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Molecular Insights into Elasmobranch Reproductive Behavior for Conservation and Management

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CONTENTS

10.1 Introduction

Molecular approaches in elasmobranch research have been used predominantly for phylogenetic inference or to define population structure. However, a variety of high-resolution molecular markers allow for the interpretation of complex patterns of molecular variance as well as the fast and accurate reconstruction of individual molecular profiles. Such techniques can be useful in augmenting current understanding of the reproductive biology of elasmobranchs and, where experimental or observational approaches may be difficult, can provide novel insights. The increased access and ease of utilizing molecular approaches makes the techniques and concepts discussed in this chapter useful for any elasmobranch researcher interested in the study of reproductive biology.

10.2 Molecular Markers

The first step toward utilizing a molecular approach to augment reproductive studies is informed selection of appropriate markers. Many choices exist but the following discussion will be limited to three types: microsatellites, single nuclear polymorphisms (SNPs), and direct DNA sequence data. All three are desirable because they can be highly polymorphic, making them more informative than markers such as restriction fragment length polymorphisms (RFLPs) and allozymes. They also are easier to interpret than amplified fragment length polymorphisms (AFLPs) and more reproducible than randomly amplified polymorphic DNAs (RAPDs). However, there are strengths and weaknesses of each of these marker types and the decision to use one or the other will be based on the type of questions being asked and the resources available to the researcher.

10.2.1 Microsatellites

Microsatellites are short stretches of repetitive DNA found in the nuclear genome, sometimes referred to as short tandem repeats (STRs) or variable number tandem repeats (VNTRs). An individual microsatellite is composed of a motif up to six base pairs that repeats n times, for example CACACACA, which often is expressed as $(CA)_4$, with less repetitive sections of DNA (flanking regions) on either side of the microsatellite repeat. Individual microsatellites and their flanking regions can be amplified using the polymerase chain reaction (PCR) and appropriate primers, which are sequences generally 18 to 24 base pairs long and designed to be complementary to areas in the flanking regions. Individual alleles at a microsatellite are scored as size polymorphisms that generally are differences in the number of individual repeats [e.g., $(CA)_4$ versus $(CA)_6$]. Microsatellite markers have the desirable properties of being highly polymorphic, codominantly inherited, and widely distributed throughout genomes (Weber 1990).

However, use of microsatellite markers relies on the assumption that all size variation is due to changes in the number of repeat units. Changes in repeat number are thought to occur via "slip-strand mispairing" where the repetitive segment aligns improperly during DNA replication, resulting in excision or addition of a repeat unit (Levinson and Gutman 1987; Weber and Wong 1993). This simple model of mutation is known as the stepwise mutational model or SMM (Kimura and Ohta 1978) and is appealing because it makes the

relationship between individual alleles easy to understand and model. However, this is generally an oversimplification, as most loci undergo some percentage of mutations that involve more than one repeat unit. The degree to which the SMM is violated depends on the individual microsatellite; consequently, analysis of microsatellite data often requires use of the more complicated two-phase model (TPM) that takes into account both stepwise and larger repeat additions and/or deletions (Di Rienzo et al. 1994; Ellegren 2004). Moreover, many microsatellites feature mutations that do not involve the repetitive unit (Angers and Bernatchez 1997), causing shifts in size that may or may not correspond to the repeat unit. Other mutations may affect the binding affinity of a PCR primer for the target sequence, leading to amplification failure during PCR (Chapuis and Estoup 2007). Loci that are highly polymorphic may feature several of these confounding problems and the risk of homoplasy (where two alleles appear identical but have different genealogical history) may be high (Balloux et al. 2000). Finally, microsatellites frequently exhibit PCR artifacts, such as stutter bands and allele size changes, which further complicate scoring (Figure 10.1). For studies where the goal is individual identification, as in parentage, kinship, or genetic tagging, the nature of mutational events is usually unimportant (as

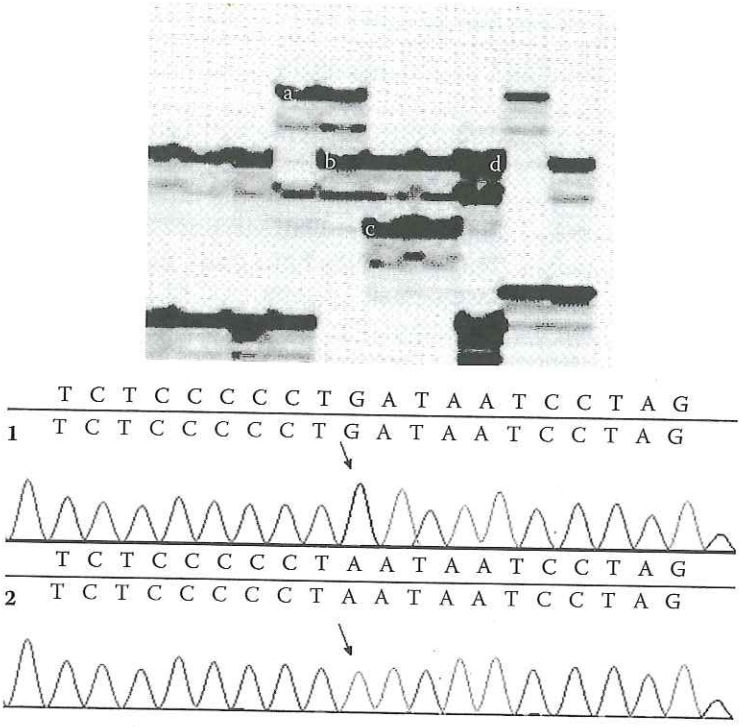

FIGURE 10.1

A color version of this figure follows page 336. A comparison of microsatellite data and SNP data. Top panel is a gel image generated on a LiCor 4200 Global IR² system. Each band is a different allele detected in an array of juvenile *Carcharhinus plumbeus* and each column is one individual. The relationship between alleles marked a, b, and c is assumed to be the result of either the loss or gain of repetitive motif units but is only scored as a size polymorphism so one cannot be sure. Allele d shows a strong stutter band, which is a PCR artifact that may make accurate scoring difficult. Bottom panel is an image of a 20 base pair read that differs by one base pair substitution. In this case the relationship between alleles is clear; 1 has a G at site 10 and 2 has an A at site 10. When scored as an SNP all individuals sampled will show one state or the other for each allele at this locus. This provides less information than a polymorphic microsatellite locus.

mutational rates tend to be low). Genotyping error, however, has the potential to seriously confound results. For other types of studies such as population genetics, where the mutational model may be more important, researchers who are aware of these problems can screen and select loci to avoid them.

Another potential pitfall associated with the use of microsatellites is the expense and time associated with creating enriched libraries and designing species-specific markers. This problem can be circumvented by the use of microsatellites designed for one species on a closely related species. The opportunity to do this is increasing in elasmobranch research, as markers are becoming available for an ever widening range of species across taxa [e.g., *Carcharadon carcharius* (Pardini et al. 2001); *Carcharhinus limbatus* (Keeney and Heist 2003); *Stegostoma fasciatum* (Dudgeon et al. 2006); *Raja clavata* (Chevolot et al. 2007)]. While this type of approach is useful, it is important to note that many microsatellites are most polymorphic in the species for which they have been isolated. For some applications where high levels of polymorphism are important, such as parentage, species-specific markers are likely to be preferable (for a more complete review of microsatellites and their use, see Goldstein and Schlötterer 1999 and Ellegren 2004).

10.2.2 SNPs (Single Nuclear Polymorphisms)

SNPs are single base pair polymorphisms, generally in the nuclear genome. Whereas microsatellite distribution in the genome is nonrandom and dependent on both motif and organism of interest (Chakraborty et al. 1997), SNPs are densely distributed throughout genomes (Vignal et al. 2002). Because SNPs are single base pair substitutions, they have a very simple mutational model and a low probability of homoplasy, due to the low frequency of these substitutions at a given site (Li, Gojobori, and Nei 1981; Martinez-Arias et al. 2001). However, this slow mutational rate means that most SNPs have only two alternate states, making them diallelic. For this reason, datasets must be composed of large numbers of SNPs or panels of linked SNPs to gain the same level of resolution available from a modest number of microsatellites (Glaubitz, Rhodes, and DeWoody 2003; Jones et al. 2009). Finally SNPs are easy to score and interpret, making experimenter error less common (Figure 10.1).

There are many cost-effective and reliable methods for amplifying and scoring SNPs (reviewed in Kwok 2001), but designing and optimizing these markers may require significant sequencing and cloning that can be cost prohibitive (methods of design are reviewed in Vignal et al. 2002). A more cost-effective strategy involves screening large numbers of sequences present on web resources such as GenBank (available at http://www.ncbi.nlm.nih.gov/). This strategy works best for researchers working on either model organisms or intensely studied taxa. Currently, this approach may be problematic for elasmobranch researchers due to the paucity of available materials; however, some exceptions exist (see Nazarian et al. 2007). In addition, much of the available sequence data have originated from functional segments of the genome, meaning that the acquisition of SNPs from neutral parts of the genome (desirable for some types of analysis) may be difficult. While these markers have not yet found wide application in elasmobranch behavioral ecology, as the number of web-available sequences increases, their use may become more common (for a more complete review of SNPs and their use, see Kwok 2001 and Vignal et al. 2002).

10.2.3 DNA Sequence Data

DNA sequence data combines many of the positive aspects of the previously discussed molecular markers and can be nuclear or mitochondrial. Sequences are typically

polymorphic, because mutations at multiple sites along the sequenced segment are assayed, and scoring the base changes is easy and accurate. Because the exact mutations that define different haplotypes can be observed directly, modeling relationship(s) between or among sequences is generally straightforward (Goldman 1993). The amount of information available from sequence data, however, is highly dependent on the portion of the genome sequenced. Coding regions (e.g., exons in nuclear genes or protein-coding mitochondrial genes) may be too conserved to be useful in behavioral ecology. Noncoding regions (e.g., introns in nuclear genes or the mitochondrial control region) are generally not constrained by selection and thus are more variable. While these regions may be more useful in behavioral ecology, too much variation may make the relationship(s) between or among sequences problematic for some applications.

There are two major concerns with sequence data. The first involves the design of appropriate primers that amplify the correct target region. This is especially important when amplifying nuclear DNA where there may be multiple, nonorthologous copies of the same gene or pseudogenes (Hillis et al. 1996). In addition, nuclear mitochondrial DNAs (numts; Lopez et al. 1994), mitochondrial DNA that has been transposed into the nuclear genome, can confound analysis of mitochondrial loci. Identification of these artifacts may require significant cloning and sequencing. The second and related concern is price. Although improved technology has made sequencing relatively inexpensive and time efficient, sequencing a large number of individuals is still far more expensive than using either microsatellites or SNPs. In addition, elasmobranchs exhibit an extremely slow rate of mtDNA sequence evolution (Martin, Naylor, and Palumbi 1992). Researchers interested in using mtDNA may therefore need long sequences to capture enough variation, further increasing expense (for a more complete review of sequence data and their use, see Avise 1987 and Ellegren 2004).

For elasmobranch researchers interested in using DNA sequence data, a variety of primers and sequences are available online for both nuclear and mitochondrial DNA. Using these resources to design primers to amplify target sequences in a species of interest may prevent some of the problems (e.g., decreasing the time needed to optimize primers) listed previously. This type of approach has been utilized for multiple phylogeographic studies involving sharks, all of which used the same primer set to amplify the mtDNA control region (Duncan et al. 2006; Keeney and Heist 2006; Schultz et al. 2008; Portnoy 2008).

10.3 Mating System Analysis

10.3.1 Introduction

The use of highly polymorphic molecular markers has revolutionized our understanding of mating systems. Polygynandry (in which both sexes mate with multiple partners) had long been considered the dominant mating system in aggregate spawners with external fertilization and molecular inquiry has confirmed this assumption (DeWoody and Avise 2001; Myers and Zamudio 2004). However, in species with internal fertilization (e.g., mammals and birds), monogamy or polygyny (in which males mate with multiple partners) were considered the dominant mating systems. Studies using high-resolution molecular markers have revealed that polyandry (in which females mate with multiple partners) is common in these species even when observational study suggested female monogamy (Gibbs et al. 1990; Carling, Wiseman, and Byers 2003; Goetz, McFarland, and Rimmer 2003;

Yamaguchi et al. 2004). That females are capable of producing offspring sired by multiple males should not be a surprise, as most animals, even humans, that ovulate multiple eggs have the potential to be inseminated by multiple males (Girella et al. 1997).

There are two major categories of benefits (direct and indirect) that can be used to explain female polyandry. Direct benefits increase the number of offspring a female can produce. These benefits may take the form of nutritive gifts that can be invested in the production of ova, increased sperm volume, or shared parental care (Gray 1997; Avise et al. 2002; Gosselin, Sainte-Marie, and Bernatchez 2005). For species where fecundity is fixed and sperm is not a limiting factor, indirect benefits that increase survivorship and eventual reproductive success of offspring have been invoked to explain polyandrous behavior (Zeh and Zeh 2001). Indirect benefits include increased additive genetic variance in progeny, bet-hedging in unstable environments, precopulatory or postcopulatory trading-up, and postcopulatory defense against genetic incompatibility (Zeh and Zeh 1997; Newcomer, Zeh, and Zeh 1999; Jennions and Petrie 2000; Tregenza and Wedell 2000). While these are appealing explanations for female behavior, they are extremely hard to demonstrate (Byrne and Roberts 2000; Garner and Schmidt 2003). Detecting indirect benefits is even more difficult in long-lived species with late maturity because such benefits can only be measured by reproduction of an individual's offspring. What's more, there is some doubt that indirect benefits can balance costs often associated with mating (Yasui 1998). This has led researchers to suggest that sometimes there may be no benefit to female remating. Instead, females remate to avoid costs associated with resistance, a phenomenon known as convenience polyandry (Alcock et al. 1978).

10.3.2 Methods

Kinship analysis, using highly polymorphic molecular markers, is a straightforward way to assess mating systems (Blouin et al. 1996; Fiumera et al. 2001; Jones and Ardren 2003). The general strategy involves collecting tissues from large numbers of juveniles of known age. Preferably, tissues also will be collected from known parents (for live-bearing animals, this is usually the mother). Arrays of multilocus genotype data can then be collected using microsatellites or SNPs (due to expense, sequencing is likely not practical). When knowledge of parental genotypes is not possible, the basic principle underlying analysis derives from Hamilton's (1964) original formulation of the genetic relatedness between relatives. Put simply, full siblings share one quarter of their mother's genes and one quarter of their father's genes, meaning they have half of their genome in common. Half siblings related maternally still share one quarter of their mother's genes but none of their father's genes. Just like full siblings, offspring and parents share half of their genome. However, offspring directly inherit genes from their parents meaning they will share one allele at every locus examined. Siblings on the other hand may share no alleles at a particular locus. This means that despite the fact both pairings share half of their genome, offspring–parent relationships are always easier to detect than sibling relationships. Individuals who are unrelated also share parts of their genome due to common inheritance in generations past and homplasy. This creates noise that can lead to incorrectly inferred relationships among juveniles. In order to account for this, algorithms must correct for the probability that shared alleles may be identical by state but not identical by descent. Increasing the number of markers and their level of polymorphism further helps increase the accuracy of assignment (see Blouin 2003 for a full review). Individuals can then be grouped by their relatedness or assigned to categories such as full sibling or half-sibling, taking into account allele frequencies, and statistical confidence can be generated for these groupings using Bayesian or

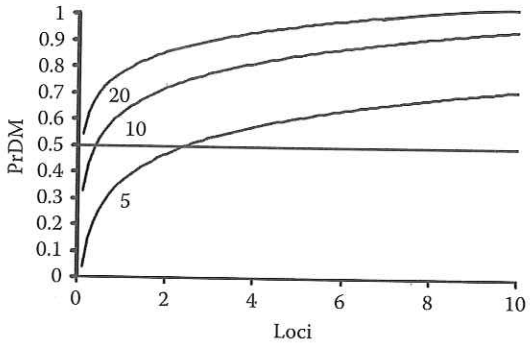

FIGURE 10.2
The probability of detecting multiple paternity (PrDM) increases as the number of loci analyzed increases. The effect is greatest when litter size is small. The three trend lines represent litters of 5, 10, and 20 pups each. PrDM was calculated using PrDM software (Neff and Pitcher 2002). Each locus was assigned five alleles of equal frequency and females were assigned a homozygous condition. If females were heterozygous PrDM would be smaller. A similar affect will occur if the variability of all loci is increased.

maximum likelihood approaches (Goodnight and Queller 1999; Emery et al. 2001). When the maternal genotype is known, reconstructing male genotypes is quite simple. Maternal alleles are identified in progeny and the remaining alleles assigned to males. The algorithms available for this type of analysis either search for the minimum number of sires or the most likely combination of sires based on allele frequencies in the population (Wang 2004; Jones 2005; Kalinowski, Taper, and Marshall 2007). Parental genotypes can also be reconstructed without knowledge of either parent's genotype but these methods rely on the assumption that one sex has greater contribution to the array of progeny than the other (Feldheim, Gruber, and Ashley 2004).

Within a litter, polyandry can be detected by looking for half-siblings that are related by an inferred maternal genotype. This conclusion will be aided if mothers have been genotyped previously. If not, the assumption that maternally related offspring are more common than paternally related offspring must be justified. This approach works well if the number of breeders contributing to offspring in a given location is small and most of the juveniles can be sampled (Feldheim, Gruber, and Ashley 2004; DiBattista et al. 2008b), but will be difficult if the number of breeders is large and only a small fraction of juveniles can be sampled (Portnoy 2008).

Ideally, the maternal multilocus genotypes will be known when litters are analyzed. In such situations, a litter from a monogamous mating will have no more than four alleles present at a given locus. The detection of extra-paternal alleles at one or more loci can be used to infer genetic polyandry. Various algorithms have been constructed that can return the most likely multilocus genotypes of the unsampled parents (Jones 2005; Kalinowski, Taper, and Marshall 2007). When the number of offspring is small, using multiple polymorphic markers (four or more) will increase the probability of detecting extra-pair matings (Figure 10.2). Using multiple markers also ensures that PCR artefacts at a single marker, mistakenly identified as extra paternal alleles, do not lead to a conclusion of polyandry.

10.3.3 Mating Systems in Elasmobranchs

Molecular tools have been used widely in studies of elasmobranch mating systems. So far, all species where multiple litters have been examined have shown genetic polyandry.

TABLE 10.1

Prevalence of Genetic Polyandry in Elasmobranch Species

Species	N	P	L	Study
Ginglymostoma cirratum	2	1.00	28.0	Ohta et al. 2000
Ginglymostoma cirratum	1	1.00	32.0	Saville et al. 2002
Ginglymostoma cirratum	2	1.00	NA	Heist 2004
Triakis scyllium	1	0.00	17.0	Ohta et al. 2000
Carcharhinus plumbeus	20	0.85	9.4	Portnoy et al. 2007
Carcharhinus plumbeus	20	0.40	5.5	Daly-Engel et al. 2007
Carcharhinus galapagensis	1	0.00	7.0	Daly-Engel et al. 2006
Carcharhinus altimus	1	1.00	9.0	Daly-Engel et al. 2006
Sphyrna tiburo	22	0.19	8.5	Chapman et al.2004
Negaprion brevrirosrris	45*	0.86	NA	Feldheim et al. 2004
Negaprion brevrirosrris	46*	0.81	NA	DiBattista et al. 2008
Squalus acanthias	10	0.30	5.0	Lage et al. 2008
Squalus acanthias	29	0.17	5.0	Portnoy unpublished data
Raja clavata	4	1.00	43.0	Chevolot et al. 2007

Note: *N* is the number of litters examined, *P* is the percentage of litters that showed polyandry, *L* is the average litter size calculated from the citation. For studies where polyandry was detected via inferred multilocus parental genotypes (*) no average litter size is presented. Litter sizes were also not reported in Heist (2004) for the two females.

However, the prevalence has differed greatly among species (Table 10.1). The majority of litters examined were genetically monogamous in bonnethead sharks, *Sphyrna tiburo* [19% (N = 22), Chapman et al. 2004] and spiny dogfish, *Squalus acanthias* [30% (N = 10) and 17% (N = 29), Lage at al. 2008; Portnoy, unpublished data]. By contrast, the majority of litters in lemon sharks, *Negaprion brevirostris*, nurse sharks, *Ginglymostoma cirratum*, and sandbar sharks, *Carcharhinus plumbeus*, had multiple sires (Saville et al. 2002; Ohta et al. 2000; Feldheim, Gruber, and Ashley 2004; Portnoy et al. 2007).

These studies were designed to detect genetic polyandry, the presence of multiple sires in a single litter. Females may engage in behavioral polyandry, mating with multiple males, without producing a multiple-sired litter. In addition, in iteroparous species, females may engage in serial monogamy, a type of polyandry in which females mate with one male per reproductive effort but change mates across years (Sugg and Chesser 1994; Karl 2008). The latter is likely a general feature of elasmobranch reproduction even when individual litters are sired by a single male because females almost certainly do not mate with the same male in successive reproductive seasons.

Given the high cost associated with reproduction in elasmobranchs (Pratt and Carrier 2001), the presence of genetic polyandry has led to questions about the benefits of this behavior. Portnoy et al. (2007) found no increase in female fecundity (a direct benefit) for genetically polyandrous female *Carcharhinus plumbeus* in the western North Atlantic. DiBattista et al. (2008a) could not detect indirect benefits, measured as increased survival of pups to age two, in *Negaprion brevirostris*. In both cases, convenience polyandry may be the best explanation for multiple paternity. However, future studies are required as there are multiple types of indirect benefits and they will be difficult to detect in species with long generation times. In *Carcharhinus plumbeus*, the prevalence of genetic polyandry varies between populations with high prevalence in the western North Atlantic (Portnoy et al.

2007) but less than 50% prevalence in Hawaii (Daly-Engel et al. 2007), suggesting that environment or demography may have an effect on female remating rate. In *Negaprion brevirostris*, however, the prevalence of genetic polyandry is consistent among nursery grounds with different environmental characteristics, within the same population (DiBattista et al. 2008a). Our understanding of patterns of genetic polyandry in elasmobranchs is still in its infancy and further study examining possible indirect benefits is necessary. Large comparative studies across multiple populations with different environmental and demographic characteristics as well as long-term studies will be needed to further examine these questions.

Parentage analysis has found an alternate application in examining apparent virgin births in captive sharks. Parthenogenesis had been suggested for both *Sphyrna tiburo* and *Carcharhinus limbatus* when single females of each species gave birth in captivity, apparently without access to males. In both cases molecular analysis failed to turn up paternal alleles, suggesting that these animals produced viable offspring with no paternal contribution (Chapman et al. 2007; Chapman, Firchau, and Shivji 2008). The relevance of these findings in wild populations, however, is unclear.

10.4 Female Philopatry

10.4.1 Introduction

In some animals male and female reproductive strategies may lead to differing dispersal potential. In particular, females, which in live-bearing animals tend to show greater parental investment in offspring than males, may show philopatry to specific areas if it will increase survival of their offspring (Perrin and Mazalov 2000). There are two main reasons to be concerned with this behavior. First, strongly philopatric animals may be at greater risk of localized extinction when exploited (Hueter 1998), making definition of the presence and strictness of philopatry important. Second, adaptive genetic variation is carried in the nuclear genome, so the presence or absence of nuclear gene flow should be used to define populations. When population structure is examined using only mtDNA, misidentification of stocks is likely in highly philopatric animals featuring male-mediated gene flow.

Traditional observational and tagging studies have demonstrated that individual females repeatedly return to the same nursery grounds over multiple years in both *Ginglymostoma cirratum* and *Negaprion brevirostris* (Pratt and Carrier 2001; Feldheim, Gruber, and Ashley 2002). Acoustic monitoring data also suggests that female *Carcharhinus plumbeus* may return to the same nursery ground in multiple years (Portnoy, unpublished data). Tagging studies involving *Carcharhinus limbatus* and *Carcharhinus plumbeus* have shown the tendency for juveniles to return to the same nursery grounds in the summer months (Heupel and Hueter 2001; Grubbs et al. 2007).

Molecular approaches may be important when the ability to tag and resample individuals is compromised by characteristics of the environment or the animal's behavior. In addition, molecular approaches can be used to augment tagging and observational studies, as they allow researchers to examine patterns of philopatry over wider spaces and longer periods of time than tagging or observational studies may allow. The researcher's interests in individual behavior versus patterns of philopatry present over time and space will determine the methodology employed.

10.4.2 Methods

10.4.2.1 Individual Identification

For researchers interested in studies of individual behavior, molecular markers, specifically microsatellites and SNPs, can be used to generate "unique" multilocus genetic identifiers for individuals (genetic fingerprinting). This approach works by genotyping individuals at a modest to large number of markers. As additional markers are assayed, the probability of catching two individuals with identical genotypes rapidly approaches zero. To give a simplified example, consider a study where a researcher employs 10 microsatellites each with 10 equally frequent alleles to genotype individual animals. The probability of sampling an unrelated individual with the same genotype as a previously genotyped individual is $1.0*10^{-17}$. While having a panel of markers that behave this way is highly unlikely, the example demonstrates the power of this methodology. This type of approach can be used to identify and track individuals when traditional tagging is not possible, or if tag shedding is a problem (Palsbøll et al. 1997; Amos, Schlötterer, and Tautz 1993) but requires resampling individuals.

The problem with the above approach is that resampling adult individuals may be inherently difficult, which is why traditional tagging approaches could not be used in the first place. Using the kinship approach, discussed in the previous section, researchers can identify philopatric females by catching and genotyping their progeny. Because this approach can be used to infer maternal genotypes, it negates the need to catch and handle adults. However, the inference of unsampled adult genotypes from juveniles will always require a greater number of markers than the detection of previously sampled adult genotypes from juveniles. In addition, a larger number of SNPs, relative to microsatellites, will be required for this type of analysis because they are diallelic (Glaubitz, Rhodes, and DeWoody 2003).

10.4.2.2 Phylogeographic Approaches

While kinship and genetic fingerprinting are powerful approaches to examining individual philopatry over short periods of time, a phylogeographic approach is needed to examine philopatry over larger geographic areas and across many generations. For this type of application the use of molecular markers with different modes of inheritance is necessary (Karl, Bowen, and Avise 1992; Palumbi and Baker 1994). Specifically, comparisons should be made between patterns of variation across samples with maternally inherited mtDNA versus bi-parentally inherited nuclear genes (microsatellites or SNPs). To accomplish this, the F-statistic or F_{ST}, derived from the inbreeding coefficient (Wright 1965), can be used to detect population structure. This is commonly expressed by examining variance in allele frequency within and between subpopulations as defined by (Weir and Cockerham 1984):

$$\Theta = \frac{s^2}{p(1-p)} \tag{10.1}$$

where p is the average sample frequency of allele A and s^2 is the variance of that allele across populations. The values obtained can be tested against the expectation of the null model (H_0), a population with panmictic mating, using permutation testing (Excoffier, Laval, and Schneider 2005).

Both mtDNA and microsatellite analyses may be enhanced by the use of F_{ST} analogues, Φ_{ST} (Excoffier, Smouse, and Quattro 1992), and R_{ST} (Slatkin 1995), respectively. These measures incorporate assumptions more appropriate for the inheritance patterns and

mutational properties of the respective markers when calculating an F_{ST} value. Statistical procedures can examine variance in a pairwise fashion or at different hierarchical levels. When Φ_{ST} estimates, calculated as both the variance within and between groupings, are tested for significance the procedure is analogous to an analysis of variance (ANOVA). For this reason it is referred to as analysis of molecular variance (AMOVA). Φ_{ST} is an unbiased analogue of F_{st} that takes into account both the divergence of alleles as well as their frequencies. It can be defined as (Excoffier, Smouse, and Quattro 1992):

$$\Phi_{st} = \frac{\sigma_a^2 + \sigma_b^2}{\sigma^2} \tag{10.2}$$

where σ_a^2 is the variance component among groups of population, σ_b^2 is the variance component among populations within groups, and σ^2 is the total variance (the sum of the first two elements plus the variance component between individuals within populations). Discordance between results obtained with these markers, in which mtDNA data suggest population structure and nuclear data do not, could be indicative of female philopatric behavior and male-mediated gene flow.

10.4.3 Female Philopatry in Elasmobranchs

The approaches discussed above have been used to explore patterns of female philopatry in elasmobranchs. Genetic tagging, using nine polymorphic microsatellite loci, was used to accompany a traditional tagging study of *Negaprion brevirostris* in Bimini, Bahamas (Feldheim et al. 2002). The results showed that the tagging study failed to detect 12% of the returning animals due to tag shedding. Using the same set of markers, individual adults and juveniles were genotyped across six years of sampling. By assigning juveniles back to sampled females, philopatric behavior was detected in four females (Feldheim, Gruber, and Ashley 2002). These results also were supported by a study in which 32 of 45 females, whose genotypes had been reconstructed from sampled juveniles, gave birth at Bimini in multiple years (Feldheim, Gruber, and Ashley 2004). Similar results were obtained using the same methodology for *Negaprion brevirostris* at Marquesas Key, Florida (DiBattista et al. 2008b). Despite evidence of strong philopatric behavior by individual female *Negaprion brevirostris*, analysis of population structure, using microsatellites, indicated no population structure for this species in the western Atlantic, suggesting male mediated gene-flow (Feldheim, Gruber, and Ashley 2001). For *Carcharhinus plumbeus* pupping in Chesapeake Bay, Delaware Bay, and the Eastern Shore of Virginia, this same approach linked several females of known genotype to their offspring caught at different times (Figure 10.3). However, attempts to infer the genotypes of nonsampled females from juveniles were largely unsuccessful. This may have been caused by a relatively small sample size, about 100 juveniles per annum, compared to a relatively large effective number of breeders, 500 to 1000 per annum (Portnoy et al. 2008a).

Several studies have used a phylogeographic approach to examine sex-biased dispersal. In white sharks, *Carcharodon carcharias*, significant divergence was found in mtDNA sequences between samples from the eastern and western Pacific, while no divergence was detected with microsatellites (Pardini et al. 2001). Likewise in mako sharks, *Isurus oxyrinchus*, population structure across the Atlantic was detected with mtDNA, while nuclear microsatellite allele frequency distributions were homogeneous (Schrey and Heist 2003). Patterns of variation obtained from mtDNA sequence data and microsatellites suggest philopatry in female *Carcharhinus limbatus* to the western North Atlantic, Gulf of Mexico,

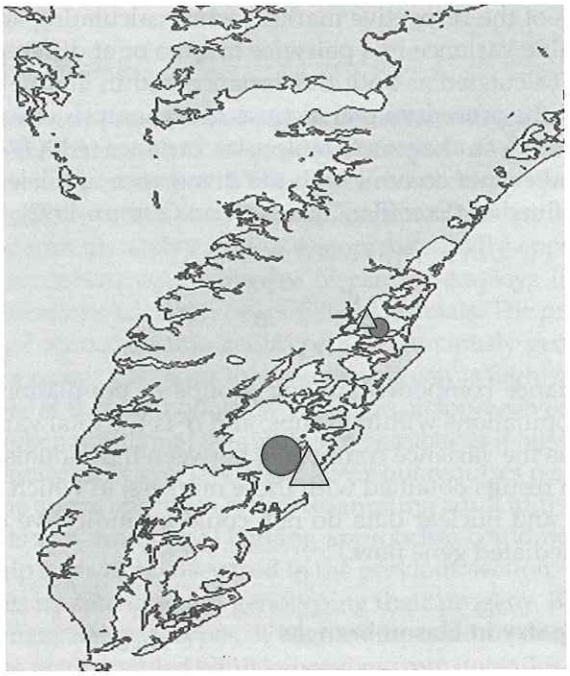

FIGURE 10.3
A color version of this figure follows page 336. The reproductive output of females of known genotype can be detected through the use of multilocus genetic fingerprinting. Here adult and juvenile *Carcharhinus plumbeus* were sampled in the lagoons of the Eastern Shore of Virginia. A mother–offspring relationship was detected using eight microsatellites between a sampled juvenile (small triangle) and a postpartum female (large triangle) sampled two years later. A postpartum female (large circle) and two juveniles (small circles) identified as the female's progeny were caught several months apart in the same year.

and Caribbean Sea, with high levels of male dispersal (Keeney et al. 2005). Long-term female philopatry also has been suggested for *Negaprion brevirostris* in the western Atlantic (Schultz et al. 2008). Similar results with *Carcharhinus plumbeus* were obtained in the Indo-West Pacific, where mtDNA divergence between samples taken from Taiwan and western Australia, and between western Australia and eastern Australia, were significant and more than an order of magnitude greater than the nonsignificant measures of microsatellite divergence (Portnoy et al. 2008a). It is important to remember that because different marker types differ in mode of inheritance, ploidy, and mutation rate, some discordance in results should be expected (see Buonaccorsi, McDowell, and Graves 2001). Sex biased dispersal should only be inferred when the magnitude of this discordance is larger than expected, as in the previous example.

10.5 Effective Population Size

10.5.1 Introduction

Small populations are of concern in conservation and management contexts because they are more susceptible to processes (demographic and genetic) that lead to extinction

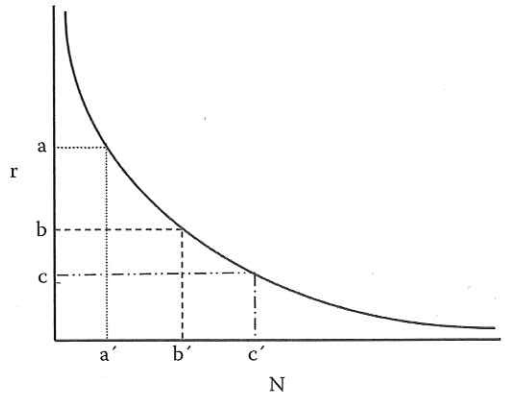

FIGURE 10.4
The size of a population (N) and its relationship with the rate of evolutionary change, in this case drift (r). While the change in N between c′ and b′ equals that between b′ and a′, the resulting change in r between c and b is less than between b and a. As populations shrink, r increases more rapidly, a phenomenon that leads to the fixation of alleles and may rapidly lead to loss of adaptive genetic variation.

(Franklin 1980; Newman and Pilson 1997). In part this is due to the inverse relationship between the number of successful breeders and genetic drift (Figure 10.4). As drift increases so does the probability of the fixation of deleterious (harmful) alleles and loss of additive adaptive variance. In addition, as the gene pool contracts the rate of inbreeding increases. In total these processes may reduce individual fitness across a population that may in turn lead to extirpation. Drift can be simply conceptualized as the sampling variance of gametes during each round of reproduction (Falconer and MacKay 1996):

$$\sigma_p^2 = \frac{pq}{2N_c} \tag{10.3}$$

where p and q are allele frequencies and N_c is the census population size in a Wright–Fisher ideal population (Wright 1931). Since true populations violate most assumptions of an ideal population (equal reproductive success, nonoverlapping generations, equal number of males and females, and random mating; for a more complete review, see Caballero 1994), N_c must be replaced with the measure effective population size (N_e).

It is important to note that N_e and N_c are rarely equal and there is no direct relationship between the two measures. In fact surveys of wild populations have found that the two measures vary greatly across taxa, with N_e/N_c ratios from 10^{-5} in many marine species to nearly 1.0 in some terrestrial vertebrates (Frankham 1995; Hedrick 2005). Therefore, N_e must be estimated from demographic and/or genetic data. Difficulty in obtaining the information required for demographic methods has led to interest in using genetic data to estimate N_e (Caballero 1994; Wang 2005). There are two broad types of N_e that can be estimated from genetic data: historical and contemporary. Understanding the distinction is very important as the meaning and use of the two measures differ greatly.

10.5.1.1 Historical Effective Population Size

Historical or long-term effective size is a backward looking measure that can be defined as:

$$N_e = \frac{\Theta}{4\mu} \quad \text{or} \quad N_e = \frac{\Theta}{2\mu} \tag{10.4}$$

for diploid autosomal loci or haploid mtDNA, respectively (Hartl and Clark 2007). The parameter θ is a function of DNA diversity (sequence data) or multilocus polymorphism (microsatellites, SNPs), which can be calculated in a number of software packages [e.g., MIGRATE (Beerli and Felsenstein 1999); DNASP (Rozas et al. 2003); ARLEQUIN (Excoffier, Laval, and Schneider 2005)]. Put simply, populations that persist over long periods of time at large size will accumulate large amounts of genetic diversity detectable in the present. This, of course, is highly dependent on μ, the mutation rate, because the larger the μ, the more quickly populations of any size accumulate diversity.

There are a number of problems with deriving and interpreting historical N_e estimates. To begin with, using an inappropriate μ will greatly bias the estimate. Because the exact mutation rate at a given locus, let alone across multiple loci, in a species of interest is seldom known, μ must be estimated from the fossil record or from known vicariant events. For many species, this type of information is lacking, so mutation rates estimated for the same locus from closely related species are used. While this does provide a rational "best guess" it does not truly solve the problem. A better way to avert this problem may be to assume that within a species μ is a constant and therefore θ can be left uncorrected. Using this strategy, θ compared across populations within a species will provide information on relative sizes over long periods of time.

A second problem that this strategy cannot overcome is that demographic changes over time, such as fluctuations in population size or past connectivity between or among populations, will lead to differences in historical N_e, making it hard to determine to what time period and population the estimate applies (Crandall, Posada, and Vasco 1999; Schwartz, Tallmon, and Luikart 1999). For example, if one were to estimate historical N_e for a population that is currently isolated but may have had significant immigration from unsampled populations in the past, the N_e estimated might be quite large, as it pertains to all of the populations together instead of the population of interest. Without significant information about connectivity in the past there is no way to tell. Past population bottle necks will have the opposite effect, resulting in historical N_e estimates that are much smaller than contemporary N_e. Finally, from a conservation and management standpoint, historical N_e provides little information about the current number of breeders in a population or that population's evolutionary potential.

10.5.1.2 Contemporary Effective Population Size

Contemporary estimates of N_e apply to generations in the recent past and estimates of the effective number of breeders (N_b) apply directly to the parents of a sampled cohort (Waples 2005). Therefore, the information is more useful for conservation and management of elasmobranchs. There are three major types of contemporary estimates that differ in the type of evolutionary change measured in order to estimate N_e. Variance N_e estimates are based on measures of drift between and within generations and are the most commonly used estimates. Less commonly used are inbreeding N_e estimates, based on the rate of inbreeding within a population, and eigenvalue N_e estimates, based on rates of allele loss within a population. While researchers should be aware of these distinctions (see Crandall, Posada, and Vasco 1999 for review), a more pressing concern is whether one sample (point estimates) or several temporal samples (temporal estimates) are required. Since contemporary

estimates are more useful to elasmobranch researchers interested in reproduction and conservation, they will be the focus of the methods section below.

10.5.2 Methods

10.5.2.1 Heterozygote Excess

Heterozygote excess is a point estimate dependent on the idea that when the number of breeders is small, allele frequencies in males and females will tend to be different because of sampling error. This means the number of heterozygote progeny will be greater than expected (Pudovkin, Zaykin, and Hedgecock 1996). N_b can, therefore, be calculated from equations that derive from the simple relationship (Luikart and Cornuet 1999):

$$N_b = \frac{H_{exp}}{2(H_{obs} - H_{exp})} \tag{10.5}$$

where H_{exp} is the expected heterozygosity and H_{obs} is the observed heterozygosity. The method can be used to estimate N_e if adults are sampled. However, heterozygote excess is most accurate and easily differentiated from infinity when the true number of breeders is relatively small, which is unlikely for a whole population (Balloux 2004). Instead, it will be most useful for estimating N_b from juveniles (Zhdanova and Pudovkin 2008). The ability to sample individuals from known cohorts at specific nursery grounds should make this method appealing to elasmobranch researchers interested in the magnitude of breeding effort. Increased sample size and larger numbers of polymorphic loci will increase the accuracy of estimates and shrink confidence intervals.

10.5.2.2 Linkage Disequilibrium

The linkage disequilibrium method is another point estimate based on the premise that when populations are small, alleles at independent loci will appear linked because of drift. It calculates the correlation among alleles at unlinked loci (r), which can be related to N_e by the formula (Hill 1981; Waples 1991):

$$N_e = \frac{1}{3 * \left(r^2 - \frac{1}{S}\right)} \tag{10.6}$$

where S is sample size. As with the heterozygote excess method, the linkage disequilibrium method can be used to calculate N_e or N_b but performs best when N_e or N_b is small. Consequently, it also may be useful for estimating N_b from samples of juveniles of known age at specific nursery grounds. In addition, there may be downward bias associated with small sample sizes (England et al. 2006). Though later formulations have accounted for this (Waples 2006), having large sample size ($S > 0.1N_e$) is still advisable.

10.5.2.3 Temporal Estimates

Temporal estimates require multiple samples separated in time and measure changes in gene frequencies caused by genetic drift. The magnitude of these changes will be affected

$$N_e = \frac{\Theta}{4\mu} \quad \text{or} \quad N_e = \frac{\Theta}{2\mu} \tag{10.4}$$

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10.5.2.3 Temporal Estimates

Temporal estimates require multiple samples separated in time and measure changes in gene frequencies caused by genetic drift. The magnitude of these changes will be affected

by two parameters: the amount of time between sampling and the effective size of the population. This relationship can be expressed simply as (Krimbas and Tsakas 1971; Nei and Tajima 1981; Pollack 1983):

$$N_e = \frac{T}{2\{F - (1/2S_0 + 1/2S_t)\}} \tag{10.7}$$

where T is the generation time between samples, F is an F_{ST} analogue that employs temporally separated samples instead of spatially separated samples, and S_0 is sample size from the first time period and S_t is the sample from a time period t generations later. In general, this is the most widely used method because it tends to produce both accurate estimates and tight confidence intervals (Waples 1989). Another advantage of this method is that, unlike the previous two methods, which require diploid data (microsatellites, SNPs), one can use mitochondrial sequence data to estimate female effective size (N_{ef}; Laikre, Jorde, and Ryman 1998). This approach requires samples be at least one generation apart to ensure accuracy of the estimate (Waples 1991; Williamson and Slatkin 1999).

This may be problematic for elasmobranch researchers because generation times tend to be long and samples that have been archived in a manner allowing for molecular analysis are rare. Instead, a modified version of the temporal method, which examines shifts in allele frequencies between consecutive cohorts, is preferable (Jorde and Ryman 1995). Drift is then related to N_e by the formula

$$N_e = \frac{C}{2GF'} \tag{10.8}$$

where G is generation time, F' is the F-statistic averaged across cohorts, and C is a parameter used to account for the probability of survival to age (l_i) and reproductive output of each age class (b_i). This methodology requires both samples from juveniles of known age and fairly accurate life history data, but these may be easier to obtain than samples separated by a generation or more. As with other methodologies, accuracy will increase dramatically with increased sample size.

10.5.3 Effective Population Size in Elasmobranchs

Estimates of effective population sizes are generally lacking for elasmobranchs. Long-term N_{ef} (female effective size) was examined in a phylogeographic context in whale sharks, *Rhincodon typus*, scalloped hammerheads, *Sphyrna lewini*, and lemon sharks, *Negaprion brevirostris* and *N. acutidens* (Duncan et al. 2006; Castro et al. 2007; Schultz et al. 2008). In these cases calculations were based on θ_1, which is the long-term effective size after a presumed population expansion. In *Rhincodon typus*, N_{ef} was estimated to be between 119,000 and 238,000 worldwide (Castro et al. 2007) and expanded from an original N_{ef} estimate between 13,000 and 26,000. For populations of *Sphyrna lewini*, N_{ef} estimates varied from 550 up to 31,000,000 females (Duncan et al. 2006). For populations of *N. brevirostris*, N_{ef} estimates varied from 13,000 to 26,000 and from 17,000 to 26,000 for N. *acutidens*. Like other forms of long-term N_e, these estimates require the use of an assumed mutation rate and are greatly affected by demographic processes in the past. In addition, these estimates are highly dependent on the model used (Beaumont 2001), in this case a population expansion model. This means that the actual estimates of N_{ef} must be treated very cautiously.

However, conclusions regarding population increases in *Rhincodon typus* and the relative population sizes in *Sphyrna lewini* and *Negaprion* are robust because they can be based on θ alone and are not affected by uncertainty regarding mutation rates.

Estimates of contemporary N_e and N_b have been made for commercially exploited *Carcharhinus plumbeus*, using linkage disequilibrium and the modified temporal method (Portnoy et al. 2008b). Juvenile sharks of known age were collected from lagoons along the Eastern Shore of Virginia and from the Delaware Bay. N_b was estimated for each cohort at each location and N_e was estimated across cohorts at each location. The results showed fairly consistent estimates of N_b across years within the Delaware Bay and Eastern Shore nursery grounds, with harmonic means of 1059 and 511, respectively. This suggests twice the reproductive effort in the Delaware Bay, likely due to a larger number of females using that nursery ground. In addition, estimate of the census number (N_c) of breeders in the Delaware Bay were compared to N_e and N_b resulting in ratios ($N_{e(b)}/N_c$) near 0.5. This closely approximates the expected relationship between N_e and N_c for species with overlapping generations where reproductive success is even (Nunney 1993). In addition, the estimated ratio is orders of magnitude higher than ratios seen in exploited bony fishes (10^{-3} to 10^{-5}; Hoarau et al. 2005), typical of species that feature large variances in reproductive success (Hedgecock 1994). This suggests that the relationship between N_e and N_c is more affected by life history characteristics than exploitation.

10.6 Summary and Conclusions

The use of molecular techniques to explore the reproductive biology of elasmobranchs has become a more viable option, even for researchers without genetics backgrounds, as the technology and analysis have become easier and more affordable. These investigations require the use of highly polymorphic markers, but each type has pros and cons that must be weighed, not the least of which will be the availability of resources.

With the appropriate markers, multiple aspects of reproductive behavior can be investigated. Kinship and parentage analysis can be used to investigate mating systems or philopatric behavior. In addition, phylogeographic techniques available to investigate philopatry can uncover long-term trends over wide geographic areas. Multiple formulations exist for estimating N_e and N_b. The most useful ones for elasmobranch researchers interested in current reproductive behavior and conservation are contemporary estimates of N_e and N_b and require either one sample or consecutively sampled cohorts.

References

Alcock J, Barrows EM, Gordh G, et al. (1978) The ecology and evolution of male reproductive behavior in the bees and wasps. Zool J Linn Soc 64: 293–326.

Amos B, Schlotterer C, Tautz D (1993) Social structure of pilot whales revealed by analytical DNA profiling. Science 260: 670–672.

Angers B, Bernatchez L (1997) Complex evolution of salmonid microsatellite locus and its consequences in inferring allelic divergence from size information. Mol Biol Evol 14: 230–238.

Avise JC, Arnold J, Ball RM, et al. (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst 18: 489–522.

Avise JC, Jones AG, Walker D, et al. (2002) Genetic mating systems and reproductive natural histories of fishes: lessons for ecology and evolution. Annu Rev Genet 36: 19–45.

Balloux F (2004) Heterozygote excess in small populations and the heterozygote-excess effective population size. Evolution 58: 1891–1900.

Balloux F, Brünner H, Lugon-Moulin N, et al. (2000) Microsatellites can be misleading: an empirical and simulation study. Evolution 54: 1414–1422.

Beaumont MA (2001) Conservation genetics. In: Balding DJ, Bishop M, Cannings C (eds) Handbook of Statistical Genetics, John Wiley & Sons, New York, pp 779–812.

Beerli P, Felsenstein J (1999) Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. Genetics 152: 763–773.

Blouin MS (2003) DNA-based methods for pedigree reconstruction and kinship analysis in natural populations. Trends Ecol Evol 18: 503–511.

Blouin MS, Parsons M, Lacaille V, Lotz S (1996) Use of microsatellite loci to classify individuals by relatedness. Mol Ecol 5: 393–401.

Buonacccorsi VP, McDowell JR, Graves JE (2001) Reconciling patterns of inter-ocean molecular variance from four classes of molecular markers in blue marlin (*Makaira nigricans*). Mol Ecol 10: 1179–1196.

Byrne P, Roberts J (2000) Does multiple paternity improve fitness of the frog *Crinia georgiana*. Evolution 54: 968–973.

Caballero A (1994) Developments in the prediction of effective population size. Heredity 73: 657–679.

Carling M, Wiseman PA, Byers JA (2003) Microsatellite analysis reveals multiple paternity in a population of wild pronghorn antelopes. J Mammal 84: 1237–1243.

Castro AL, Stewart BS, Wilson SG, et al. (2007) Population genetic structure of the earth's largest fish, the whale shark (*Rhincodon typus*). Mol Ecol 16: 5183–5192.

Chakraborty R, Kimmel M, Stivers DN, et al. (1997) Relative mutation rates at di-, tri-, and tetranucleotide microsatellite loci. Proc Natl Acad Sci USA 94: 1041–1046.

Chapman DD, Firchau B, Shivji MS (2008) Parthenogenesis in a large-bodied requiem shark, the blacktip *Carcharhinus limbatus*. J Fish Biol 73: 1473–1477.

Chapman DD, Prodohl PA, Gelsleichter J, et al. (2004) Predominance of genetic monogamy by females in a hammerhead shark, *Sphyrna tiburo*: implications for shark conservation. Mol Ecol 13: 1965–1974.

Chapman DD, Shivji MS, Louis E, et al. (2007) Virgin birth in a hammerhead shark. Biol Lett 3: 425–427.

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. Mol Biol Evol 24: 621–631.

Chevolot M, Ellis JR, Rijnsdorp AD, et al. (2007) Multiple paternity analysis in the thornback ray *Raja clavata* L. J Hered 98: 712–715.

Crandall KA, Posada D, Vasco D (1999) Effective population sizes: missing measures and missing concepts. Anim Conserv 2: 317–319.

Daly-Engel TS, Grubbs RD, Bowen BW, Toonen RJ (2007) Frequency of multiple paternity in an unexploited tropical population of sandbar sharks (*Carcharhinus plumbeus*). Can J Fish Aquat Sci 64: 198–204.

Daly-Engel TS, Grubbs RD, Holland K, et al. (2006) Assessment of multiple paternity in single litters from three species of carcharhinid sharks in Hawaii. Environ Biol Fishes 76: 419–424.

DeWoody JA, Avise JC (2001) Genetic perspectives on the natural history of fish mating systems. J Hered 92: 167–172.

DiBattista JD, Feldheim KA, Gruber SH, Hendry AP (2008a) Are indirect genetic benefits associated with polyandry? Testing predictions in a natural population of lemon sharks. Mol Ecol 17: 783–795.

DiBattista J, Feldheim KA, Thibert-Plante X, et al. (2008b) A genetic assessment of polyandry and breeding-site fidelity in lemon sharks. Mol Ecol 17: 3337–3351.

Di Rienzo A, Peterson A, Garza J, et al. (1994) Mutational processes of simple-sequence repeat loci in human populations. Proc Natl Acad Sci USA 91: 3166–3170.
Dudgeon CL, Feldheim KA, Schick M, Ovenden JR (2006) Polymorphic microsatellite loci for the zebra shark *Stegostoma fasciatum*. Mol Ecol Notes 6: 1086–1088.
Duncan KM, Martin AP, Bowen BW, De Couet HG (2006) Global phylogeography of the scalloped hammerhead shark (*Sphyrna lewini*). Mol Ecol 15: 2239–2251.
Ellegren H (2004) Microsatellites: simple sequences with complex evolution. Nature Rev Genet 5: 435–445.
Emery A, Wilson I, Craig S, et al. (2001) Assignment of paternity groups without access to parental genotypes: multiple mating and developmental plasticity in squid. Mol Ecol 10: 1265–1278.
England PR, Cornuet JM, Berthier P, et al. (2006) Estimating effective population size from linkage disequilibrium: severe bias in small samples. Conserv Genet 7: 303–308.
Excoffier L, Laval G, Schneider S (2005) ARLEQUIN (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1: 47–50.
Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491.
Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Pearson Education, Harlow, UK.
Feldheim KA, Gruber SH, Ashley MV (2001) Population genetic structure of the lemon shark (*Negaprion brevirostris*) in the western Atlantic: DNA microsatellite variation. Mol Ecol 10: 295–303.
Feldheim KA, Gruber SH, Ashley MV (2002) The breeding biology of lemon sharks at a tropical nursery lagoon. Proc R Soc London (Biol) 269: 1655–1661.
Feldheim KA, Gruber SH, Ashley MV (2004) Reconstruction of parental microsatellite genotypes reveals female polyandry and philopatry in the lemon shark, *Negaprion brevirostris*. Evolution 58: 2332–2342.
Feldheim KA, Gruber SH, de Marignac JRC, Ashley MV (2002) Genetic tagging to determine passive integrated transponder loss in lemon sharks. J Fish Biol 61: 1309–1313.
Fiumera AC, DeWoody YD, DeWoody JA, et al. (2001) Accuracy and precision of methods to estimate the number of parents contributing to a half-sib progeny array. J Hered 92: 120–126.
Frankham R (1995) Effective population size/adult population size ratios in wildlife: a review. Genet Res 66: 95–106.
Franklin IR (1980) Evolutionary changes in small populations. In: Soule ME, Wilcox BA (eds) Conservation biology: an evolutionary-ecological perspective. Sinauer Associates, Sunderland, MA, pp 135–150.
Garner TWJ, Schmidt BR (2003) Relatedness, body size and paternity in the alpine newt, *Triturus alpestris*. Proc R Soc London (Biol) 270: 619–624.
Gibbs H, Weatherhead P, Boag P, et al. (1990) Realized reproductive success of polygynous red-winged blackbirds revealed by DNA markers. Science 250: 1394–1397.
Girela E, Lorente J, Alvarez J, et al. (1997) Indisputable double paternity in dizygous twins. Fertil Steril 67: 1159–1161.
Glaubitz JC, Rhodes EJ, DeWoody JA (2003) Prospects for inferring pairwise relationships with single nucleotide polymorphisms. Mol Ecol 12: 1039–1047.
Goetz J, McFarland KP, Rimmer CC (2003) Multiple paternity and multiple male feeders in Bicknell's thrush (*Catharus bicknelli*). Auk 120: 1044–1053.
Goldman N (1993) Statistical tests of models of DNA substitution. J Mol Evol 36: 182–198.
Goldstein DB, Schlötterer C (eds) (1999) Microsatellites: evolution and applications. Oxford University Press, New York.
Goodnight KF, Queller DC (1999) Computer software for performing likelihood tests of pedigree relationship using genetic markers. Mol Ecol 8: 1231–1234.
Gosselin T, Sainte-Marie B, Bernatchez L (2005) Geographic variation of multiple paternity in the American Lobster, *Homarus americanus*. Mol Ecol 14: 1517–1525.

Gray EM (1997) Female red-winged blackbirds accrue material benefits from copulating with extra-pair males. Anim Behav 53: 625–639.

Grubbs RD, Musick JA (2007) Spatial delineation of summer nursery areas for juvenile sandbar sharks in Chesapeake Bay, Virginia. Amer Fish Soc Symp 50: 63–85.

Grubbs RD, Musick JA, Conrath C, Romine J (2007) Long-term movements, migration and temporal delineation of a summer nursery for juvenile sandbar sharks in the Chesapeake Bay region. Amer Fish Soc Symp 50: 87–107.

Hamilton WD (1964) The genetical evolution of social behaviour. I. J Theor Biol 7: 1–16.

Hartl DL, Clark AG (2007) Principles of population genetics, 4th ed. Sinauer Associates, Sunderland, MA.

Hedgecock D (1994) Does variance in reproductive success limit effective population sizes of marine organisms? In: Beaumont MA (ed) Genetics and evolution of aquatic organisms. Chapman and Hall, London, pp 122–134.

Hedrick PW (2005) Large variance in reproductive success and the Ne/N ratio. Evolution 59: 1569–1599.

Heist EJ (2004) Genetics of sharks, skates, and rays. In: Carrier JC, Musick JA, Heithaus MR (eds) Biology of sharks and their relatives. CRC Press, Boca Raton, FL, pp 471–486.

Heupel MR, Hueter RE (2001) Use of an automated acoustic telemetry system to passively track juvenile blacktip shark movements. In: Sibert JR, Nielsen JL (eds) Electronic tagging and tracking in marine fisheries. Kluwer Academic Publishers, Boston, pp 7–64.

Hueter RE (1998) Philopatry, natal homing and localized stock depletion in sharks. Shark News 12.

Hill WG (1981) Estimation of effective population size from data on linkage disequilibrium. Genet Res 38: 209–216.

Hillis DM, Mable BK, Larson A, et al. (1996) Nucleic acids, IV: Sequencing and cloning. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics. Sinauer Associates, Sunderland, MA, pp 321–381.

Hoarau G, Boon E, Jongma DN, et al. (2005) Low effective population size and evidence for inbreeding in an overexploited flatfish, plaice (*Pleuronectes platessa L.*). Proc R Soc London (Biol) 272: 497–503.

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. Biol Rev 75: 21–64.

Jones AG (2005) Gerud 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Mol Ecol Notes 5: 708–711.

Jones AG, Ardren WR (2003) Methods of parentage analysis in natural populations. Mol Ecol 12: 2511–2523.

Jones B, Walsh D, Werne L, Fiumera A (2009) Using blocks of linked SNPs as highly polymorphic genetic markers for parentage analysis. Mol Ecol Res 9: 487–497.

Jorde PE, Ryman N (1995) Temporal allele frequency change and estimation of effective size in populations with overlapping generations. Genetics 139: 1077–1090.

Kalinowski S, Taper M, Marshall T (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16: 1099–1106.

Karl SA (2008) The effect of multiple paternity on the genetically effective size of a population. Mol Ecol 17: 3973–3977.

Karl SA, Bowen BW, Avise JC (1992) Global population genetic structure and male-mediated gene flow in the green turtle (*Chelonia mydas*): RFLP analyses of anonymous nuclear loci. Genetics 131: 163–173.

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.

Keeney DB, Heupel M, Hueter RE, Heist EJ (2005) Microsatellite and mitochondrial DNA analyses of the genetic structure of blacktip shark (*Carcharhinus limbatus*) nurseries in the Northwestern Atlantic, Gulf of Mexico, and Caribbean Sea. Mol Ecol 14: 1911–1923.

Kimura M, Ohta T (1978) Stepwise mutation model and distribution of allelic frequencies in a finite population. Proc Natl Acad Sci USA 75: 2868–2872.

Krimbas CB, Tsakas S (1971) The genetics of *Dacas oleae V.* changes of esterase polymorphism in natural population following insecticide control: selection or drift? Evolution 25: 454–460.

Kwok PY (2001) Methods for genotyping single nucleotide polymorphisms. Annu Rev Genomics Hum Genet 2: 235–258.

Lage CR, Petersen CW, Forest D, et al. (2008) Evidence of multiple paternity in spiny dogfish (*Squalus acanthias*) broods based on microsatellite analysis. J Fish Biol 73: 2068–2074.

Laikre L, Jorde PE, Ryman N (1998) Temporal change of mitochondrial DNA haplotype frequencies and female effective size in a brown trout (*Salmo trutta*) population. Evolution 52: 910–915.

Levinson G, Gutman G (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol 4: 203–221.

Li WH, Gojobori T, Nei M (1981) Pseudogenes as a paradigm of neutral evolution. Nature 292: 237–239.

Lopez JV, Yuhki N, Modi W, et al. (1994) Numt, a recent transfer and tandem amplification of mitochondrial DNA in the nuclear genome of the domestic cat. J Mol Evol 39: 171–190.

Luikart G, Cornuet JM (1999) Estimating the effective number of breeders from heterozygote excess in progeny. Genetics 151: 1211–1216.

Martin AP, Naylor GJP, Palumbi SR (1992) Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357: 153–155.

Martinez-Arias R, Calafell F, Mateu E, et al. (2001) Sequence variability of human pseudogene. Genome Res 11: 1071–1085.

Myers EM, Zamudio KR (2004) Multiple paternity in an aggregate breeding amphibian: the effect of reproductive skew on estimates of male reproductive success. Mol Ecol 13: 1952–1963.

Nazarian J, Hathout Y, Vertes A, Hoffman EP (2007) The proteome survey of an electricity-generating organ (*Torpedo californica* electric organ). Proteomics 7: 617–627.

Nei M, Tajima F (1981) Genetic drift and estimation of effective population size. Genetics 98: 625–640.

Neff BD, Pitcher TE (2002) Assessing the statistical power of genetic analyses to detect multiple mating in fishes. J Fish Biol 61: 739–750.

Newcomer SD, Zeh JA, Zeh DW (1999) Genetic benefits enhance the reproductive success of polyandrous females. Proc Natl Acad Sci USA 96: 10236–10241.

Newman D, Pilson D (1997) Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. Evolution 51: 354–362.

Nunney L (1993) The influence of mating system and overlapping generations on effective population size. Evolution 47: 1329–1341.

Ohta Y, Okamura K, Mckinney EC, et al. (2000) Primitive synteny of vertebrate major histocompatibility complex class I and class II genes. Proc Natl Acad Sci USA 97: 4712–4717.

Palsbøll PJ, Allen J, Berube M, et al. (1997) Genetic tagging of humpback whales. Nature 388: 767–769.

Palumbi SR, Baker CS (1994) Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. Mol Biol Evol 11: 426–435.

Pardini AT, Jones CS, Noble LR, et al. (2001) Sex-biased dispersal of great white sharks. Nature 412: 139–140.

Perrin N, Mazalov V (2000) Local competition, inbreeding, and the evolution of sex-biased dispersal. Amer Nat 155: 116–127.

Pollak, E (1983) A new method for estimating the effective population size from allele frequency changes. Genetics 104: 531–548.

Portnoy DS (2008a) Understanding the reproductive behavior and population condition of the sandbar shark (*Carcharhinus plumbeus*) in the western North Atlantic: a molecular approach to conservation and management. PhD Dissertation, College of William and Mary.

Portnoy DS, McDowell JR, McCandless CT, et al. (2008b) Effective size closely approximates the census size in the heavily exploited western Atlantic population of sandbar sharks, *Carcharhinus plumbeus*. Cons Genet DOI 10.1007/s10592-008-9771-2.

Portnoy DS, Piercy AN, Musick JA, et al. (2007) Genetic polyandry and sexual conflict in the sandbar shark, *Carcharhinus plumbeus*, in the western North Atlantic and Gulf of Mexico. Mol Ecol 16: 187–197.

Pratt HL, Carrier JC (2001) A review of elasmobranch reproductive behavior with a case study on the nurse shark, *Ginglymostoma cirratum*. Environ Biol Fish 60: 157–188.

Pudovkin AI, Zaykin DV, Hedgecock D (1996) On the potential for estimating the effective number of breeders from heterozygote excess in progeny. Genetics 144: 383–387.

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.

Saville KJ, Lindley AM, Maries EG, et al. (2002) Multiple paternity in the nurse shark, *Ginglymostoma cirratum*. Environ Biol Fish 63: 347–351.

Schrey AW, Heist EJ (2003) Microsatellite analysis of population structure in the shortfin mako (*Isurus oxyrinchus*). Can J Fish Aquat Sci 60: 670–675.

Schultz JK, Feldheim KA, Gruber SH, et al. (2008) Global phylogeography and seascape genetics of the lemon sharks (genus *Negaprion*). Mol Ecol 17: 5336–5348.

Schwartz MK, Tallmon DA, Luikart G (1999) Using genetics to estimate the size of wild populations: many methods, much potential, uncertain utility. Anim Conserv 2: 321–323.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457–462.

Stepien C (1995) Population genetic divergence and geographic patterns from DNA sequences: examples from marine and freshwater fishes. Amer Fish Soc Symp 17: 263–287.

Sugg DW, Chesser RK (1994) Effective population sizes with multiple paternity. Genetics 137: 1147–1155.

Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage: Invited Review. Mol Ecol 9: 1013–1027.

Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. Genet Select Evol 34: 275–305.

Wang J (2004) Sibship reconstruction from genetic data with typing errors. Genetics 166: 1963–1979.

Wang, J (2005) Estimation of effective population sizes from data on genetic markers. Philos Trans R Soc London (Biol) 360: 1395–1409.

Waples RS (1989) A generalized approach for estimating effective population size from temporal changes in allele frequency. Genetics 121: 379–391.

Waples RS (1991) Genetic methods for estimating the effective size of cetacean populations. Rep Int Whal Comm (special issue) 13: 279–300.

Waples RS (2005) Genetic estimates of contemporary effective population size: to what time periods do estimates apply? Mol Ecol 14: 3335–3352.

Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 7: 167–184.

Weber JL (1990) Informativeness of human $(dC\text{-}dA)_n$, $(dG\text{-}dT)_n$ polymorphisms. Genomics 7: 524–530.

Weber JL, Wong C (1993) Mutation of human short tandem repeats. Hum Mol Gen 2: 1123–1128.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38: 1358–1370.

Williamson EG, Slatkin M (1999) Using maximum likelihood to estimate population size from temporal changes in allele frequencies. Genetics 152: 755–761.

Wright S (1931) Evolution in Mendelian populations. Genetics 16: 97–159.

Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19: 395–420.

Yamaguchi N, Sarno RJ, Johnson WE, et al. (2004) Multiple paternity and reproductive tactics of free-ranging American minks. J Mammal 85: 432–439.

Yasui Y (1998) The "genetic benefit" of female multiple mating reconsidered. Trends Ecol Evol 13: 246–250.

Zeh JA, Zeh DW (1997) The evolution of polyandry, II: post-copulatory defenses against genetic incompatibility. Proc R Soc Lond (Biol) 264: 69–75.

Zeh JA, Zeh DW (2001) Reproductive mode and the genetic benefits of polyandry. Anim Behav 61: 1051–1063.

Zhdanova OL, Pudovkin AI (2008) Nb_HetEx: a program to estimate the effective number of breeders. J Hered 99: 694–695.