

MOLECULAR APPROACHES IN NATURAL RESOURCE CONSERVATION AND MANAGEMENT

Recent advances in molecular genetics and genomics have been embraced by many scientists in natural resource conservation. Today, several major conservation and management journals are using the “genetics” editors of this book to deal solely with the influx of manuscripts that employ molecular data. The editors have attempted to synthesize some of the major uses of molecular markers in natural resource management in a book targeted not only at scientists but also at individuals actively making conservation and management decisions. To that end, the text features contributors who are major figures in molecular ecology and evolution – many having published books of their own. The aim is to direct and distill the thoughts of these outstanding scientists by compiling compelling case histories in molecular ecology as they apply to natural resource management.

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Molecular Approaches in Natural Resource Conservation and Management

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Preface

The world would be a wonderful place if our natural resources (e.g., forests, fish, and wildlife) needed no management and conservation was not a concern. In a world with a global human population approaching 7 billion and where most developed nations overconsume these resources, however, conservation is a concern and management is necessary for sustainable use. Historically, natural resource management strategies were determined by the collection and interpretation of basic field data. Today, as challenges to the sustainability and conservation of our natural resources arise, managers often need data that cannot be acquired using conventional methods. For example, a natural resource manager might want to know the number of successful breeders in a population or if genetic variation was being depleted because of a management practice. Traditional field craft alone cannot directly address such questions, but the answers can be determined with some precision if the field work is coupled with modern molecular genetic techniques.

Molecules can enlighten us about biological attributes that are virtually impossible to observe in the field (Avise 2004). Parentage analysis is one such arena in which genetic data can inform management practices (DeWoody 2005), but there are a host of others. For example, molecular data have revealed deep evolutionary splits in stocks at one time thought to be homogeneous. This finding has concomitant management implications (Hoffman et al. 2006). Similarly, molecules can enlighten us about biologies that are virtually impossible to observe in the field, such as pollen flow (Hamrick, this volume) or the physiology of migration (Nichols et al. 2008).

Recent advances in molecular genetics and genomics have been embraced by many scientists in natural resource conservation. Today, several major conservation and management journals (e.g., *Journal of Wildlife Management*, *North American Journal of Fisheries Management*, *Plant Breeding Reviews*) are now using “genetics” editors to deal solely with the influx of manuscripts that employ molecular data. We have attempted to synthesize some of the major uses of molecular markers in natural resource management in a book targeted not only at scientists but also at individuals actively making conservation and management decisions. To that end, we have identified contributors who are major figures in molecular ecology and evolution; many have published books of their own. Our aim has been to direct and distill the thoughts of these outstanding

scientists by compiling compelling case histories in molecular ecology as they apply to natural resource management.

Clearly, we hope this book will appeal to academics interested in conservation genetics, molecular ecology, and the quantitative genetics of wild organisms. We think this book could be used as an educational tool – as a text for graduate ecology/genetics courses but also, perhaps, in advanced undergraduate courses. Furthermore, we hope this book will be useful to audiences in natural resource management, education, and research by clarifying how genetic approaches can be used to answer resource-related questions.

ABOUT THE EDITORS

Our collective expertise spans from molecular population genetics in the wild to genomics and quantitative genetics of managed or cultured species. We all study the genetics of natural resources, however, and we find that similar issues arise in wildlife, forestry, and fisheries. For example, when the forest geneticists began asking how many sires contributed pollen to a nut-bearing hardwood tree, it turns out that fisheries geneticists had already studied this problem from the perspective of a male fish guarding a nest full of developing embryos, and they had created computer programs to estimate the number of parents contributing gametes to a nest (DeWoody et al. 2000). Another such intersection of research across disciplines lies in the study of genetic processes in small populations; the same conceptual and analytical approaches being used to elucidate the genetic consequences of wildlife reintroductions (Latch & Rhodes 2005) are employed to evaluate genetic diversity in hardwood tree species subjected to severe habitat fragmentation (Victory et al. 2006). Our desire to produce a book stems from our mutual interests in understanding how molecular genetics can be used to inform and improve natural resource management.

In addition to our research interests, we teach several courses that directly pertain to this book. These courses include *Conservation Genetics* (DeWoody), *Molecular Ecology and Evolution* (DeWoody), and *Evolutionary Quantitative Genetics* (Nichols). Furthermore, several of us (DeWoody, Michler, Rhodes) have served as “genetics” editors for conservation and management journals, including *Journal of Wildlife Management*, *North American Journal of Fisheries Management*, and *Plant Breeding Reviews*.

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Book contributors at an October 2008 meeting, held at the John S. Wright Forestry Center (Purdue University). Row 1: Krista Nichols, Kelly Zamudio, Charles Michler, Yousry El-Kassaby, Tom Whitham, Jamie Ivy, Emily Latch, Lisette Waits, and Marjorie Matocq. Row 2: Lee Shugart, Dave Neale, Dave Hillis, John Avise, Andrew DeWoody, Robin Waples, Rodney Honeycutt, Paul Leberg, and John Bickham. Row 3: Kermit Ritland, Antoine Kremer, Stan Wullschleger, Keith Woeste, Peter Waser, Jim Hamrick, Gene Rhodes, and John Patton. Photo credit: Caleb D. Phillips. See *Color Plate I*.

individual chapters and boxes, and we trust that this book has been enhanced by their efforts.

This volume was largely possible because of the financial and logistical support of the Department of Forestry and Natural Resources at Purdue University. In particular, the department sponsored an October 2008 meeting at Purdue where many of the book contributors congregated for three days of scientific discourse and fellowship before finalizing their respective chapters or boxes.

Our own research programs have been supported by a variety of organizations, including the National Science Foundation (DeWoody, Bickham, Michler, Nichols), the U.S. Department of Agriculture (DeWoody, Michler, Nichols, Rhodes, Woeste), the State of Indiana (DeWoody, Michler, Rhodes), the National Oceanic and Atmospheric Administration (Bickham), the Great Lakes Fishery Trust (DeWoody, Nichols), and the U.S. Forest Service (Michler, Woeste). We thank them all for investing in science.

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4 Vertebrate sex-determining genes and their potential utility in conservation, with particular emphasis on fishes

J. Andrew DeWoody, Matthew C. Hale, and John C. Avise

GENETIC MARKERS IN WILDLIFE CONSERVATION

Individual identification

Often, animals leave clues that can provide some information about their individual identities. These may be conventional fingerprints, which are extremely useful in courts of law but can be physically altered or removed and are restricted to humans. In contrast, deoxyribonucleic acid (DNA) fingerprinting (Avise 2004) is important not only in human forensics and paternity analysis but also in conservation biology and resource management (Table 4–1). When monitoring DNA fingerprints, biologists are capitalizing on the permanent genetic tags by which nature has labeled each individual. In principle, DNA fingerprints (e.g., from leaves, root tips, blood, hair, or feathers) can be traced over space and time, thereby yielding insights into organismal behavior, population structure, and population demography.

Sexing assays and conservation

In concert with individual identification via DNA fingerprinting, molecular sexing has proven valuable in conservation and management (see Box 4 by Lisette Waits). Molecular assays that distinguish males from females can be informative in many ways. For example, the sexes of most dioecious plants are indistinguishable prior to sexual maturity, yet molecular assays have revealed that sex ratios change as seeds develop into reproductively mature plants (Korpelainen 2002; Korpelainen & Kostamo 2008). For vertebrates, most efforts in molecular sexing have focused on mammals and birds in part because of the special interest in these animals, but also because the mode of sex determination

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Table 4–1. DNA fingerprinting plays a major role in the modern management of our natural resources

Organism	Insights	References
Polar bear (<i>Ursus maritimus</i>)	Long annual migrations, but little gene flow	Paetkau et al. 1995
Atlantic salmon (<i>Salmo salar</i>)	Geographic origin of fraudulent fish	Primmer et al. 2000
Painted turtle (<i>Chrysemys picta</i>)	Genetic mark-recapture estimates of population size	Pearse et al. 2001
Passerine birds	Genetic promiscuity despite social monogamy	Griffith et al. 2002
Gray (<i>Halichoerus grypus</i>) and harbor (<i>Phoca vitulina</i>) seals	Dietary preferences via scatology	Reed et al. 1997
Imperial eagles (<i>Aquila heliaca</i>)	Monogamy, turnover, population size	Rudnick et al. 2005, 2008

is known and conserved within each of these two taxonomic groups. Thus, reliable molecular-sexing assays have been relatively straightforward to develop and also to transfer across species within each group.

To date, DNA-based sexing has been most valuable in the conservation and management of mammals. For instance, Zhan and colleagues (2007) discovered via molecular sexing that dispersal in giant pandas (*Ailuropoda melanoleuca*) is female-biased, driven largely by female competition for birthing dens. In another mammalian example, Blejwas and coworkers (2006) used DNA from the saliva of attack wounds to determine that male coyotes (*Canis latrans*) are most often responsible for sheep kills. Finally, Bradley and colleagues (2008) used fecal DNA to assess the demography of gorillas (*Gorilla gorilla*) at nesting sites and showed that individual age and/or sex determinations based on dung size alone may be misleading.

With regard to such studies in birds, Millar and coworkers (1997) sexed monomorphic individuals of the highly endangered New Zealand black stilt (*Himantopus novaezelandiae*), thus allowing captive individuals to be paired on the basis of gender. Clout and colleagues (2002) showed by molecular sexing that supplemental feeding of critically endangered kakapos (*Strigops habroptilus*) in the wild led to male-biased clutches because of the ability of female birds to manipulate the sex ratio of their offspring. Finally, Rudnick and coworkers (2005) coupled DNA fingerprinting with sexing assays to infer genetic parentage, demographic turnover, and variance in chick sex ratios over six years in a population of imperial eagles (*Aquila heliaca*).

The utility of DNA-based sexing assays among mammals and birds is due largely to the conserved mode of sex determination within each of these taxonomic groups, thus allowing sex assays developed in particular species to be applied with only slight modifications to many taxa. The evolution of sex-determining mechanisms has been addressed by Bull (1983) and several others (e.g., Mank et al. 2006). As we will see, genetic sex determination (GSD) is nearly universal

BOX 4: SEX IDENTIFICATION AND POPULATION SIZE OF GRIZZLY BEARS BY USING NONINVASIVE GENETIC SAMPLING

Lisette Waits

Problem

Globally, the numbers of large carnivores have declined due to human persecution and loss of habitat. As a result, there are many vulnerable and endangered populations of conservation concern. To manage these populations, researchers need to collect basic data on population size, population trend, and sex ratios. Historically, this type of data was collected by capturing and marking animals. Capture rates are generally low for large carnivores, however, and capture techniques pose a risk for humans and the study organism. The application of molecular tools in the form of noninvasive genetic sampling of hair and fecal samples has provided an alternative approach for counting individual animals and determining sex.

Case Study

Grizzly bears (*Ursus arctos horribilis*) were listed as threatened under the U.S. Endangered Species Act in 1975, and six recovery zones were identified. The grizzly bear population of the Northern Continental Divide Ecosystem (NCDE) Recovery Zone in northwestern Montana, including Glacier National Park, is particularly important to the long-term viability of grizzly bears in the lower forty-eight states due to its connection to grizzly bear populations in Canada.

In 1998, bear managers launched a large-scale noninvasive genetic sampling effort to estimate the number of bears in a 7,933 km² area in the northern one-third of the NCDE (Boulanger et al. 2008; Kendall et al. 2008). In 1998 and 2000, almost 15,000 hair samples were collected using barbed wire hair traps (with a scent lure attractant) systematically distributed using a 125-unit grid of 8 × 8 km cells and naturally occurring bear rub trees distributed along 1,185 km of trails. Grizzly bear samples were separated from black bear samples by amplifying a section of the mitochondrial DNA control region with a diagnostic length variant. Individual bears were identified by genotyping six microsatellite loci. Sex identification was initially conducted on all unique individuals using the SRY marker and was verified using a size polymorphism in the amelogenin gene. Both methods produce a single PCR fragment for females and two PCR fragments for males. These data were used to estimate population size and sex ratio (Boulanger et al. 2008; Kendall et al. 2008).

Capture probability varied by sex and by sampling method. Males were captured more frequently at rub trees, and females were captured more frequently at hair traps (Box Table 4–1). Sex ratio estimates varied depending on the sampling method, but results from hair trapping were closest to the estimates obtained from a mark-recapture population estimate using the full data set (Box Table 4–1).

Box Table 4–1. Minimum (Min) count of male (M) and female (F) grizzly bears, sex ratio, and mark-recapture population estimate determined using DNA analysis of hair samples collected using hair traps and rub trees in the Greater Glacier Ecosystem (from Kendall et al. 2008)

Year	Min count hair traps			Min count rub trees			Population estimate		
	F	M	F:M	F	M	F:M	F	M	F:M
1998	91	56	1.63	25	37	0.68	125.4	115.3	1.09
2000	85	70	1.21	54	81	0.67	146.0	94.6	1.54

Molecular sex identification has become an important tool for the conservation and management of wildlife species. This study demonstrates the value of noninvasive sampling and molecular sex identification to the conservation and management of grizzly bears and highlights the importance of considering potential biases introduced by sampling methods when using noninvasive genetic approaches to estimate sex ratios.

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in mammals and birds but occurs far more sporadically in reptiles, amphibians, and fishes (Table 4–2).

Sex determination is chromosomal (with males being XY and females XX) in most of the 5,000 extant therian mammals. Exceptions include some voles in which both sexes are XX or XO (Just et al. 1995, 2007) and the monotremes (platypus and echidnas) in which sex determination includes five X and five Y chromosomes; the Xs are homologous to the bird Z chromosome (Grützner et al. 2004; Veyrunes et al. 2008). Sex determination is probably chromosomal in most if not all of the 10,000 extant species of birds (Mank & Ellegren 2007), but males are the homogametic sex (ZZ) and females are heterogametic (ZW).

SEX-DETERMINING GENES

Mammals and birds

The molecular mechanism of sex determination is also conserved across most mammals. The mammalian sex-determining gene *Sry* encodes a transcription factor (SRY) that initiates the male cascade of sexual differentiation (Table 4–3; Sinclair et al. 1990; Ferguson-Smith 2007). Known exceptions (species that do not have the *Sry* gene or that do not express SRY) include the five monotremes

Table 4–2. Vertebrate sex-determining mechanisms are diverse

Taxa	Sex determination	Examples	Citations
Mammals	XY	Nearly all therians	Schafer & Goodfellow 1996
	XO in both sexes	<i>Ellobius lutescens</i>	Just et al. 1995
	XX in both sexes	<i>Ellobius tancrei</i>	Just et al. 1995
	XXXXX/YYYYY	Monotremes (platypus and echidnas)	Grützner et al. 2004
Birds	ZW	Presumably all	Smith & Sinclair 2004
Reptiles	XY	<i>Bassiana duperreyi</i> ¹	Radder et al. 2007
	ZW	<i>Pogona vitticeps</i> ¹	Ezaz et al. 2005
	ESD (TSD)	<i>Amphibolurus muricatus</i>	Warner & Shine 2008
	GSD+ESD	<i>Gekko japonicus</i>	Valenzuela et al. 2003
Amphibians	XY	<i>Rana rugosa</i> ²	Ogata et al. 2008
	ZW	<i>Rana rugosa</i> ²	Ogata et al. 2008
	XY (with reversals ³)	<i>Rana temporaria</i>	Matsuba et al. 2008
Fishes	XY	<i>Takifugu rubripes</i>	Kikuchi et al. 2007
	ZW	<i>Characidium cf. gomesi</i>	Vicari et al. 2008
	X ₁ X ₁ X ₂ X ₂ /X ₁ X ₂ Y	<i>Lutjanus quinquefasciatus</i>	Ueno & Takai 2008
	XX/XY ₁ Y ₂	<i>Ancistrus</i> sp. 1 Balbina	Ribeiro de Oliveira et al. 2008
	Z ₁ Z ₁ Z ₂ Z ₂ /Z ₁ Z ₂ W ₁ W ₂	<i>Ancistrus</i> sp. 2 Barcelos	Ribeiro de Oliveira et al. 2008
	Autosomal modifiers ⁴	<i>Oreochromis niloticus</i>	Mair et al. 1991
	GSD+ESD	<i>Menidia menida</i>	Conover & Kynard 1981

¹ Environmental factors can also influence sex determination in these species, so they might also be categorized as GSD+ESD.

² Some populations of *R. rugosa* have XY sex determination, whereas others are ZW.

³ Reversals may be caused by environmental factors.

⁴ Sex determined chromosomally but can be subsequently reversed depending on the autosomal gene complement.

(Graves 2002), the mole vole (*Ellobius lutescens*; Just et al. 2007), and the spiny rat (*Tokudaia osimensis*; Arakawa et al. 2002).

Molecular sexing assays exploit the presence or absence of *Sry* as revealed by polymerase chain reaction (PCR) and electrophoresis (e.g., Pomp et al. 1995). They yield definitive results, at least in principle, because *Sry* normally determines maleness. Furthermore, recombination typically is suppressed

near the master sex-determining gene, so *Sry* is nearly always in strong linkage disequilibrium with nearby loci that also may be sound indicators of gender. For example, length differences between zinc finger genes (*Zfx* and *Zfy*), often in conjunction with *Sry*, can be used to determine mammalian sex (Pomp et al. 1995; Cathey et al. 1998). For loci more distant from *Sry*, the categorical error rate in molecular sexing should increase proportionally with the recombination rate (i.e., vary as a function of the history of recombination).

Table 4–3. Some of the primary gene products involved in mammalian sex determination

Gene	Role
SRY	Primary testis-determining factor
SOX9	Main target of SRY
DAX1	Antagonist to SRY
AMH	Activated by SOX9
WNT4, WNT7	Required by AMH
DMRT1	Required by Sertoli and germ cells
ATRX	Required for testis development
LHX9, M33	Required for gonad development
TDA1,2,3	Promote ovarian development

Many vertebrates share the same cascade of genes for sexual differentiation, but the primary switch that initiates the cascade (e.g., *SRY* in mammals) varies. Modified from Ferguson-Smith (2007).

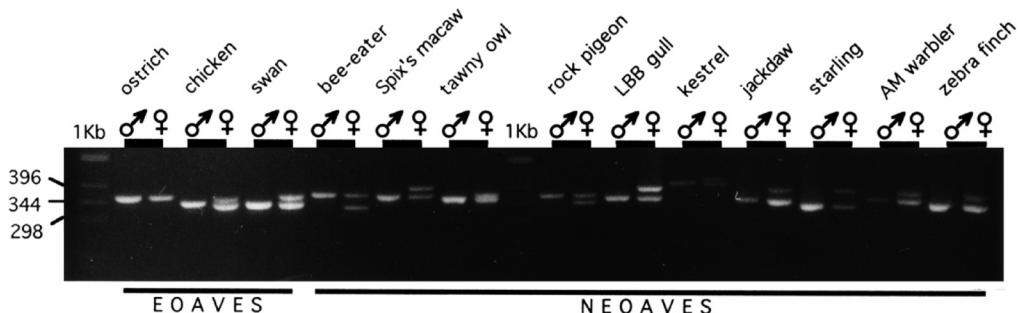


Figure 4–1: A sex-determining assay based on the avian *Chd* gene. This test can be used to sex most nonratite birds (Griffiths et al. 1998). Such broad assays are of great general utility, as evidenced by the more than 800 citations to Griffiths et al. (1998) in the last decade. Reprinted from Griffiths et al. 1998.

In birds, it has long been unclear how genes on the Z or W chromosome cause sexual differentiation (Ferguson-Smith 2007). Some evidence suggested that Z-linked genes such as *Dmrt1* may masculinize chicks, but there is also evidence for feminization by W genes like *Asw* and/or *Fet1* (Smith & Sinclair 2004). Recently, Smith and colleagues (2009) proposed that a Z-linked *DMRT1* gene is the master sex-determinant in the chicken (*Gallus gallus*), such that two doses of the DMRT1 protein produces roosters (ZZ) and one dose gives rise to hens (ZW). It seems very likely that *DMRT1* is indeed the master sex-determining gene in chickens, but there is still a possibility that *DRMT1* is a downstream subordinate of a yet-unidentified master switch (Kuroiwa, 2009; Smith et al. 2009).

Regardless of whether *DMRT1* is the master sex-determining gene, *Chd* (the avian chromo-helicase-DNA-binding gene) seems to be tightly linked to the master control gene in most birds and thus can be used to differentiate the sexes. When amplified via PCR, distinct amplicons emerge for males and females because the introns differ in size between the Z and W chromosomes (Fig. 4–1). Various recent conservation studies on birds have used this or similar sexing assays (Clout et al. 2002; Rudnick et al. 2005; Jarvi & Farias 2006).

Reptiles and amphibians

As we will see, sex determination is more complicated in reptiles and amphibians than in mammals and birds. That said, some species have systems of chromosomal sex determination (XY or ZW) that are similar to those seen in mammals or birds. For instance, snake sex is determined by ZW chromosomes that, depending on the species, may be morphologically similar to or quite differentiated from the autosomes (Matsubara et al. 2006). The Australian central bearded dragon (*Pogona vitticeps*) also has a ZW chromosome system (Ezaz et al. 2005). Unlike mammals, in which sex is determined by the dominance of *Sry*, sex in *P. vitticeps* is determined by the dosage of gene(s) on the Z chromosome (one dose gives rise to a ZW female, two doses produce a ZZ male) (Quinn et al. 2007).

Many reptiles have environmental sex determination (ESD) rather than GSD (Shine 1999). ESD is thought to occur in all crocodilians, many turtles, and some

lizards. ESD in reptiles is often manifested as temperature-dependent sex determination (TSD) whereby eggs incubated at high temperatures yield mostly females and those incubated at low temperatures produce mostly males (Bull 1981; Valenzuela & Lance 2004). Conversely, in many turtles, high incubation temperatures tend to produce females and low incubation temperatures bias toward males. Sex determination is not always straightforward, however (Valenzuela et al. 2003). For example, genotypic male (ZZ) lizards reared at high temperatures reverse to phenotypic females, so in some cases, ESD in effect overrides what normally is GSD (Quinn et al. 2007).

The evolution of GSD from TSD has been studied in Testudines (Janzen & Krenz 2004; Valenzuela et al. 2006). The loss of TSD in GSD species is thought to be caused by downstream genes that override the signal from the TSD switch and thus prevent phenotypic response to temperature (Valenzuela 2008). Temperature-sensitive genes in *Chrysemys picta* (painted turtle) include the Wilms' tumor-suppressor gene *Wt1* and the steroidogenic factor 1 gene *Sf1* (Valenzuela 2008). In mammals, *Wt1* encodes a transcription factor necessary for the maintenance of Sertoli cells and the development of testes, and *Sf1* is a nuclear receptor that controls the expression of *Wt1* (Goa et al. 2006). In the GSD species *Apalone mutica* (smooth softshell turtle), both *Dax1* (which targets downstream *Sf1*) and *Wt1* are differentially expressed according to temperature, but this temperature sensitivity does not seem to skew the sex ratio (Valenzuela 2008). The broader role (if any) of *Sf1* in reptilian sex determination needs further investigation.

Among amphibians, GSD is the rule. Although extreme temperatures can skew sex ratios, they seldom seem to do so in nature (Nakamura 2009; see also Valenzuela et al. 2003). Anthropogenic agents including exogenous steroids (Hayes 1998; Nakamura 2009) and some herbicides (Hayes et al. 2002, 2010) can also induce alterations (including intersexuality) in many amphibians, but here we will restrict our discussion to natural means of sex determination.

Amphibians generally lack morphologically distinguishable sex chromosomes, but sex reversal and breeding experiments indicate that the mechanism of GSD in various species may be XY, ZW, or OO/OW (Eggert 2004; Ezaz et al. 2006). This evolutionary plasticity sometimes is reflected within a single species. For instance, some local populations of the Japanese wrinkled frog *Rana rugosa* are XY (heterogametic males) whereas others are ZW (heterogametic females) (Ogata et al. 2008). Presumably, mutations in the primary sex-determining gene could cause phenotypic sex reversals in frogs. A similar situation occurs in medaka (*Oryzias latipes*), a fish normally displaying an XY system of sex determination but in which XX males and XY females occur infrequently (Shinomiya et al. 2004; Otake et al. 2008).

The master sex-determining gene in amphibians has proven elusive, but several genes involved in sexual differentiation have been cloned. These genes include *Sf1*, *Dmrt1*, and various *Sox* genes (reviewed in Nakamura 2009), all of which appear to be downstream loci controlled by a master switch. In the African clawed frog *Xenopus laevis*, the master switch may be the *DM-W* gene (Yoshimoto et al. 2008). Because the mode of GSD is evolutionarily labile in amphibians, it is likely that – as in fishes – different master genes have evolved in different lineages.

Table 4–4. Sex-determining systems across more than twenty taxonomic orders of teleost fishes

Order	GSD			Hermaphroditism			
	XY	ZW	Protandry	Protogyny	Simul.	Unisexuality	ESD
Anguilliformes	X	X		X	X		
Atheriniformes					X		X
Aulopiformes		X			X		
Beloniformes	X						X
Beryciformes	X						
Characiformes	X	X					
Clupeiformes	X		X				
Cypriniformes	X	X		X		X	X
Cyprinodontiformes	X	X		X	X	X	X
Gasterosteiformes	X	X					
Gymnotiformes	X						
Myctophiformes	X						
Osteoglossiformes	X						
Perciformes	X	X	X	X	X		X
Pleuronectiformes	X	X					X
Salmoniformes	X						X
Scorpaeniformes	X						
Siluriformes	X	X	X				X
Stephanoberyciformes	X						
Stomiiformes	X		X				
Synbranchiformes	X			X			
Tetraodontiformes	X						
Zeiformes	X						

After Mank et al. (2006); some of these orders, most notably Perciformes, are polyphyletic.

SEX DETERMINATION IN FISHES

Sex determination in fishes is complicated, and interested readers are urged to consult Devlin and Nagahama (2002) for more detail than can be provided here. Their 173-page monograph covers gonad development, differentiation, sex-determining systems, environmental effects (EE), and marker development. Sex-determination modes in fishes include male-heterogametic (XY-like) and female-heterogametic (ZW-like) systems, various forms of hermaphroditism (protandry, protogyny, and simultaneous hermaphroditism; Avise & Mank 2010), unisexuality (in which clonal or hemiclonal taxa consist solely of females; Avise 2008), and various forms of ESD (Table 4–4). Alternative mechanisms often are found even in closely related fish taxa (e.g., confamilial species, congeners, and sometimes even conspecifics), thus indicating a high level of evolutionary lability in how sex is determined (Mank et al. 2006; Mank & Avise 2009). Here, we briefly summarize several of these mechanisms.

ESD as determined by behavior

In many fishes, sex determination is environmental (Penman & Piferrer 2008). For example, individuals in species that are sequentially hermaphroditic may switch sex in response to changes in the behavior or social status of conspecifics.

The change of sex can be from female to male (protogynous) or from male to female (protandrous), and the process of sex reversal often involves complete reorganization of the gonads, hormones, and behaviors (Devlin & Nagahama 2002). Such phenotypic changes can be completed in a few days or may take several years, depending on the species (Hattori 1991; Godwin 1994). In fishes that exhibit this mode of ESD, the social structure normally consists of many individuals of one sex that are submissive to one or a few members of the opposite sex. When the dominant individual departs or dies, the individual that ranks highest in the new hierarchy may switch sex and fill the vacated role. Most species that exhibit behaviorally induced ESD live in small social groups or relatively confined spaces (e.g., reef fishes).

Behaviorally induced ESD is exhibited by some gobies (family Gobiidae), in which both protogyny and protandry occur. In protogynous species, the removal of a dominant male can induce sex reversal of the largest female, but that individual retains both ovarian and testicular tissues and can revert back to a female state if the social settings again change (e.g., if a larger male immigrates from a nearby population; Sunobe & Nakazono 1993). Some gobies possess both ovarian and testicular tissues simultaneously. In the “female phase,” the ovaries are well developed and functional whereas the testes are shrunken and inactivated; the reverse applies to individuals in the “male phase” (Kobayashi et al. 2005). In aquarium experiments involving pairs of female Okinawa rubble gobies (*Trimma okinawae*), the larger female always changed to a functional male within five days; when males were paired, the smaller male changed to a functional female within ten days (Kobayashi et al. 2005).

Wrasses provide another example of behaviorally induced ESD. In *Thalassoma bifasciatum*, all individuals first develop a rudimentary female gonad that subsequently differentiates into ovaries or testes. Fish that differentiate as males remain male for life and are called primary males (Warner & Swearer 1991). Fish that differentiate as females either remain female or subsequently become male; the latter are known as secondary males. The gonads of primary males show no evidence of ovarian tissue, whereas those of secondary males do (Munday et al. 2006). Similarly, in the bluestreak cleaner wrasse (*Labroides dimidiatus*), sexual differentiation is under behavioral control. Bluestreak cleaner wrasses are protogynous; each social group consists of about six to eight individuals, including one adult male plus females and juveniles. If the male dies, the dominant female changes sex. Coral reef fish seem particularly prone to behavior-induced ESD (Munday et al. 2006).

ESD as determined by temperature

In homeothermic mammals and birds, a cascade of genes normally dictates an embryo's sex. Fish, by contrast, are poikilothermic, so their embryos are exposed to a wide range of environmental conditions, including temperature. TSD has been studied closely in reptiles, where a temperature change as little as 2°C can alter the sex ratio in a clutch from 100% female to 100% male (Bull & Vogt 1979). These effects arise in part through the increased production of aromatase and subsequent estradiol synthesis at elevated temperatures (Crews 1996). Estradiol secretion in the common carp can range 20-fold over a 5°C change in temperature.

Temperature also affects steroid production in trout and carp (Manning & Kimme 1985). TSD appears to be widespread in fishes, having been documented provisionally in at least nine taxonomic families (Devlin & Nagahama 2002). Valenzuela and colleagues (2003) argue, however, that some of these purported cases may not be true TSD but rather instances of GSD with EE.

One such well-documented case involves the Atlantic silverside (*Menidia menidia*). In some populations, early-season offspring (which are reared in lower temperatures) are mostly female, whereas late-season offspring (reared in warmer temperatures) are mostly male (Conover & Kynard 1981). Presumably, embryos exposed to colder environments have the additional time that is needed to develop ovarian tissues (Conover 1984). TSD is not uniform across the range of *M. menidia*, however. For example, fish in some Canadian waters do not respond to fluctuations in temperature, whereas populations from South Carolina do (Lagomarsino & Conover 1993). Sex determination in *M. menidia* also appears to have a genetic component because progeny from different females respond differently to the same temperature fluctuations and different sires seem to have a strong effect on the sensitivity of their offspring to temperature (Conover & Kynard 1981).

In another such case, sex determination in various tilapias normally has a genetic basis (involving either XY or ZW chromosomal systems depending on the species), yet temperature can influence sex ratios as well. In the Nile tilapia (*Oreochromis niloticus*), for example, high temperature induces masculinization that can override genetic influences (in an XY system) on sexual development (Baras et al. 2001). In Mozambique tilapia (*O. mossambicus*), crosses between sex-reversed males (genotype XX) and normal females (also XX) should produce 100% female progeny, but approximately 90% of embryos reared at low temperatures develop as males (Mair et al. 1990). Temperature also influences sex ratio in *O. aureus* (Desperz & Melard 1998). The differences between the sex chromosomes in tilapias are minor and may reflect the recent evolution of heteromorphic sex chromosomes from a TSD ancestor. Baras and coworkers (2001) suggested that remnant temperature sensitivity might be retained if it provides a selective advantage, such as a greater capacity for dispersal via males.

Sex determination can range from pure GSD to GSD+EE to pure TSD (Valenzuela et al. 2003; Penman & Piferrer 2008), so it can be difficult to categorize sex-determining mechanisms using published data from fishes. For example, some species (e.g., *Poeciliopsis lucida* and *Ictalurus punctatus*) have heteromorphic sex chromosomes – often considered a defining feature of GSD – but temperature nevertheless seems to influence progeny sex ratios in at least some cases (Schultz 1993; Patino et al. 1996). Overall, sex determination in fishes is extremely complex and evolutionarily labile.

Genetic sex determination

Despite the prevalence of ESD in fishes, many species exhibit strict GSD (Penman & Piferrer 2008). Furthermore, several genes involved in sex determination have been uncovered in various fish species (Table 4–5). Unfortunately, several primary piscine genetic models – the zebrafish (*Danio rerio*) and two pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*) – lack known sex chromosomes and sex-determining markers (although see Cui et al. 2006).

Table 4–5. Loci involved in sex determination of fishes

Species	Locus/Gene	Comments	Citations
<i>Gasterosteus aculeatus</i> (stickleback)	LG19	XX–XY system, master gene near <i>Idh</i>	Peichel et al. 2004
<i>Oreochromis</i> spp. (tilapia)	LG23; <i>Amh</i>	Master gene?	Shirak et al. 2006
<i>Oreochromis</i> spp. (tilapia)	LG23; <i>Dmrt2</i>	Master gene?	Shirak et al. 2006
<i>Oreochromis</i> spp. (tilapia)	SOX14	Minor effect gene	Cnaani et al. 2007
<i>Oreochromis aureus</i> (tilapia)	LG1 and LG3	Both loci sex associated	Cnaani et al. 2008
<i>Oreochromis karongae</i> (tilapia)	LG3	WZ-ZZ system	Cnaani et al. 2008
<i>Oreochromis mossambicus</i> (tilapia)	LG1 and LG3	Both loci sex associated	Cnaani et al. 2008
<i>Oreochromis niloticus</i> (tilapia)	LG1	XX–XY system	Cnaani et al. 2008
<i>Oryzias dancena</i> (medaka)	LG10	XY system, but master gene is not DMY	Takehana et al. 2007a
<i>Oryzias hubbsi</i> (medaka)	LG5	ZW system, unlike <i>O. latipes</i> and <i>O. dancena</i>	Takehana et al. 2007b
<i>Oryzias latipes</i> (medaka)	DMY (<i>dmrt1bY</i>)	Master gene, derived from <i>DMRT1</i>	Matsuda et al. 2002
<i>Takifugu rubripes</i> (pufferfish)	LG19	<i>amhrll</i> or <i>inhbb</i> master genes?	Kikuchi et al. 2007
<i>Tilapia mariae</i> (tilapia)	LG3	WZ-ZZ system	Cnaani et al. 2008
<i>Tilapia zillii</i> (tilapia)	LG1	XX–XY system	Cnaani et al. 2008
<i>Xiphophorus maculatus</i> (platyfish)	LG24	X, Y, Z system	Volff et al. 2007

Where known, the candidate master gene is indicated.

The best-characterized example of a sex-determining gene in fish involves the medaka (*Oryzias latipes*). In that species, a protein variously designated as DMRT1bY or DMY – encoded by a gene (*DMY/DMRT1bY*) housed on the sex-determining portion of the Y chromosome – is expressed specifically in Sertoli cells of the testis (Matsuda et al. 2002; Nanda et al. 2002). The gene shows 90% nucleotide sequence similarity to *DMRT1*, which is autosomal but is not itself a master sex-determining gene (Brunner et al. 2001). In mammals and birds, a comparable gene is expressed differentially in males and females. It produces a protein with a DNA-binding domain – also called the DM domain – that is involved in testis determination and differentiation (Volff et al. 2003). Similarly, in the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster*, homologous DM domain proteins are integral components of the male sex-determining cascade (Raymond et al. 1998). Clearly, proteins with DM domains play an evolutionarily conserved role in the testis-forming pathway in a wide variety of animals.

The medaka was the first nonmammalian vertebrate in which the primary sex-determining gene has been characterized (Volff & Schartl 2002; Matsuda 2005; Volff et al. 2007). *DMY/DMRT1bY* is not the master sex-determining gene in all fishes, however. The duplication that gave rise to the nascent *DMY/DMRT1bY* gene was recent (~10 million years ago; Kondo et al. 2004) as *DMY/DMRT1bY* is absent in more distantly related members of the genus (Kondo et al. 2004), and the sex-determining region in medaka corresponds to an autosomal linkage group (LG) in *Oryzias dancena* (Takehana et al. 2007a). In the Asian swamp eel (*Monopterus albus*), some of the variants of four alternatively spliced transcripts of *DMRT1* are expressed more in one sex than the other (Huang et al. 2005).

Alternative splicing and differential expression of DM domain proteins also have been reported in the zebrafish (Guo et al. 2005), honeycomb grouper (Alam et al. 2008), Nile tilapia (Wang & Tsai 2006), channel catfish (Liu et al. 2007), and lake sturgeon (Hale et al. 2010). In all cases, *DMRT1* (or its alternative transcripts) is expressed in both sexes but substantially more so in males than in females. These results again suggest a key role for this gene in testis differentiation. On its initial discovery, *DMRT1* was believed to be the master sex-determining gene in both birds and fish, analogous to *SRY* in mammals. Current thought is that although *DMRT1* may not be the master switch in all fishes, it probably plays a key role in the sex-determining pathway (Volff et al. 2003; Ferguson-Smith 2007).

Many other fish species exhibit GSD. For example, the platyfish (*Xiphophorus maculatus*) has three sex chromosomes; males can be either XY or YY, and females can be XX, XW, or YW. One model to explain this pattern proposes that male-determining genes are present on all three chromosomes, but only the Y chromosome allele is active (and perhaps the YW females arise because the W chromosome carries a suppressor for the master sex-determining gene on the Y chromosome) (Kallman 1984). A different model proposes that sex determination depends on gene dosage; a male-determining gene is present in different copy numbers on the Y (two copies), X (one copy), and W (no copies) chromosomes (Volff & Shartl 2001). The higher the copy number, the higher the gene dosage, which in turn leads to the male phenotype (Volff et al. 2007). The sex chromosomes in platyfish are barely distinguishable from each other or from autosomes, and recombination occurs along most of their length. The sex-determining region has been located, but no sex-determining genes have been documented to date (Volff & Schartl 2001; Schultheis et al. 2006).

Some other fishes with GSD have simpler sex-determining systems. For example, the three-spined stickleback (*Gasterosteus aculeatus*) is male heterogametic (Peichel et al. 2004), with the sex chromosomes being cytogenetically indistinguishable from autosomes, and is thus probably young evolutionarily. As expected, recombination rates are reduced on the sex chromosomes, particularly in the region suspected to contain the master sex-determination gene. Various salmonids including rainbow trout (*Oncorhynchus mykiss*), arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*), and Atlantic salmon (*Salmo salar*) also are XY male heterogametic (Woram et al. 2003). Mapping studies have shown that the sex-determining region of the sex chromosomes is short and is located on different LGs in each salmonid species (in contrast to the extensive synteny exhibited across most of their genomes). These data suggest that novel Y chromosomes have evolved in each species (Phillips et al. 2001; Woram et al. 2003). Despite extensive efforts, a master sex-determining gene has not yet been discovered in either the sticklebacks or the salmonids (although see Griffiths et al. 2000 for the description of a stickleback sexing marker).

Sex determination in tilapia has been studied in detail. Both genetic and linkage maps are available for *Oreochromis niloticus*, among others (Katagiri et al. 2005; Cnaani et al. 2008). As previously mentioned, *O. niloticus* has an XY system with influences also from autosomal loci and rearing temperature (Mair et al. 1991; Griffin et al. 2002). Sex chromosomes are indistinguishable, and YY males are viable, suggesting recent and limited divergence between the X and Y chromosomes (Cnaani et al. 2008). Mapping studies have localized the

sex-determining region to LG1, an area with reduced recombination in males. Thirteen candidate genes known to be involved in the sex-determining pathway in other species were mapped, but none occurred in LG1 (Lee & Kocher 2007), so the master sex-determining gene remains unknown. GSD in two other tilapias (*T. mariae* and *O. karongae*) entails female heterogamety. Markers on LG3 appear to be associated with gender in that specific alleles at several loci are seen only in the maternal line, suggesting the presence of female heterogamety (Cnaani et al. 2008). These results indicate that at least two major loci are involved in sex determination in tilapiine fish.

In some fish species, sex determination is “autosomal” and occurs in the absence of well-differentiated sex chromosomes (Mank et al. 2006). The number and identity of genes involved are unknown. Polygenic sex determination, however, is thought to be evolutionarily unstable and easily invaded by single-locus sex determination (Rice 1986). Thus, autosomal mechanisms of sex determination seem unlikely to persist over evolutionary timescales, and this may explain their relative scarcity in fishes compared to GSD involving differentiated sex chromosomes.

Each particular sex chromosome characterized to date in extant fish lineages seems evolutionarily young (perhaps arising no more than approximately 10 million years ago; Kondo et al. 2004; Peichel et al. 2004), especially compared to the ancient X and Y chromosomal systems (and the *Sry* gene) of mammals. Furthermore, fishes with GSD have evolved a region of suppressed recombination around the sex-determining region, a direct result of heteromorphic sex-chromosome evolution (Charlesworth et al. 2005). The suppression of recombination permits the joint inheritance of genes that are advantageous for one sex. It also avoids the transfer of genes from one sex chromosome to the other, which could have detrimental effects (Bull 1983; Rice 1987). Another consequence of the suppression of recombination is the genetic degeneration of the hemizygous chromosome (Charlesworth et al. 2005). For homeothermic vertebrates, this degeneration is demonstrated by the Y chromosome of mammals and the W chromosome of birds, which are both much smaller and contain far fewer genes than their X and Z counterparts. The small size and reduced gene content of the heteromorphic sex chromosome mean that YY and WW genotypes are lethal in mammals and birds (a situation that contrasts with the viability and often fertility of such genotypes in fishes).

In summary, an emerging theme with regard to GSD fishes is a repeated, independent evolution of sex chromosomes in different lineages, most likely accompanied or followed by the emergence of different sex-determining genes (Mank et al. 2006; Volff et al. 2007). This greatly complicates the scientific search for master sex-determining loci in this largest vertebrate class.

ISOLATION OF SEXING MARKERS FOR FISH MANAGEMENT

We envision many potential uses of sexing assays in fish conservation and management. A few of these uses have already been achieved, mostly in salmonids. For example, molecular sexing assays have latent utility in aquaculture. Devlin and

colleagues have developed a culture methodology, based on a molecular sexing assay, for the production of monosex salmon populations. After removing immature juvenile XY males from the population, females produce substantially more roe than do mixed-sex cultures (Devlin et al. 1994; Henry et al. 2004). Monosex cultures are not only of interest to salmon culturists but probably also to caviar producers who would prefer to rear only females if the fish somehow could be sexed during early-life-history stages (e.g., from fin clips). Likewise, tilapia mature and breed before they reach marketable size, so aquaculturists often wish to rear monosex cultures so that fish expend less energy on reproduction (Lee et al. 2004).

Molecular sexing markers could also inform studies of population structure, which can differ between the sexes because of sex-biased dispersal (e.g., in cichlids and brook trout; Knight et al. 1999; Hutchings & Gerber 2002). Such markers also could facilitate estimates of the operational sex ratio (OSR). [The OSR influences effective population size (N_e) so that as the ratio of $N_f:N_m$ departs from unity, N_e decreases according to the expression: $N_e = (4N_f N_m)/(N_f + N_m)$.] In many fishes, spawning aggregations are markedly male-biased, so N_e may be much smaller than expected if sex ratios were near unity. For instance, in red drum (*Sciaenops ocellatus*), N_e is only 1/1,000 of the adult census size (Turner et al. 2002) and, in theory, this could be partly due to the OSR.

There are few empirical examples that illustrate the potential utility of fish-sexing markers better than the OSR. In some Pacific salmonids, the OSR is skewed toward males. Biologists wondered if this skewing was because of sex reversals or differential survivorship between the sexes. This question has since been answered in large part because of the fortuitous discovery of a Y-linked pseudogene (GH- Ψ) that contains a restriction site not found in the functional gene (Du et al. 1993). The presence of GH- Ψ was used to evaluate sex-specific survivorship in chinook salmon (*Oncorhynchus tshawytscha*) and in coho salmon (*O. kisutch*). In both species, freshwater survivorship was similar between juvenile males and females, but male survivorship was substantially greater than female survivorship in the marine realm (Spidle et al. 1998; Olsen et al. 2006). This is a fundamental biological insight that was realized only when biologists applied molecular sexing assays to juvenile salmon that could not be phenotypically sexed. Unfortunately, the phylogenetic distribution of GH- Ψ appears to be constrained to a few Pacific salmonids (Devlin et al. 2001), and thus its utility is limited.

Biased OSRs might be due to differential survivorship, but they might also be due to sex reversals (Olsen et al. 2006). For example, the sex ratio in a young GSD species might be near 1:1, but if one sex predominates among adults, then we must consider environmental factors, such as contaminants. Williamson & May (2002) employed a Y-specific sexing marker to determine that sex reversals are likely in chinook salmon, perhaps due to endocrine-disrupting chemicals. In contrast, Fernandez and colleagues (2007) found no such evidence for sex reversals in a different population.

In principle, the molecular identification of sexing markers like GH- Ψ is straightforward in species with GSD; one need only isolate and exploit the genomic region found in one sex but not the other. Occasionally, such markers are identified fortuitously (Du et al. 1993). One example is the discovery that *Idh* is sex-linked in the three-spined stickleback (Withler et al. 1986; Peichel

et al. 2004). Subsequent efforts with amplified fragment length polymorphisms (AFLPs) produced a PCR-based sexing assay in that species (Griffiths et al. 2000). Sometimes sexing markers can be isolated by subtractive hybridization, whereby male and female portions of the genome are compared and the similar portions are discarded (Devlin et al. 1991; Wallner et al. 2004). In theory, this should reveal the region in linkage disequilibrium with the master sex-determining gene. Alternatively, the candidate gene approach might seem to be an effective means of characterizing novel markers of gender (as in the use of medaka *DMY/DMRT1bY* as a probe for another fish species). Master sex-determining genes evolve so rapidly in fishes, however, that this approach is likely to work only for closely related taxa.

Next, we focus on yet another possible approach for the isolation of sexing markers: comprehensive transcriptome analyses involving the characterization of genes expressed in the gonads of males and females. We are now using next-generation sequencing technologies to evaluate pools of complementary DNA (cDNA) synthesized from messenger ribonucleic acid (RNA) isolated from fish gonads, in anticipation that comparisons of genes transcribed in testes versus those transcribed in ovaries will help to elucidate the sex-determining cascade in a primitive fish, the lake sturgeon.

CASE STUDY: THE IDENTIFICATION OF SEX-DETERMINING GENES IN LAKE STURGEON

Sturgeons comprise an ancient lineage of benthic fishes characterized by cartilaginous skeletons and bony plates called scutes. The lake sturgeon (*Acipenser fulvescens*) is a freshwater North American species whose historic distribution ranged from Hudson Bay drainages down through the lower reaches of the Mississippi River, but whose range today is much more restricted (Peterson et al. 2007). Mature individuals are large bottom feeders; specimens can grow to more than 100 kg and more than 2 m in length.

Since the arrival of Europeans, lake sturgeon populations have declined precipitously because of habitat degradation and overfishing (Peterson et al. 2002, 2007). Dams now prevent natural spawning migrations, and pollution has impacted many watersheds. Other sturgeon species are famous for their eggs (caviar), but lake sturgeon also were harvested for isinglass to clarify wine and beer, for fish oil that purportedly was used to fuel steamboats, and for animal (pig) feed. According to the U.S. Fish and Wildlife Service (2008), “In Lake Michigan, commercial harvest was closed in 1929 after the catch declined to only 2000 pounds compared to 3.8 million pounds harvested in 1879.” Similarly, the Mississippi River harvest declined from 113,000 kg in 1894 to 3,100 kg in 1922 to 0 kg in 1931 (Peterson et al. 2007).

Today, lake sturgeon in Great Lakes watersheds are of significant conservation concern (Jackson et al. 2002; Peterson et al. 2002, 2007). The restoration of lake sturgeon populations is complicated by their biology, including delayed sexual maturity, infrequent spawning, and sexual monomorphism. Lake sturgeon are long-lived, as evidenced by wild centenarians that live to be older than

150 years (Peterson et al. 2007). Males become sexually mature at approximately twelve to twenty years of age and females at eighteen to thirty years. Males may spawn every second or third year, but most females probably spawn on a four- to nine-year cycle (Peterson et al. 2007). Males arrive on the spawning grounds before females, and a half-dozen or so males flank spawning females and simultaneously attempt to fertilize her eggs. There is no parental care, as females depart soon after spawning and males wait for the next available female.

The sex of a nonbreeding lake sturgeon cannot be determined reliably without invasive surgical examination or ultrasound (Vecsei et al. 2003). Furthermore, even for individuals that are sexually mature but nonspawning, current hormonal assays require anesthetization and a substantial blood sample (Feist et al. 2004). Thus, a general conservation and management problem is that gender cannot be determined easily or consistently in most specimens.

Studies on sex reversal and artificial gynogenesis strongly suggest that at least some sturgeon have GSD (Van Eenennaam et al. 1999; Hett & Ludwig 2005). Furthermore, cytogenetic analyses indicate that female white sturgeon have two different sex chromosomes (Z and W) that are similar in appearance, whereas males have two identical sex chromosomes (Z and Z). Thus, the female genome has all of the components of the male genome, plus some components that are found only on the W chromosome (Van Eenennaam et al. 1999). The gene(s) that differentiate females from males presumably are found somewhere on the W chromosome. Thus, consistent DNA-based differences should exist in the chromosomal makeup of males and females.

Sturgeon species are polyploid, and chromosome numbers (including ~250 microchromosomes) range from 120 to approximately 500. Most lake sturgeon microsatellites are inherited in a tetrasomic (4n) fashion, but some loci are disomic (2n) and others are octasomic (8n); thus, the overall ploidy level is unclear (Ludwig et al. 2001). (Our unpublished single-nucleotide polymorphism [SNP] data generally support the microsatellite data [Hale et al. forthcoming].) The DNA content per cell (c-value) is 4.45 pg in lake sturgeon, considerably larger than the 3.50 pg in humans (Gregory et al. 2006). The large size and apparent complexity of sturgeon genomes have conspired to make the search for sex-determining genes extremely challenging. As summarized later and in McCormick and coworkers (2008), we have used a variety of molecular approaches in attempts to isolate sex-specific markers in lake sturgeon because breeding studies designed to evaluate the possibility of ESD would be difficult in this long-lived species with protracted spawning periodicity.

Candidate genes

We first considered genes known to be involved in the sex-determination pathway of other fishes. We started with the medaka *DMY* gene, which failed to amplify in lake sturgeon. We then designed locus-specific primers for the high-mobility group domain of *Sox2*, *Sox3*, *Sox4*, *Sox11*, *Sox17*, *Sox19*, and *Sox21* genes as *Sox* genes are often involved in sex determination (Hett & Ludwig 2005). Of these seven genes, four (*Sox2*, *Sox4*, *Sox17*, and *Sox21*) amplified in lake sturgeon but none was sex-specific.

Subtractive hybridization

Representational difference analysis (RDA) is a technique often employed to characterize the differences between two genomes (Lisitsyn et al. 1993) and has been used to clone sex-specific markers in mammals (Perez-Perez & Barragan 1998; Wallner et al. 2004). We performed RDA in lake sturgeon first by using known female DNA as the “tester” and male DNA as the “driver” and then by reversing the sex of the tester and the driver. We cloned DNA fragments using this approach, then sequenced them and designed PCR primers for each putative sex-specific fragment, and then screened them against male and female DNA samples. One hundred fifty-nine clones were sequenced; several genes and many previously uncharacterized DNA sequences were isolated using this procedure, but no clone proved to be sex-specific.

In addition to RDA, we performed subtractive hybridization between male and female lake sturgeon genomes using streptavidin-coated magnetic beads. This procedure was carried out twice using various restriction enzymes; after nine rounds of subtractive hybridization, the enriched fragments were cloned and sequenced. Three hundred sixteen clones, representing approximately thirty unique DNA fragments, were analyzed, but no primer set developed from these clones yielded a sex-specific marker.

Random markers

We also attempted to generate a sex-specific lake sturgeon marker using random primers. Randomly amplified polymorphic DNA (RAPD) fragments were generated using a commercial kit. We first iteratively assessed fifty RAPD primers singly before proceeding to evaluations of primer pairs. This procedure was used first on genomic DNA, then on ovary and testis cDNA samples, and finally on the RDA and subtractive hybridization products. More than 120 unique PCRs were performed and thousands of discrete amplicons were generated. Random primers produced no sex-specific DNA fragments, however.

Alternatives to genomic DNA?

De novo characterization of a sturgeon sexing marker has proven difficult for other research groups as well. Hett and Ludwig (2005) evaluated candidate genes in Atlantic sturgeon (*Acipenser sturio*), Wuertz and coworkers (2006) used RAPDs, AFLPs, and inter-sample sequence repeat markers in four *Acipenser* species, and Keyvanshokooh and colleagues (2007) tried RAPDs in beluga sturgeon (*Huso huso*). All of these attempts relied primarily on genomic DNA, and all failed to identify sex-specific markers. Might such searches be enhanced if they focused on candidate genes expressed in gonads? In principle, a focus on expressed genes (i.e., cDNA) might simplify otherwise fruitless searches for sex-linked markers in these extraordinarily complex sturgeon genomes.

Pyrosequencing of transcriptomes

To narrow our search to expressed genes, we sampled gonads from lake sturgeon collected in Lake Oneida, New York, in June 2007 and May 2008. Based on stocking records and established length-at-age relationships (Jackson et al. 2002),

lake sturgeon collected in 2007 were most likely twelve years old and those in 2008 were thirteen years old. The males expressed milt during handling and were apparently ready to mate, whereas gametes from the two females were not fully developed (Colombo et al. 2007). From these fish, we surgically collected gonad biopsies and froze them immediately in liquid nitrogen for subsequent RNA extraction and cDNA synthesis (Hale et al. 2009, 2010).

These cDNA fragments were sequenced using the Roche 454 platform (Margulies et al. 2005). The Roche 454 platform does not use conventional dideoxy sequencing, but instead uses a massive sequencing-by-synthesis approach in which photons of light are emitted during elongation (i.e., pyrosequencing). Briefly, DNA is fragmented using air pressure, adapters are ligated to both ends of fragments, and single-stranded DNA is attached to microbeads (one unique fragment per bead). All beads with a unique genomic DNA fragment are contained in a single reaction tube but are subsequently separated into hydrophobic bubbles for emulsion PCR (ePCR). Each unique DNA fragment is amplified via ePCR to coat each bead with a single sequencing template. The four nucleotides (ACGT) are sequentially washed over the entire plate for chain extension, and then CCD cameras detect photon emissions from each of 1.6×10^6 wells on a plate the size of a microscope slide.

Pyrosequencing generates a vast amount of data. We have generated more than 500,000 sequences from lake sturgeon gonads, and they span some 125 MB (Fig. 4–2; Hale et al. 2010). These lake sturgeon sequences include (among others) genes involved in protein binding, DNA repair, translation, and apoptosis. One major advantage to pyrosequencing is that unlike subtractive hybridization or random priming, useful data are recovered regardless of whether the gene or genes of interest are identified. For example, our 454 surveys of lake sturgeon gonads have identified hundreds of single nucleotide polymorphisms (SNPs) in functional genes as well as in genes that are differentially expressed between the sexes (Hale et al. 2009, 2010). Furthermore, our transcriptome surveys have uncovered a number of reproductive proteins that are apparently expressed on the surface of gametes. A number of these reproductive proteins bear signatures of strong selection, suggesting that genes encoding these proteins may have played a key role in the diversification (speciation) of sturgeons. Finally, our surveys of genes expressed in lake sturgeon gonads suggest that exogenous parasites (schistosomes) and pathogens (trichomonads) apparently infect lake sturgeon. The schistosomes horizontally transferred their genes to the lake sturgeon genome, perhaps through the ingestion of snail hosts, whereas the trichomonad sequences were probably derived from a contemporary infection (Hale et al. 2010).

With regard to the search for candidate sex-determining genes in lake sturgeon, we have used a multipronged approach (Fig. 4–3; Hale et al. 2010). By comparing the sequences of known sex-determining genes in other organisms (e.g., *DMY/DMRT1bY*, *SOX9*, *Sry*) to the lake sturgeon gene sequences, we generated a list of candidate genes that were then evaluated as sexing markers using PCR. We also compared sequences derived from lake sturgeon testes to those derived from ovaries (and vice versa). Those genes expressed in only one sex were considered as candidate sex-determining genes and were again evaluated as sexing markers with PCR. Presumably, this set of genes includes those that are

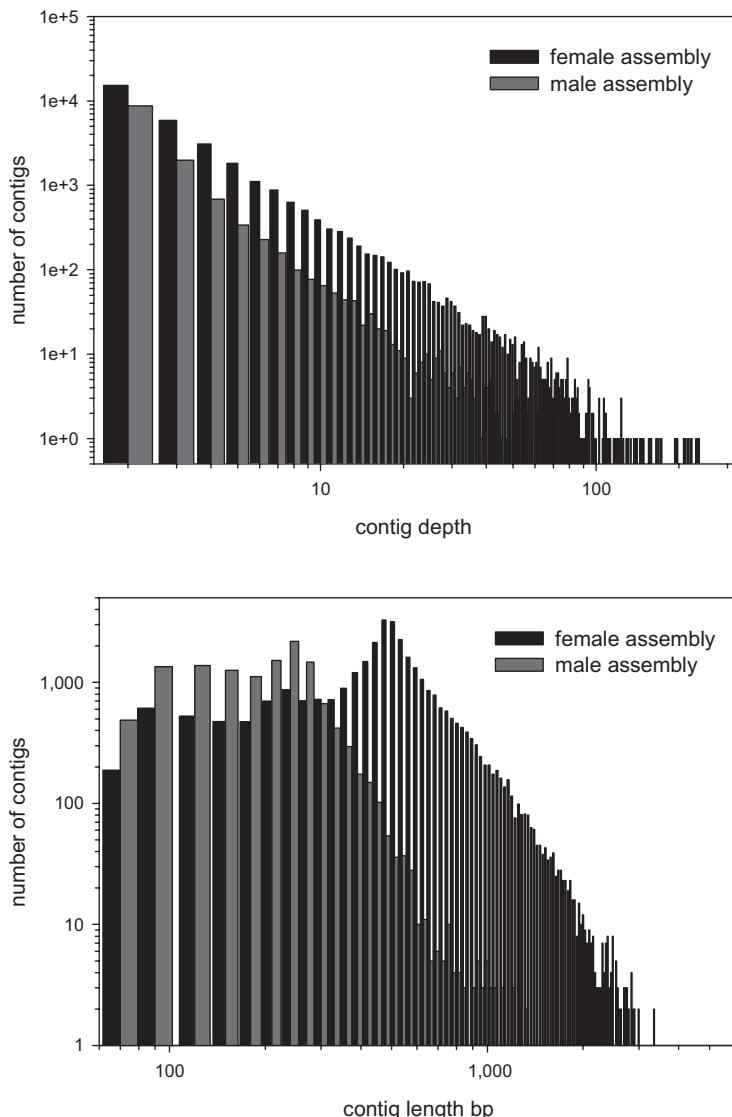


Figure 4-2: The data from 454 runs can be used to generate thousands of long contigs that each consists of many individual sequencing reads. From Hale et al. (2010). See Color Plate IV.

expressed in one sex but not the other, as should occur if the genes exist in both sexes but are differentially regulated.

The 454 sequence data indicate that at least two lake sturgeon genes (*DMRT1* and *Tra-1*) are differentially expressed between the sexes (Hale et al. 2010). These expression differences are interesting from an evolutionary perspective as they suggest that gene dosage might play a role in lake sturgeon sex determination. Our *DMRT1* data, considered in light of similar data from chickens, medaka, and lizards, further point to *DMRT1* as a key determinant of vertebrate sex (Marshall Graves 2009). It remains to be determined if sex in lake sturgeon is determined simply by gene dosage (e.g., *DMRT1*; Hale et al. 2010), but at this point, it seems likely. Unfortunately, gene expression assays based on dosage have little practical

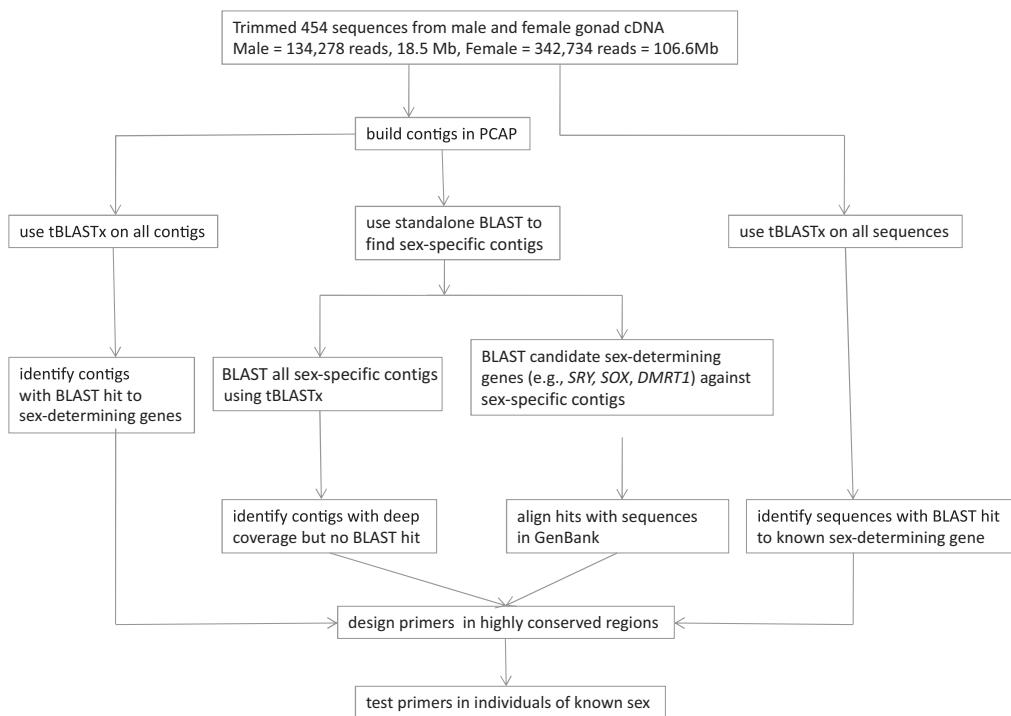


Figure 4–3: Methods employed to search for candidate sex determining. From Hale et al. (2010).

utility for conservation efforts because they require gonad tissue. We uncovered no evidence for a master gene that determines sex by its presence or absence, but we remain optimistic that the evaluation of gonad transcriptomes will generally prove to be a profitable approach for such endeavors in fishes and in various other animals.

Of course, the possibility still remains that lake sturgeon sex determination is not genetic (Hale et al. 2010). An absence of GSD in this species would be consistent with other recent molecular work (Hett & Ludwig 2005; Wuertz et al. 2006; Keyvanshokooh et al. 2007) but apparently inconsistent with cytogenetic evidence from a related species (Van Eenennaam et al. 1999) and with unpublished observations that lake sturgeon hatcheries produce sex ratios near parity.

Lessons from lake sturgeon

The development of sexing assays in reptiles, amphibians, and fishes is not trivial. First and foremost, one needs to know that sex determination is genetic. For the reasons outlined in this chapter, GSD is suspected in lake sturgeon but not confirmed because of their delayed sexual maturity, tiny homomorphic chromosomes, and polyploidy. Second, we suspect that the development of robust sexing assays might be easier in species with small simple genomes than in polyploids. Finally, the rapid evolution of fish sex chromosomes and of master sex-determining genes means that candidate loci will typically be useful only if they are assessed in species closely related to the prototype species from which the gene originally was described. Thus, the approaches described herein need to be

refined if the scientific community is to more efficiently identify and characterize various master sex-determining genes among the diversity of fishes.

Prospective

In species with GSD, molecular markers can assign sex to unconventional tissue samples such as hair, feathers, blood, or fin snips. The utility of molecular sexing assays is yet to be realized in fishes because sex-determining genes and chromosomes evolve rapidly in this taxonomic group. When such assays *are* developed – and, in species with pure GSD, there is no reason to think they will not be developed – they will likely be of greatest interest and utility in long-lived species of management or conservation concern, such as sturgeon, tunas, billfishes, paddlefish, and rockfishes. As the taxonomic distribution of complete genome sequences increases, speeded in part by new technologies such as pyrosequencing, our ability to use comparative genomics to help elucidate mechanisms of sex determination in various vertebrate lineages, including fishes, will be greatly enhanced.

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