *C. tilstoni* in New South Wales, and suggested an expansion of its geographical range of >1000 km into temperate waters. Boomer *et al.* [19], however, relied on two mtDNA genes to identify these individuals. Despite the accuracy of these two regions to identify *C. tilstoni* from *C. limbatus*, mtDNA cannot identify patterns of admixture. Thus, the idea that those five individuals were actually hybrids cannot be ruled out and the extension of Australian blacktip's distribution into temperate waters will require further validation using diagnostic SNPs.

The current approach of solely relying on a few microsatellite markers, mtDNA or PCV counts for species identification of *C. tilstoni* and *C. limbatus* implies that an inherent error has been present in all records of these two species. This error reiterates the urgent need to reevaluate the status of these two species, particularly as they are an important component of many fisheries in Australia. Hence, future studies should aim at increasing the number of individuals and localities sampled. This should include the complete geographic range of both species in Australia.

Reliable catch data are essential for effective fisheries management. Thus, accurate species identification is vital for determining stock structure and population subdivision, particularly as sharks are experiencing unsustainable levels of exploitation pressure. The power of the species diagnostic markers developed in this study represent a tool to aid fishery observer programs which operate in Australian states where the species are landed [20]. The SNP panels are not only valuable tool for accurate species identification but will be of use to tackle IUU fishing and finning in Australia (and worldwide).

Conclusions

DArT-generated SNP markers shared between populations of *C. tilstoni* and *C. limbatus* were used to infer the overall level admixture between these species complex. Despite ongoing hybridization and introgression we discovered that these two populations are highly differentiated. While the amount of genetic resources for both species was initially limited to five microsatellite markers and three mitochondrial genes, the development of such a large panel of novel SNPs represents an unprecedented genomic coverage also available to aid studies in conservation genetics. Particularly, to facilitate the development of informative marker panels for stock traceability, population genomic studies and the identification of candidate genes or genomic regions associated with ecologically important traits. Here, these genomic resources were used to identify species diagnostic markers to reveal the level of hybridization and introgression between these two blacktip sharks. After applying a combination of criteria to select these markers we discovered that the most informative SNPs, with capacity to detect even low levels of admixture where those with high differentiation and a call rate of 100%. These diagnostic SNPs managed to identify hybrids up the third generation with high assignment power. These novel SNP markers will allow a reliable assessment of hybridization through the geographic range of these species in Australia. This in turn, will provide crucial information for management and conservation of these two species.

Country-specific information (Belgium)

The current study was performed during a three year appointment in Australia to work on Marine Conservation and Fisheries Genomics applications. Given the limited number of Conservation/Population Genomics studies applied in Belgian Fisheries (with only 60 km coastline), I present here results from exploited top-predators in Australia, but discuss the funding sources for fisheries genomics in Belgium (home country).

Fisheries genomics work funding sources

As Belgium is split into various semi-independent governments (Federal, Flemish, Walloon, Brussels and German), the funding bodies and fisheries regulations are different at the Belgian or regional level. Marine fisheries is entirely managed by the Flemish government, while freshwater fisheries is managed by each regional government independently. Hence, Marine Fisheries Research (including genetics and genomics) is mainly funded by the Flemish Government (through the Institute for agricultural and fisheries research – ILVO), FWO-Flanders (Flemish Scientific Research Fund; www.fwo.be), in parallel to BELSPO (Belgian Science Policy) at the federal level (www.belspo.be). Freshwater Fisheries Genomics can be funded by the same funding bodies as above, but the ANB (www.anb.be) and INBO (www.inbo.be) can access specific funding to investigate species important for sport fishing.

Funding sources accessed to support Genomics research

Over the past 15-20 years, our laboratory received national funding resources for various projects using molecular tools, mainly concerning freshwater fish management in Flanders (eels, pike, chub, loach, burbot, brown trout, bullhead, gibel carps, …) from ANB, FWO, IWT and INBO. IWT (now part of FWO since 2016) has been a major funding source for PhD student grants, running in parallel with more substantial EU projects on exploited marine fish natural populations, Fisheries induced evolution or farmed escapee traceability (FishPopTrace, FinE, AquaGen, AquaTrace).

Examples of genetic/genomic information to inform fisheries management and/or policy decisions in Belgium

As stated above, various freshwater fishery supportive breeding, connectivity, migration barriers alleviation, population structure background studies have been funded and used as complementary resources to improve population resilience to harvesting. Results were used to discard exogenous restocking material, replenish natural populations or avoid further hybridisation between local and introduced species. For marine species, the implementation of genetics/genomics has been quite poor in the last years, despite relevant EU project outcomes (see references below). Neighbouring countries did however take up results more easily to manage overexploited stocks, control geographic origin of landed fishes or just take into account the genetic/biological discreteness of stocks within ICES squares. A novel research and monitoring tool used in freshwater fish conservation is Environmental DNA (eDNA) to assess density and occurrence of various commercial or endangered fish species or to develop monitoring program for the Water Framework Directive (WFD). INBO is responsible for these tasks in Flanders.

Relevant websites:

- AquaTrace: https://aquatrace.eu/
- FishPopTrace: http://fishpoptrace.jrc.ec.europa.eu/
- AquaGen: http://aquagen.jrc.ec.europa.eu/
- FinE (Fisheries Induced Evolution): http://www.iiasa.ac.at/Research/EEP/FinE/
- eDNA of freshwater fishes: https://data.inbo.be/pureportal/en/projects/edna-monitoring-voor-pasdpb(51c8c034-a9d7-4dd1-95fb-80804ded8f1c).html

Relevant articles:

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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. The Australian Fisheries Research and Development Corporation (project 2007/035) and JCU starting grant supported this study. We thank the OECD Co-operative research Program for the financial support to attend the World Fisheries Conference (Busan, Korea).

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