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Molecular markers: progress and prospects for understanding reproductive ecology in elasmobranchs

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Application of modern molecular tools is expanding the understanding of elasmobranch reproductive ecology. High-resolution molecular markers provide information at scales ranging from the identification of reproductively isolated populations in sympatry (*i.e.* cryptic species) to the relationships among parents, offspring and siblings. This avenue of study has not only augmented the current understanding of the reproductive biology of elasmobranchs but has also provided novel insights that could not be obtained through experimental or observational techniques. Sharing of genetic polymorphisms across ocean basins indicates that for some species there may be gene flow on global scales. The presence, however, of morphologically similar but genetically distinct entities in sympatry suggests that reproductive isolation can occur with minimal morphological differentiation. This review discusses the recent findings in elasmobranch reproductive biology like philopatry, hybridization and polyandry while highlighting important molecular and analytical techniques. Furthermore, the review examines gaps in current knowledge and discusses how new technologies may be applied to further the understanding of elasmobranch reproductive ecology.

Key words: cartilaginous fishes; cryptic taxa; gene flow; philopatry; polyandry; stock structure.

INTRODUCTION

Studying the reproductive biology of sharks, skates and rays is inherently difficult due to the highly migratory nature of many species and difficulties associated with *in situ* observation of behaviour (Speed *et al*., 2010). Although for a few species a great deal of observational data is available in well-characterized nursery areas (Carrier *et al*., 1994; Feldheim *et al*., 2002), for the vast majority no such data exist and observations of mating behaviour are rare or non-existent (Pratt & Carrier, 2001). These problems are compounded by complex behaviour patterns such as female philopatry, sexual segregation and differing adult and juvenile migration patterns and home ranges that lead to complex population structure, which may be difficult to detect by tagging studies alone. Finally, conserved morphology across taxa has led to difficulty in properly identifying both cryptic and known species, which may consequently lead to difficulties in defining ranges (Quattro *et al*., 2006; Ovenden *et al*., 2010).

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Currently a variety of high-resolution molecular markers are available that can be used in a time and cost-efficient manner for the interpretation of complex patterns of molecular variation as well as for accurate reconstruction of individual genetic fingerprints. These techniques have proved useful in expanding the current understanding of reproductive ecology in a number of elasmobranch species while providing novel insights into behaviour and biology and at the same time suggesting new avenues of research. As the cost and difficulty with which molecular information can be obtained continues to decrease, the utility of high-resolution molecular data as complementary information to traditional studies will increase.

MOLECULAR MARKERS

Molecular inquiries into population structure and behaviour primarily seek to characterize and understand the partitioning of genetic variation at multiple levels (from intra-individual to intraspecific) using nuclear or mitochondrial DNA (mtDNA) or both. Early molecular work on elasmobranchs examined components of nuclear variation using allozymes (enzymes which possess allelic variation at a single locus) or mitochondrial (mt) variation using restriction fragment length polymorphisms (RFLP: fragments of DNA which differ in size depending on the presence or absence of recognition sites for enzymes which cleave DNA). While allozymes have been applied to a number of species, *e.g.* spotted estuary smoothhound *Mustelus lenticulatus* Phillipps 1932 (Smith, 1986), gummy shark *Mustelus antarcticus* Günther 1870 (MacDonald, 1988), sandbar shark *Carcharhinus plumbeus* (Nardo 1827) (Heist *et al*., 1995), Australian blacktip shark *Carcharhinus tilstoni* (Whitley 1950) (Lavery & Shaklee, 1989) and *Squatina* sp. (Gaida, 1997), they suffer from a lack of resolution caused by low heterozygosity, with an average of 0·064 for marine fishes (Ward *et al*., 1994), and differing allele counts that are dependent on experimental procedures (Heist, 2004). Similarly, RFLP analyses of mtDNA, while yielding some informative results in elasmobranch species, *e.g. C. plumbeus* (Heist *et al*., 1995) and Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Richardson 1836) (Heist *et al*., 1996*a*), suffer from a lack of observable variation, as only mutations affecting recognition sites can be visualized.

While allozyme and RFLP techniques score only a fraction of the genetic variation present in a DNA region, direct sequencing provides resolution of all polymorphisms present in an amplified fragment. The majority of sequencing, as it applies to the study of elasmobranch population structure and behaviour, has been mtDNA sequencing. As mtDNA is inherited maternally, it provides information about female behaviour, which is quite useful because elasmobranchs often exhibit female philopatry (Hueter *et al*., 2005). In addition, because mtDNA is haploid, it typically exhibits only one sequence within an individual, known as a haplotype, while nuclear DNA, which is diploid, may have two different nucleotides at a single position, one from each parent. Assessing differences between haplotypes is easy and accurate, modelling the relationships between different haplotypes is fairly straightforward and the likelihood of homoplasy between recently diverged haplotypes is remote (Goldman, 1993). A number of mitochondrial regions have been utilized, but the majority of work has involved the non-coding control region (Duncan *et al*., 2006; Keeney & Heist, 2006; Schultz *et al*., 2008; Portnoy *et al*., 2010), which typically features variable regions less constrained by selection than mitochondrial genes that code for

proteins. Even so the rate of mtDNA sequence evolution appears to be extremely slow in elasmobranchs and finding appropriate variation can be difficult (Martin *et al*., 1992).

The two most utilized nuclear DNA marker types are microsatellites and single nucleotide polymorphisms (SNP). Microsatellites are short stretches of nuclear DNA composed of a motif, up to six bp in length, repeated *n* times (*e.g.* CA_4 or $GATA_7$) centred between less repetitive flanking regions. Most size polymorphisms occur with the addition or excision of a repeat unit caused by improper alignment of the repetitive segment of DNA during replication (Levinson & Gutman, 1987; Weber & Wong, 1993). These size polymorphisms can be visualized using locus-specific polymerase chain reaction (PCR) primer pairs, each designed to anneal to a specific portion of one flanking region. Microsatellites are widely used in population genetics applications because they are often highly polymorphic (Weber, 1990) and can be analysed using relatively simple mutational models (Kimura & Ohta, 1978; Di Rienzo *et al*., 1994). Some problems associated with microsatellites are the presence of mutational events in the flanking regions that may cause shifts in allele size, which do not correspond to a change in the length of the repeat region being assayed (Angers & Bernatchez, 1997), homoplasy, where alleles appear the same size but are not identical by decent (Balloux *et al*., 2000) and null alleles, where mutations occur in primer regions causing allele-specific amplification failure during PCR (Chapuis & Estoup, 2007). Nonetheless, experienced researchers can take measures to avoid such problems and these markers have already been widely utilized in many species of elasmobranch (Table I). Because of their high levels of polymorphism, microsatellites remain the most widely used markers for inferring familial relatedness and polyandry.

SNPs on the other hand are single base pair substitutions distributed throughout the nuclear genome (Vignal *et al*., 2002). Unlike microsatellites, the chances of homoplasy in SNPs are low because the frequency of substitutions at any given site is low (Li *et al*., 1981; Martinez-Arias *et al*., 2001). Due to their slow mutation rate, the majority of SNPs are diallelic, meaning there is less information per locus and consequently large numbers of loci may be required (Glaubitz *et al*., 2003; Jones *et al*., 2009), however, they can be scored more efficiently than microsatellites and may eventually replace microsatellites for population-level studies in species for which a sufficient number of SNPs have been identified. These markers have yet to be widely utilized in elasmobranch research in large part due to the current paucity of nuclear DNA data in elasmobranchs.

While sequencing has advantages over SNPs and microsatellites, it still remains more expensive when a large number of samples are being assayed. DNA sequencing technology is, however, advancing rapidly and there may soon be complete genomes for thousands of species (Allendorf *et al*., 2010). Future population-level studies will probably involve comparing polymorphisms spread throughout the nuclear genome as opposed to genotypes at a handful of loci or mtDNA haplotypes.

INSIGHTS INTO REPRODUCTIVE BEHAVIOUR

MATING SYSTEMS

Traditionally, the understanding of mating systems in elasmobranchs came from direct observational data of mating in only a few species (Pratt & Carrier, 2001).

TABLE I. Elasmobranch species for which microsatellite resources are available, with number (*n*) of species-specific markers described and citation

Order	Family	Common name	Species	*n*	Citation
Pristiformes	Pristidae	Smalltooth sawfish	*Pristis pectinata*	11	Feldheim *et al.* (2010*b*)
Rajiformes	Rajidae	Little skate	*Leucoraja erinacea*	13	El Nagar *et al.* (2010)
		Thornback ray	*Raja clavata*	5	Chevolot *et al.* (2005)
Myliobatiformes	Myliobatidae	Longhead eagle ray	*Aetobatus flagellum*	8	Yagishita & Yamaguchi (2009)
		Spotted eagle ray	*Aetobatus narinari*	10	Sellas *et al.* (2011)
Hexanchiformes	Hexanchidae	Sixgill shark	*Hexanchus griseus*	14	Larson *et al.* (2009)
Squaliformes	Somniosidae	Portuguese dogfish	*Centroscymnus coelolepis*	10	Veríssimo *et al.* (2011*b*)
		Longnose velvet dogfish	*Centroselachus crepidater*	7	Helyar *et al.* (2011)
	Squalidae	Spiny dogfish	*Squalus acanthias*	8, 4	McCauley *et al.* (2004); Veríssimo *et al.* (2010)
Orectolobiformes	Orectolobidae	Nurse shark	*Ginglymostoma cirratum*	9	Heist *et al.* (2003)
	Hemiscylliidae	Whitespotted bambooshark	*Chiloscyllium plagiosum*	12	Ding *et al.* (2009)
	Rhincodontidae	Whale shark	*Rhincodon typus*	9	Ramírez-Macías *et al.* (2009)
	Stegostomatidae	Zebra shark	*Stegostoma fasciatum*	14	Dudgeon *et al.* (2006)
Carcharhiniformes	Triakidae	Tope	*Galeorhinus galeus*	13	Chabot & Nigenda (2011)
		Gummy shark	*Mustelus antarcticus*	12	Boomer & Stow (2010)
		Dusky smoothhound	*Mustelus canis*	15	Giresi *et al.* (2011*a*)
	Carcharhinidae	Blacknose	*Carcharhinus acronotus*	23	Giresi *et al.* (2011*b*)
		Blacktip shark	*Carcharhinus limbatus*	16	Keeney & Heist (2003)
		Sandbar shark	*Carcharhinus plumbeus*	3, 5	Heist & Gold (1999*b*); Portnoy *et al.* (2006)
		Spot-tail shark	*Carcharhinus sorrah*	12	Ovenden *et al.* (2006)
		Australian blacktip shark	*Carcharhinus tilstoni*	9	Ovenden *et al.* (2006)
		Lemon shark	*Negaprion brevirostris*	4, 3	Feldheim *et al.* (2001*a*, *b*)
		Blue shark	*Prionace glauca*	10	Fitzpatrick *et al.* (2011)
		Scalloped hammerhead	*Sphyrna lewini*	15	Nance *et al.* (2009)
		Bonntehead shark	*Sphyrna tiburo*	3	Chapman *et al.* (2004)
Lamniformes	Lamnidae	White shark	*Carcharodon carcharias*	5	Pardini *et al.* (2000)
		Shortfin mako	*Isurus oxyrinchus*	5	Schrey & Heist (2002)
	Odontaspididae	Sand tiger shark	*Carcharias taurus*	8	Feldheim *et al.* (2007)

Early research on nurse sharks *Ginglymostoma cirratum* (Bonnaterre 1788), for example, noted male mobbing behaviour, in which multiple males aggressively approached a single female (Carrier *et al*., 1994). The significance of such behaviour, in terms of male contribution to a single litter, has only recently been described by molecular inquiry, which indicated that five to seven sires had contributing to each of three *G. cirratum* litters of 29 to 39 pups (Heist *et al*., 2011).

While the number of sires contributing to those three litters was the largest reported, all species in which multiple litters have been examined have shown some level of genetic polyandry. This finding is important because in species with internal fertilization, such as elasmobranchs, monogamy or polygyny have traditionally been considered the dominant mating systems. In addition, direct observation of elasmobranch mating behaviour in the field is scant and the prevalence of polyandry suggests that the group courtship behaviour observed in nurse sharks and in whitetip reef sharks *Triaenodon obesus* (Rüppell 1837) by Whitney *et al*. (2004) is probably not atypical. Interestingly, the prevalence of genetic polyandry and the estimated number of sires contributing to individual litters differ greatly among species. For example, the majority of litters examined were genetically monogamous in bonnethead sharks *Sphyrna tiburo* (L. 1758) (17%; Chapman *et al*., 2004), shortspine spurdog *Squalus mitsukurii* Jordan & Snyder 1903 (11%; Daly-Engle *et al*., 2010) and spiny dogfish *Squalus acanthias* L. 1758 (30 and 17%; Lage *et al*., 2008; Veríssimo *et al*., 2011*a*). By contrast, the majority of litters in lemon sharks *Negaprion brevirostris* (Poey 1868), *G. cirratum* and thornback rays *Raja clavata* L. 1758 feature multiple sires (Ohta *et al*., 2000; Saville *et al*., 2002; Feldheim *et al*., 2004; Chevolot *et al*., 2007*a*). In addition, there is evidence that the prevalence of genetic polyandry may vary within species between populations. For *C. plumbeus* litters in Hawaii, only 40% (8/20) were found to be genetically polyandrous, whereas 85% (17/20) of *C. plumbeus* litters in the western North Atlantic Ocean had multiple sires (Daly-Engel *et al*., 2007; Portnoy *et al*., 2007).

There are several important caveats that must be considered when using molecular techniques to assess levels of genetic polyandry. First, the techniques employed in such studies detect genetic polyandry by looking for the presence of more than two paternal alleles in multiple loci in a single litter. This means that detecting multiple sires will be more difficult in species with small litter size (Fiumera *et al*., 2001) and thus the power of such analysis will be highly dependent on the number and variability of the markers employed (Neff & Pitcher, 2002). In addition, females may mate with multiple males (behavioural polyandry) without producing a multiply sired litter. Finally, even if genetically monogamous females are also behaviourally monogamous within a year, they probably change mates across years, a behaviour known as serial monogamy, which is a type of temporal polyandry (Sugg & Chesser, 1994; Karl, 2008).

Despite these considerations, the ubiquitous presence of genetic polyandry to varying degrees across elasmobranch species has led to questions about the benefits of this behaviour. Portnoy *et al*. (2007) failed to find any increase in litter size (realized fecundity) for genetically polyandrous female *C. plumbeus* in the western North Atlantic Ocean, suggesting that multiple mating does not provide direct benefits. DiBattista *et al*. (2008*a*) found no indication that *N. brevirostris* pups from multiply sired litters had increased survival rates or genetic diversity (both proxies for indirect benefits), as compared to pups from singly sired litters. Given the costs

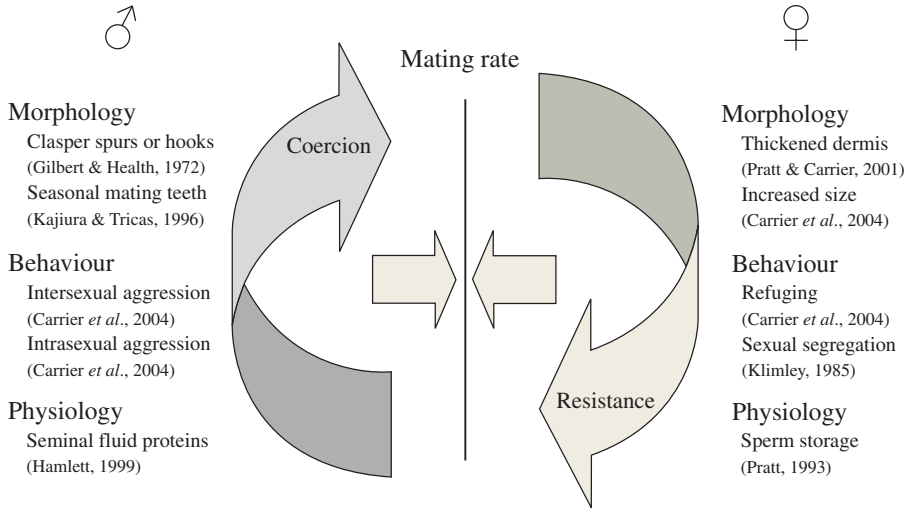

FIG. 1. Diagrammatic representation of sexually antagonistic co-evolution in which conflict over mating rate between the sexes leads to the evolution of secondary characteristics that aid in male aggression and female resistance. Listed for each sex are aspects of morphology, behaviour and physiology, which may have been influenced by such a dynamic along with citations that discuss them further.

associated with mating for female sharks, *i.e.* blood loss associated with male biting and internal trauma to the female lower reproductive tract (Pratt, 1979; Pratt & Carrier, 2001), the inability to detect any benefits for females has been somewhat surprising. This has led several authors to conclude that females may be engaging in convenience polyandry (Portnoy *et al*., 2007; DiBattista *et al*., 2008*a*; Daly-Engel *et al*., 2010), where females increase their situational fitness by engaging in superfluous matings, thereby avoiding costs associated with resistance (Alcock *et al*., 1978; Arnqvist & Nielsen, 2000). Observational data suggests that female resistances and male harassment are common behaviours in elasmobranchs (Pratt & Carrier, 2001). When increases in fitness are associated with opposing male and female reproductive strategies, a Fisherian runaway may occur in which extreme phenotypes evolve to aid in male aggression and female resistance (Chapman *et al*., 2003). Aspects of the reproductive morphology, behaviour and physiology of many elasmobranchs support the notion that this type of sexual antagonistic co-evolution may be occurring (Fig. 1). While convenience polyandry is an appealing hypothesis, it is difficult to test for and does not rule out cryptic female benefits. Comparative studies across populations that differ in environmental and demographic characteristics are probably needed to further address these questions.

An alternative way to address questions about mating systems is to collect tissues from large numbers of juveniles of known ages in a single nursery area. When samples can be taken from adults as well, assigning individual progeny back to sampled parents using multilocus genotype data is straightforward. This approach can be followed in many cases as elasmobranch researchers often have access to some percentage of the females who enter nursery grounds for parturition. When no parental data are available, polyandry can be detected by looking for full sibling or half-sibling relationships using an inferred maternal genotype. The resulting analyses

can provide information about genetic polyandry roughly equivalent to that obtained by sampling pregnant females and their embryos but in a far less invasive manner.

NUMBER OF BREEDERS

Methodologies that involve nearly exhaustive sampling of juveniles in fairly closed nursery systems may allow researchers to estimate the number of breeders responsible for a group of progeny, which is of interest both from the standpoint of ethology and conservation and management. Feldheim *et al.* (2004), for example, was able to take multilocus genotypes from 735 juvenile *N. brevirostris* caught at Bimini Islands, The Bahamas, and assign 96% back to single females and 66% back to single males. The resulting count of 129 breeding adults (45 females and 84 males) should be taken as an underestimate of the true number breeders but provides insight into the importance of this nursery area. A similar methodology assigned *N. brevirostris* pups at Marquesas Key, FL, U.S.A., to 46 females and 163 males (DiBattista *et al.*, 2008*b*). For *C. plumbeus*, using Delaware Bay, DE, U.S.A and the Eastern Shore lagoons, VA, U.S.A. as nursery grounds, this same approach was largely unsuccessful. In this case, it was probably caused by a relatively small sample size of juveniles per year ($n = 100$) compared to a large number of breeding adults (Portnoy, 2010). Instead, estimates of the effective number of breeders (N_b) were made using both a modified temporal method and a linkage disequilibrium method (Portnoy *et al.*, 2009). Estimates of N_b were consistent across years within each nursery ground, with harmonic means of 1059 for Delaware Bay and 511 for Eastern Shore. These data were used to demonstrate the importance of Delaware Bay as a nursery. It is important to note that effective size (N_e)and census size (N_c) are not equal, and there is no direct relationship between the two measures. In fact, the measures often vary greatly with N_e:N_c ratios from 10^{-5} in marine species to nearly 1·0 in terrestrial vertebrates (Frankham, 1995; Hedrick, 2005). While N_b:N_c calculated for *C. plumbeus* pupping in Delaware Bay, where an independent estimate of N_c was available, was close to 0·5 (Portnoy *et al.*, 2009), it should not be assumed that this relationship will hold across elasmobranch species.

REPRODUCTIVE ODDITIES

Molecular methods have also been used to uncover previously unknown aspects of elasmobranch reproductive biology. Parentage analysis, for example, has also been used to describe parthenogenesis in three species of captive sharks. Virgin birth was originally suggested for a female *S. tiburo* that produced a single offspring, although it had never been in the presence of a conspecific male shark while sexually mature. The pup was genotyped at four microsatellite loci, and no non-maternal alleles were detected (Chapman *et al.*, 2007). Since then the phenomena has been observed in two other species: a blacktip shark *Carcharhinus limbatus* (Müller & Henle 1839) and a whitespotted bamboo shark *Chiloscyllium plagiosum* (Bennett 1830) (Chapman *et al.*, 2008; Feldheim *et al.*, 2010*a*). More in-depth analysis will be needed to understand whether the capability to produce offspring without paternal contribution is important in wild populations.

Hybridization in sharks species has received little attention in part because the conserved morphology of sister species may make identification of hybrids difficult (Heist, 2004). Recently, it has been suggested that there is extensive hybridization

along the Australian coast between *C. limbatus* and *C. tilstoni* (Morgan *et al*., 2011). Previous work had noted taxonomic uncertainty and confusion in species identification for these closely related species (Keeney & Heist, 2006; Ovenden *et al*., 2010). Determining how widespread a phenomenon hybridization is will require further work on closely related species pairs with overlapping distributions.

POPULATION STRUCTURE AND SUB-STRUCTURE

DISPERSAL CAPABILITIES

Elasmobranchs vary greatly in activity and vagility from pelagic ram-irrigating species that travel great distances, *e.g.* shortfin mako *Isurus oxyrinchus* Rafinesque 1810, to benthic active branchial-irrigating species, *e.g.* zebra shark *Stegostoma fasciatum* (Hermann 1783), which are more sedentary. Large pelagic species have broader distributions and smaller numbers of species than do benthic forms indicating that isolation leading to speciation is more common in smaller and more benthic species (Musick *et al*., 2004). Thus, current populations of smaller more benthic forms would be expected to exhibit greater genetic heterogeneity among populations than do larger pelagic species, and this is generally the case. In marine teleosts, even species that are highly sedentary as adults may be genetically homogeneous over vast regions because of passive larval drift (Shulman & Bermingham, 1995). On the other hand, elasmobranchs either hatch or are born with precocious development and thus gene flow occurs through the active movement of juveniles or adults (Heist, 2008). The amount of gene flow among regions necessary to reduce genetic heterogeneity to levels that are barely detectable is small, on the order of a few individuals per generation (Waples, 1998). Members of pelagic species may migrate across entire ocean basins, *e.g.* blue shark *Prionace glauca* (L. 1758) (da Silva *et al*., 2010), and are likely to be genetically homogeneous across vast regions even if populations are demographically discrete. This creates difficulties for identifying populations in elasmobranchs because even modern molecular tools and sophisticated analytical methods have relatively low power to identify demographically discrete populations in the presence of gene flow on the order of several to tens of individuals per generation (Waples & Gaggiotti, 2006). Thus, failure to reject the null hypothesis that samples come from a single panmictic population should not be taken as proof that only a single population exists (Heist, 2008). The power of the analyses in earlier studies (Heist *et al*., 1995, 1996*b*) should also be considered, even some recent studies (Stow *et al*., 2006) examined relatively small numbers of individuals and few loci; thus a failure to reject the hypothesis of panmixia may also be due to a lack of power. Power analyses can be useful to determine whether a given level of differentiation could be detected using the panel of markers employed (Schrey & Heist, 2003).

Where active species are continuously distributed along coastlines there is generally negligible heterogeneity over geographic distances <1000 km, although there are notable exceptions in sedentary or philopatric species. Studies of relatively large active species that found no heterogeneity along continuous or nearly continuous stretches of coastline within oceans include: studies of *C. plumbeus* from the western North Atlantic Ocean (Heist *et al*., 1995), dusky shark *Carcharhinus obscurus* (LeSueur 1818) and scalloped hammerhead shark *Sphyrna lewini* (Griffith & Smith 1834) in Australia and Indonesia, (Ovenden *et al*., 2009) and *N. brevirostris*, from

the Bahamas to Brazil (Feldheim *et al*., 2001*a*). Even small species are generally homogeneous along the continental margins. For example, *R. terraenovae* from the U.S.A. and Mexico, (Heist *et al*., 1996*a*), Brazilian sharpnose sharks *Rhizoprionodon lalandii* (Müller & Henle 1839) in Brazil (Mendonca *et al*., 2009) and narrownose shark *Mustelus schmitti* Springer 1939 from Uruguay (Pereyra *et al*., 2010) all exhibited either no heterogeneity or negligible heterogeneity across the sampled range. Chevolot *et al*. (2007*b*) found similar mtDNA haplotype frequencies of thorny skate *Amblyraja radiata* (Donavan 1808) from the North Sea, Iceland and Newfoundland.

While the previous examples are of active species, benthic species that are sedentary may exhibit genetic heterogeneity across relatively small geographic ranges, *i.e.* <1000 km). Examples include studies of *S. fasciatum* (Dudgeon *et al*., 2009), which found significant differences in mtDNA and microsatellites among samples collected in north-eastern Australia and Papua New Guinea and leopard sharks *Triakis semifasciata* Girard 1855 (Lewallen *et al*., 2007), which exhibited heterogeneity in mtDNA and nuclear markers along the coast of California, U.S.A. Gaida (1997) found allozyme heterogeneity in Pacific angel sharks *Squatina californica* Ayres 1859 from the Channel Islands off the coast of California. The explanation for heterogeneity among islands, some of which were <100 km apart, was that the channels >500 m deep between the islands prevented gene flow. Studies of the round stingray *Urolophus halleri* (Cooper 1863) in the same region found much greater heterogeneity between the California mainland and offshore islands than was seen over comparable distances along the mainline coast (Plank *et al*., 2010). Phillips *et al*. (2011) detected significant mtDNA heterogeneity in three species of sawfishes (*Pristis*) between the Gulf of Carpentaria and the west coast of Australia and concluded that dispersal in sawfishes was limited, at least for females. Some species that are homogeneous at smaller geographic scales exhibit heterogeneity at larger scales, even in continuously distributed populations. *Carcharhinus limbatus* exhibited no heterogeneity in mtDNA haplotype frequencies among nursery areas along the west coast of Florida but did exhibit heterogeneity in more broadly dispersed samples from South Carolina, Florida, Texas and the Yucatan (Keeney *et al*., 2005). While there is only slight heterogeneity in *R. clavata* populations in British waters, populations from the Mediterranean Sea, Azores and European mainland coast were all heterogeneous (Chevolot *et al*., 2006).

Highly vagile coastal species often exhibit heterogeneity across ocean basins indicating that the open ocean can be a barrier for even large, active species. *Carcharhinus plumbeus* (Portnoy *et al*., 2010), *S. lewini* (Duncan *et al*., 2006), tope *Galeorhinus galeus* (L. 1758) (Chabot & Allen, 2009) and *N. brevirostris* (Schultz *et al*., 2008) all exhibit heterogeneity between Atlantic and Pacific Ocean populations. Sand tiger sharks *Carcharias taurus* Rafinesque 1810 exhibit mtDNA patterns consistent with distinct populations between ocean basins, although there was some sharing of haplotypes between eastern and western Australia and between Brazil and South Africa (Ahonen *et al*., 2009). Among pelagic sharks both whale sharks *Rhincodon typus* Smith 1828 (Castro *et al*., 2007) and *I. oxyrinchus* (Heist *et al*., 1996*b*; Schrey & Heist, 2003) exhibit small, but significant, levels of genetic heterogeneity among ocean basins. Hoelzel *et al*. (2006) found no heterogeneity among basins in basking sharks *Cetorhinus maximus* (Gunnerus 1765). Little is known about dispersal in deep-sea sharks, but Veríssimo *et al*. (2011*b*) detected no heterogeneity

among Portuguese dogfish *Centroscymnus coelolepis* Barbosa du Bocage & de Brito Capello 1864 from the North and South Atlantic Oceans.

PHILOPATRY

Philopatry, which is the tendency of an animal to remain or return to a particular location, can result in genetic population structure among units that mix at other locations. Examples include sea turtle, whales and salmonids, all of which have reproductively discrete units that overlap during feeding. Many sharks use nursery areas that are distinct from adult habitat to deposit their young (Knip *et al*., 2010; Speed *et al*., 2010). If juveniles of both sexes do not mix with other populations or segregate, population structure may be detected using both nuclear and mitochondrial markers. This is analogous to what happens in anadromous salmonids in which genetic heterogeneity at nuclear and mitochondrial markers is maintained among spawning areas despite the mixtures of stocks in the open ocean (Allendorf & Waples, 1996). If females return to natal nursery areas to deliver their young, after mating with males from multiple nursery areas, nuclear markers will remain homogeneous among regions while mitochondrial markers will diverge among natal nurseries. A similar phenomenon is seen in sea turtles (Karl *et al*., 1992; Bowen & Karl, 1997) and whales (Palumbi & Baker, 1994; Gladden *et al*., 1997; Lyrholm *et al*., 1999), which exhibit higher levels of mitochondrial than nuclear heterogeneity among reproductive areas. Pardini *et al*. (2001) observed much greater levels of mtDNA heterogeneity than microsatellite heterogeneity among white sharks *Carcharodon carcharias* (L. 1758) collected from South Africa and from Australia and New Zealand. The authors argued that in this respect *C. carcharias* are more similar to whales than they are to other large fishes. Since that publication, a *C. carcharias* tagged in South Africa was tracked in Australia and was later observed back in South Africa (Bonfil *et al*., 2005), indicating that the high degree of mtDNA structure is maintained in the presence of movement among regions. Differences between nuclear and mitochondrial estimates of genetic heterogeneity among geographic locations have also been attributed to philopatry in *C. limbatus* (Keeney *et al*., 2005), *I. oxyrinchus* (Schrey & Heist, 2003), *R. clavata* (Chevolot *et al*., 2006), *N. brevirostris* (Schultz *et al*., 2008), *C. plumbeus* (Portnoy *et al*., 2010) and bull shark *Carcharhinus leucas* (Müller & Henle 1839) (Karl *et al*., 2011). Care must be taken when interpreting these results because mtDNA has a smaller effective population size and thus faster rate of genetic drift and coalescence among populations (Birky, 2001). In addition, microsatellite heterogeneity is limited by high levels of within-population variation (Hedrick, 1999). Because of these differences in drift and variation, there are scenarios in which equal rates of migration of males and females can result in divergent estimates of heterogeneity using mtDNA and microsatellites (Buonaccorsi *et al*., 2001).

TAXONOMY AND SPECIES COMPOSITION

IDENTIFICATION OF CRYPTIC SPECIES

Elasmobranchs are morphologically conserved, and differences between species are often subtle and confounded by variation within species. Thus, molecular markers are proving very useful for identifying cryptic species even in areas where the

faunas are well studied. For example, Quattro *et al*. (2006) found evidence for an unrecognized species of hammerhead shark (*Sphyrna*) in the south-eastern U.S.A. based on a combination of nuclear and mitochondrial markers. Some of the earliest studies on elasmobranch molecular genetics (Solé-Cava *et al*., 1983; Solé-Cava & Levy, 1987) found evidence for three cryptic species of *Squatina* in Brazil. Sandoval-Castillo & Rocha-Olivares (2011) suggested cryptic speciation in golden cownose ray *Rhinoptera steindachneri* Evermann & Jenkins 1891 from the Baja California Peninsula, Mexico based on the presence of two highly divergent mtDNA lineages, one of which was restricted to the Pacific Ocean coast, whereas the other was found in 92% in the Gulf of California, Mexico specimens. On the basis of a combination of morphological and genetic data, Gardner & Ward (2002) detected the presence of two unrecognized species of *Mustelus* in Australia. Other examples of cryptic elasmobranchs discovered or confirmed with genetic markers include two species of ornate wobbegong [*Orectolobus* spp.; Corrigan *et al*. (2008)], two genetically differentiated and spatially segregated forms of common skate [*Dipturus* spp.; Griffiths *et al*. (2010)] and an additional species of skate found in the Antarctic Ocean [*Bathyraja* sp.; Smith *et al*. (2008)]. Some groups, *e.g*. lanternsharks of the genus *Etmopterus* (Straube *et al*., 2011), are likely to undergo significant revisions as populations thought to be distinct species are synonymized while additional species are recognized.

A number of elasmobranchs have multiple allopatric populations isolated by continents or deep ocean basins. Sometimes these populations exhibit phylogenetically distinct mtDNA profiles and may be considered distinct species. For example, *S. acanthias* populations from the North Pacific Ocean are very distinct from those in the Atlantic and South Pacific Oceans and may warrant recognition as a distinct species (Hauser, 2009; Veríssimo *et al*., 2010). Chabot & Allen (2009) suggested that *G. galeus* populations from the North Pacific Ocean are heterospecific to those in other ocean basins and that perhaps Atlantic Ocean and southern hemisphere populations may be further divided into additional taxa. Shovelnose guitarfish *Rhinobatos productus* Ayres 1854 from the Atlantic Ocean and Gulf of California exhibit distinct mtDNA profiles and may be heterospecific (Sandoval-Castillo *et al*., 2004). Richards *et al*. (2009) found that differences in mtDNA and nuclear ribosomal internal transcribed spacer (ITS) sequences between populations of spotted eagle ray *Aetobatus narinari* (Euphrasen 1790) were greater than those between other pairs of batoid taxa and recommended that either three species (Atlantic, eastern Pacific, and western and central Pacific Oceans) be recognized or, alternately, two species be recognized with eastern Pacific and Atlantic Ocean populations as subspecies. The later scenario was confirmed through morphological analysis and the species *Aetobatus ocellatus* (Kuhl 1823) redescribed (White *et al*., 2010). Determining the amount of variation between populations that is sufficient to warrant recognition of distinct species is controversial. Proposed criteria for recognizing distinct species include reciprocal monophyly (Wiens & Penkrot, 2002), benchmark levels of sequence variation (Lefébure *et al*., 2006) and variation between populations (species) that is 10-fold that of mean intraspecific variation (Hebert *et al*., 2004).

SPECIES COMPOSITION AND FISHERIES FORENSICS

The way that elasmobranchs are processed before landing or sale, typically with fins and heads removed and sometimes with only fins retained, makes DNA analysis

useful for species identification and for tracking trade of shark parts (Shivji, 2010). Due to the difficulty in accurately identifying specimens and the similarity between many prohibited and harvestable species, forensic identification of parts is necessary for species-specific monitoring. *Carcharhinus obscurus* is protected in the U.S.A. but is very similar to several other large carcharhinid sharks, *e.g. C. plumbeus* and bignose shark *Carcharhinus altimus* (Springer 1950), that are commercially harvested. Fortunately, these species are easily distinguished by distinct mtDNA profiles (Heist & Gold, 1999*a*; Pank *et al*., 2001). Using isoelectric focusing, which is a protein-based technique, Smith & Benson (2001) found that of shark fillets labelled as *M. lenticulatus* in New Zealand, 40% were from other species, some of which were prohibited species. Shivji *et al*. (2002) described a rapid and streamlined approach for distinguishing among six species of sharks [*I. oxyrinchus*, longfin mako *Isurus paucus* Guitart 1966, porbeagle *Lamna nasus* (Bonnaterre 1788), *C. obscurus*, silky shark *Carcharhinus falciformis* (Müller & Henle 1839) and *P. glauca*] likely to be encountered in North Atlantic Ocean fisheries. The method could not, however, distinguish between *C. obscurus* and oceanic whitetip *Carcharhinus longimanus* (Poey 1861). The streamlined methods described by Shivji *et al*. (2002) work for a limited and pre-determined set of taxa. When the species identity of a specimen is truly unknown, sequencing a portion of the mitochondrial genome and comparing that sequence to published data from reliably identified specimens is an effective means of species identification (Ward *et al*., 2008; Wong *et al*., 2009) and may even identify the population of origin (Shivji, 2010).

DNA barcoding (Hebert *et al*., 2003) is an approach that is useful for both species identification (Holmes *et al*., 2009) and the discovery of new species. Typically this involves obtaining DNA sequence data from all or part of the mitochondrial cytochrome oxidase I gene and comparing the data to the Barcoding Of Life Database (BOLD; www.barcodinglife.org) (Ratnasingham & Hebert, 2007). Ward *et al*. (2009) determined that DNA barcodes successfully identify >98% of marine fishes and 93% of freshwater fishes. DNA barcodes will often fail to resolve species that have diverged relatively recently and have large effective population sizes (Hickerson *et al*., 2006) and maternal inheritance will confound the results in cases of hybridization because all offspring will exhibit only the mtDNA profile of the maternal parent. If backcrossing occurs, the mtDNA originating in one of the parent species may be transferred to members of the other species, a phenomenon known as introgression. This can further exacerbate problems with species identification, as individuals carrying introgressed mtDNA will have the morphological characteristics of one species and the DNA barcode of another. Ward *et al*. (2009) were unable to resolve whether hybridization resulted in the sharing of DNA barcodes among species of *Urolophus, Carcharhinus* and *Pristiophorus* or whether some of the data on the BOLD database were from misidentified specimens. As mtDNA is a single haploid marker, conclusions about the presence of cryptic species should be confirmed through the examination of morphology and nuclear markers and ideally barcode data should be based on vouchered specimens (Ward *et al*., 2009). Examples of the use of DNA barcodes to demonstrate the presence of heterospecific lineages include studies of river sharks [*Glyphis*, Wynen *et al*. (2009); sharpnose sharks *Rhizoprionodon* (Mendonca *et al*. (2011)] and three confusing species of carcharhinid sharks [graceful shark *Carcharhinus amblyrhynchoides* (Whitley 1934)*, C. limbatus and C. tilstoni* in Australia (Ward *et al*., 2008; Ovenden *et al*., 2010)].

CONCLUSIONS

Molecular tools have provided a wealth of data, complimentary and novel, concerning the reproductive behaviour, population structure and species status of elasmobranchs. With the availability of technologies such as next-generation sequencing, which literally produces millions of reads per run (Mardis, 2008), researchers in the near future will be able to address many of the questions raised in this review with a more comprehensive genomic approach. Using these technologies large numbers of neutral markers, such as microsatellites or SNPs, can be easily obtained. With a sampling of loci distributed throughout the genome, neutral markers linked to loci under selection can be detected (Luikart *et al*., 2003). This type of approach may be especially important for detecting important aspects of localized adaptive variation specific to populations or nursery areas, which can persist despite high levels of gene flow (King & Lawson, 1995; McKay & Latta, 2002). Furthermore, mRNAs can be efficiently preserved in the field allowing researcher to characterize and explore differences in proteomes between the sexes, within species across their distribution and between closely related species. Finally, as the price bp^{-1} of large-scale sequencing continues to decrease, comparative genomic approaches will become a more viable option for researchers interested in understanding the evolution of elamobranch reproductive behaviour and the differences between cryptic taxa.

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