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Genetic sex determination, gender identification and pseudohermaphroditism in the knobbed whelk, *Busycon carica* (Mollusca: Melongenidae)

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We report perhaps the first genic-level molecular documentation of a mammalian-like 'X-linked' mode of sex determination in molluscs. From family inheritance data and observed associations between sex-phenotyped adults and genotypes in *Busycon carica*, we deduce that a polymorphic microsatellite locus (*bc2.2*) is diploid and usually heterozygous in females, hemizygous in males, and that its alleles are transmitted from mothers to sons and daughters but from fathers to daughters only. We also employ *bc2.2* to estimate near-conception sex ratio in whelk embryos, where gender is indeterminable by visual inspection. Statistical corrections are suggested at both family and population levels to accommodate the presence of homozygous *bc2.2* females that could otherwise be genetically mistaken for hemizygous males. Knobbed whelks were thought to be sequential hermaphrodites, but our evidence for genetic dioecy supports an earlier hypothesis that whelks are pseudohermaphroditic (falsely appear to switch functional sex when environmental conditions induce changes in sexual phenotype). These findings highlight the distinction between gender in a genetic versus phenotypic sense.

Keywords: microsatellites; sex ratio; sex-linked marker; dioecy; hermaphroditism; molluscs

1. INTRODUCTION

Molluscs collectively display diverse reproductive systems ranging from parthenogenesis to dioecy (separate sexes) to sequential as well as simultaneous hermaphroditism (Coe 1943; Heller 1993). Modes of sex determination, although poorly characterized, are also thought to be diverse: a mammalian-like X/Y system in several gastropods (see Vitturi *et al.* 1998) and the surfclam *Mulinia lateralis* (Guo & Allen 1994); an X/O system in *Theodoxus* and *Littorina* snails (Vitturi & Catalano 1988; Vitturi *et al.* 1988, 1995); a *Drosophila*-like mechanism of X/autosome balance in the clam *Mya arenaria* (Allen *et al.* 1986); a suspected multi-locus genetic arrangement in the oyster *Crassostrea virginica* (Haley 1977, 1979); a system seeming to involve a dominant allele for maleness and another allele for protandric femaleness in *Crassostrea gigas* (Guo *et al.* 1998); and a system wherein maternal nuclear genotypes appear to influence gender in *Mytilus* mussels (Kenchington *et al.* 2002).

We use a combination of genetic parentage analyses and association studies (between microsatellite genotype and sexual phenotype) to document that at least some melongenid whelks have an X/Y- or X/O-like genetic system of sex determination with male hemizygosity. We also employ these gender-associated DNA markers to identify sex in more than 750 full-sib and half-sib embryos within two maternal broods of knobbed whelk embryos, and compare these near-primary sex ratios with those reported in adult populations.

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2. MATERIAL AND METHODS

(a) *The animals*

Our focal species is the knobbed whelk, *Busycon carica*, for which 139 adults plus samples of 447 and 321 juveniles from two large half-sib families were genetically screened. Other melongenids analysed are the lightning whelk *Busycon sinistrum* (41 adults), channelled whelk *Busycotypus canaliculatus* (28 adults), pear whelk *Busycotypus spiratus* (two adults) and three adult crown conch *Melongena corona* (a phylogenetic outlier within the family). Mating whelk adults and associated egg cases were collected from intertidal flats adjoining Wassaw Island, Chatham Co., GA, during March and November 2002. Crown conchs were collected in Hillsborough Co. near St Petersburg, FL, in July 2002. Each adult was inspected for presence or absence of a penis (prominently located behind the head). Adults appeared to be sexually mature, but their absolute ages are unknown and in general are extremely difficult to estimate in this species (A. J. Power, unpublished data).

Two female knobbed whelks (identification numbers 564F and 523F) captured during egg laying supplied embryos that were the basis of family analyses. Whelks lay dozens of closed leathery capsules in long strings, each capsule housing several to many developing embryos (Power *et al.* 2002). By comparing each embryo's genotype at each microsatellite locus with that of its known mother, we deduced each embryo's paternally derived allele by subtraction (see Avise *et al.* 2002). An accumulation of such genetic data across loci and embryos revealed paternity within broods, which in turn facilitated analysis of inheritance modes of the molecular markers.

(b) *Molecular assays*

From each adult, DNA was extracted from foot or siphon tissue by using a standard phenol : chloroform protocol. For each embryo (2–4 mm in length and fully shelled), DNA was

Table 1. Whelk microsatellite loci and assay conditions.

locus	PCR primers[a]	repeat unit	annealing temperature (°C)	[MgCl] (mM)
bc2.2	CTGTTTCTTTATTCATATTTCTTTCTCATTATGATTGTA ACAGTGCCT	AC/TAA (irregular)	52	1.0
bc3.4	ATCCATCTAAATACACTTATGTGATGAGTTAGGTTTGGG AAAAGTGA	$[AG]_{20}$	56	1.2
bc3.12	AAATATGATATAGAATACAGATAATAACTTGACAGGACT TACTAATATG	$[AG]_{43}$	56	1.2

[a] GenBank accession numbers are AY318862 to AY318864.

isolated by removing the specimen from its egg capsule and extracting total DNA in 150 µl of Gloor & Engels (1992) buffer following the procedures described in Jones & Avise (1997).

To construct a genomic library, DNA from a *B. carica* adult was digested with *Mbo*I. After electrophoresis of digested DNA through a 2% agarose gel, fragments 200–800 bp long were purified by Prep-A-Gene Purification System (BioRad) and inserted into phagemid vectors (pBluescript II SK, Stratagene) that had been cut with *Bam*HI, and dephosphorylated. Ligations were transformed into *E. coli* (XL2-Blue MRF′ Ultracompetent Cells, Stratagene) and plated on LB-ampicillin plates for overnight growth. Colonies were screened for microsatellite sequences following Jones & Avise (1997). Positive clones were sequenced in the Molecular Genetics Instrumentation Facility at the University of Georgia, using T7 or T3 primers. Specific primers flanking each microsatellite locus were designed, optimized and labelled with γ-P^{32}, and the resulting PCR products were run through 6% acrylamide gels.

After this initial assessment, reverse primers for polymorphic markers were labelled with 6-FAM fluorescent dye (Integrated DNA Technologies, Inc.). PCR reactions were performed in 10 µl total volume containing 1× Promega *Taq* buffer, 0.25 U of *Taq* polymerase, 0.1 mM of each dNTP, 0.3 µM of each primer, and 1.0–1.2 mM of $MgCl_2$ (table 1). Thermocycling conditions consisted of an initial denaturation at 95 °C for 2 min followed by 30 cycles at 95 °C for 1 min, annealing for 1 min (temperatures in table 1), and 72 °C extension for 1 min, with a final extension step of 3 min at 72 °C. Each PCR product (0.8 µl) was electrophoresed through a 4.2% acrylamide gel with 2.1 µl of deionized formamide, 0.5 µl of loading buffer and 0.3 µl of ROX 500 Size Standard (Applied Biosystems). Software GeneScan 3.1 and Genotyper 2.5 were used to score data collected from runs on an ABI377 automated sequencer.

3. RESULTS

Microsatellite loci developed and employed in the current study are described in table 1. Locus *bc2.2*, the main focus of this report, is a complex trinucleotide motif composed of $(TAC)_n$ at the 5′ end and $(TAA)_n$ at the 3′ end flanking a unique non-repeat region 46 bp long in our clone. PCR-amplified fragments at *bc2.2* ranged in size from 279 to 330 bp in our sample of adult knobbed whelks, with all alleles differing by integer multiples of 3 bp units. Population genetic features of *bc2.2* and the other two loci scored (*bc3.4* and *bc3.12*) are summarized in table 2.

(a) *Sex linkage*

At locus *bc2.2*, among 139 adult knobbed whelks assayed, all 73 individuals with a penis displayed a single primary DNA band on gels, whereas 62 out of 66 individuals (94%) lacking a penis displayed two bands (table 3; figure 1). No gender associations characterized the other two microsatellite loci, which instead behaved like standard autosomal loci with Mendelian inheritance (table 2).

Genotypes implied by DNA-band profiles at *bc2.2* fit closely with a genetic model entailing sex linkage. If we provisionally assume that each two-band female is a diploid heterozygote and that each male is hemizygous, then 'observed' allele frequencies in the adult population sample can be readily calculated. Based on these frequencies and Hardy–Weinberg reasoning (see footnote to table 3), approximately 7.4 diploid females in our adult sample are expected to display a single band (i.e. be homozygous) at *bc 2.2*, and 58.6 females should show two bands each (i.e. be heterozygous). We observed four and 62 females in these two respective categories. These numbers do not depart significantly from a genetic model of sex linkage with female diploidy and male hemizygosity ($\chi^2 = 1.76$, d.f. = 1, $p > 0.1$; table 3).

Further support for this genetic model came from analysis of a large family of knobbed whelks. In the egg string of female 564F, which was heterozygous for alleles '303' and '306' at *bc2.2*, all 447 assayed progeny displayed either allele '303' or allele '306', and in frequencies not departing significantly from a 1 : 1 ratio (221 embryos carried '303', and 226 embryos carried '306'; $\chi^2 = 0.06$, d.f. = 1, $p > 0.5$). Thus, each embryo inherited one or the other maternal allele at *bc2.2* in a fashion consistent with Mendelian segregation from a diploid locus. Furthermore, 203 progeny (45.4%) displayed only the one band of maternal origin, whereas the remaining 244 progeny (54.6%) displayed an additional allele evidently from its father. Four such paternally derived alleles at *bc2.2* were detected: allele '285' (120 embryos), allele '300' (14 embryos), allele '309' (53 embryos) and allele '312' (57 embryos). This implies that *at least* four different fathers had sired the brood.

Table 2. Population genetic data for the adult collection of knobbed whelks.

locus	size range (bp) of amplified product	number of specimens genotyped	number of different alleles	heterozygosity		probability[b]
				observed	expected	
bc2.2	279–330	66[a]	15[a]	0.939[a]	0.888[a]	$0.5 > p > 0.1$
bc3.4	209–228	126	12	0.738	0.814	$0.05 > p > 0.025$
bc3.12	106–188	132	36	0.947	0.964	$0.5 > p > 0.1$

[a] Number here refers only to assayed females (because males are hemizygous).
[b] Results of χ^2-tests (d.f. = 1 in each case) of difference between observed and expected heterozygosity. The significant departure at locus *bc3.4* is probably due to the fact that several alleles at this locus differed by 1 bp, so some heterozygotes may have been missed.

Table 3. Gel-banding profiles observed at *bc2.2* in adult knobbed whelks.
(Shown in parentheses are expected numbers of individuals in each category, under a simple genetic model of sex linkage with male hemizygosity (see text and footnote a). The departure from this genetic model is not statistically significant $\chi^2 = 1.76$, d.f. = 1, $0.5 > p > 0.1$.)

whelk morphology	DNA gel profile	
	one band	two bands
male	73 (73.0)	0 (0.0)
female	4 (7.4)[a]	62 (58.6)[a]

[a] Expected number of one-band (presumably homozygous) females is calculated as $\Sigma f_i^2(n)$, where f_i is the estimated frequency of the ith allele in the adult population, and n is the number of females sampled. The expected number of two-band females is then $n - \Sigma f_i^2(n)$.

(b) *Sex identification and sex ratio estimation*

Given that knobbed whelk males are hemizygous and females are usually, but not invariably, heterozygous at *bc2.2*, an opportunity arises to identify sex (otherwise undeterminable) in individual embryos. A caveat should be: homozygous daughters would display a single *bc2.2* band and, at face value, could be misinterpreted as sons.

However, such scoring errors can be statistically addressed. Suppose that a brood's dam and sire(s) are known, either from controlled breeding experiments or through multi-locus paternity analysis as in the current study. Let N_2 be the observed number of progeny in a brood that show two bands at *bc2.2*; let P be the total proportion of the brood fathered by sire(s) that carry allele(s) at that locus that are identical to one or the other of those in the heterozygous dam; and let N_F be the 'corrected' number of females in the total sample of N_T embryos from that brood. Then, $N_F = N_2 + P(N_T / 4)$.

For brood 564F, this statistical correction proved to be unnecessary. Using data from *bc2.2* with genotypes at autosomal loci *bc3.4* and *bc3.12*, we determined with near certainty that the examined progeny of female 564F had been sired by precisely five males whose multi-locus genotypes were specified explicitly in a genetic paternity analysis (D. Walker, A. J. Power and J. C. Avise, in preparation). None of those fathers carried either the '303' or '306' allele, so all daughters should appear heterozygous (barring a *de novo* mutation). Thus, $P = 0$, and a face-value estimate of sex ratio in this brood (from two-band gel profiles at *bc2.2*) is identical to the actual genetic estimate: 244 daughters and 203 sons.

However, in a second whelk family (523F) that we similarly genotyped at all three microsatellite loci, only 89 among 321 surveyed embryos (28%) showed two bands at *bc2.2*. Multi-locus paternity analysis revealed that 62% of those embryos (i.e. $P = 0.62$) were sired by male(s) that shared either allele '300' or '312' at *bc2.2* with the known heterozygous dam. Using the formula given above, the corrected estimate of number of female embryos for that brood then becomes 139, or 43% of the offspring surveyed. Thus, altogether for the two broods examined (564F and 523F), we estimate that 383 out of 768 embryos (49.9%) were genetic females.

(c) *Other whelk species*

Our sample of adult channelled whelks consisted of 18 phenotypic males (penis present) and 10 females (penis absent). Locus *bc2.2* amplified well in this species also, and showed six different alleles. All 18 phenotypic males exhibited a one-band *bc2.2* profile and all 10 females displayed two bands. This is strong evidence that males again are hemizygous, females typically heterozygous, and that *bc2.2* is sex linked in this species also.

In lightning whelks the situation is less clear. All four specimens with a penis showed a single *bc2.2* band, but so too did 20 out of 37 individuals (54%) morphologically typed as females (the others showed two bands each). Some one-band females might have been homozygous at *bc2.2*, but using our provisional frequency estimates for the 14 observed alleles, and assuming Hardy–Weinberg equilibrium (procedurally as in table 3), the expected number of homozygous females (4.4) was significantly lower than the observed number of one-band females ($\chi^2 = 62.8$, d.f. = 1, $p < 0.001$). We do not know the reason for this partial departure from a simple sex-linked genetic model, but one possibility is that some alleles failed to amplify, thereby increasing apparent (but spurious) homozygosity in females. In many other taxonomic groups, PCR amplifications are known to fail with higher likelihood when heterospecific primers are employed (see FitzSimmons *et al.* 1995; Jarne & Lagoda 1996; Lowe *et al.* 2002).

For pear whelk and crown conch, efforts to amplify *bc2.2* were unsuccessful. We used a variety of PCR

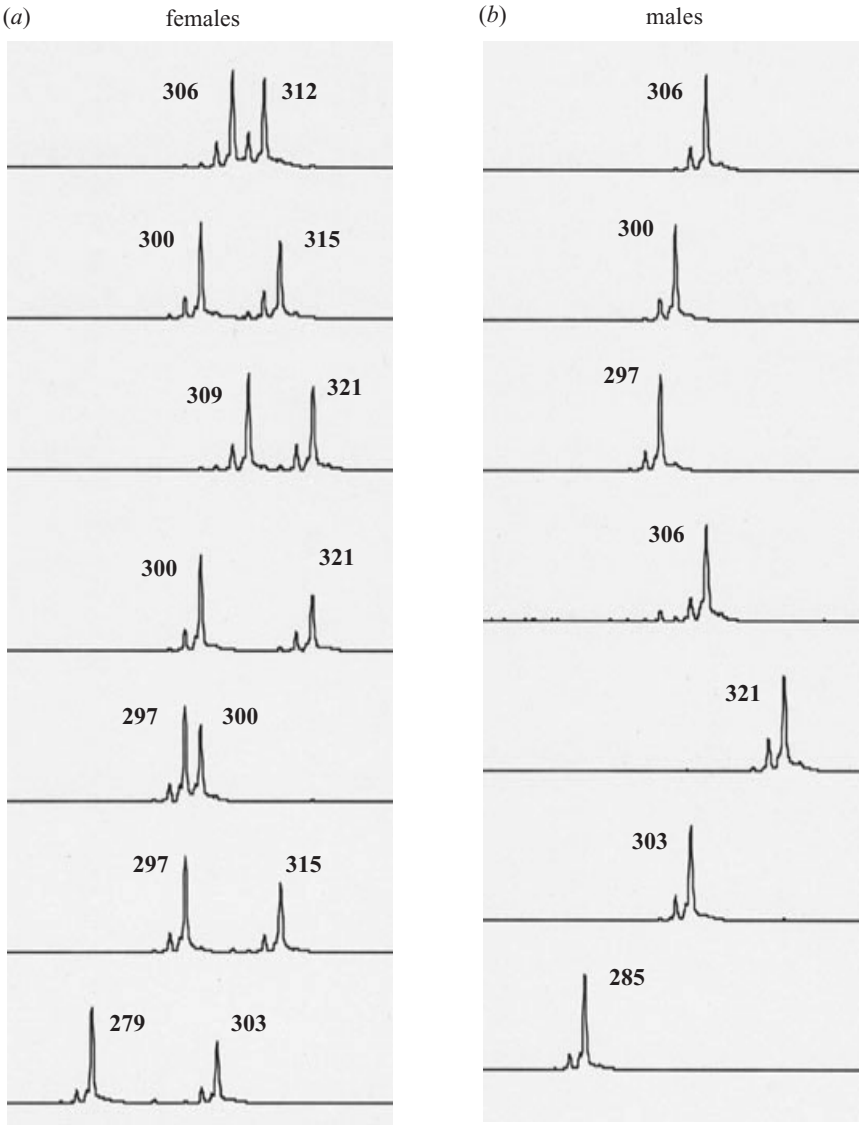

Figure 1. DNA banding patterns at sex-linked locus *bc2.2* in representative adult knobbed whelks. Shown are GENOTYPER plots displaying DNA band peaks from fluorescently labelled PCR products in seven females and seven males. Numbers indicate allelic sizes (lengths of PCR-amplified products). Note that heterozygous females display two primary peaks (alleles) each, whereas hemizygous males display only one major peak.

conditions, so it appears that the knobbed whelk primers failed to anneal effectively to DNA from these species.

4. DISCUSSION

Gender-associated DNA markers can help reveal modes of sex determination in particular taxa, provide markers for identifying an individual's sex at any stage of life and allow estimates of population sex ratios when these are not otherwise evident. All of these utilities are illustrated here by the hereditary and population features of a sex-linked molecular-genetic polymorphism in *B. carica* and relatives.

(a) *Sex-determination mode*

According to karyotypic analyses, several marine gastropods representing Neritopsina and Caenogastropoda (two out of five subclasses of Gastropoda; molluscan taxonomy is controversial, and nomenclature here follows Tudge (2000)) have either an 'X/Y' or an 'X/O' mode of sex determination (Thiriot-Quiévreux & Ayraud 1982; Vitturi & Catalano 1988; Vitturi *et al.* 1988, 1995, 1998; Rolan-Alvarez *et al.* 1996). However, few species have been assayed. Melongenid whelks are caenogastropods, so our findings extend reports of an X-linked system to another family and suggest that the phenomenon is widespread in this subclass.

Genic-level evidence alone cannot distinguish X/Y from X/O systems because 'X' and 'Y' by definition refer to karyotypes. Regardless of whether the system is 'genic' or 'chromosomal', we are not suggesting that *bc2.2 per se* mechanistically affects gender. Rather, we interpret this locus to mark, through linkage, either an entire chromosome or a smaller subset of gene(s) that causally influence sexual differentiation.

(b) *Pseudohermaphroditism*

Based on the observation that most large specimens seem to be females, previous authors speculated that *Busycon* whelks are protandrous hermaphrodites (males first, changing later to functional females) under normal

ecological conditions (Magalhaes 1948; Walker 1988). Our genetic findings cast serious doubt on this possibility, at least for the populations surveyed. Nonetheless, the protandric hypothesis is difficult to eliminate completely. A 1 : 1 sex ratio in embryos does not rule out the possibility that some juveniles mature as males first and then switch to functional females occasionally. Also, it is conceivable that some of the single-band females at *bc2.2* are actually hemizygous genetic males that lost or never developed a penis.

Environmental factors can impinge on sexual anatomy in whelks and other molluscs. For example, *Busycon* males reared for many years in the laboratory reportedly sometimes experience pronounced reductions in penis size (Castagna & Kraeuter 1994). Trematode parasites can 'castrate' molluscs (Køie 1969) and are known to prompt reproductive abnormalities in some crustaceans (Pung *et al.* 2002) and vertebrates (Johnson *et al.* 2002). In some Prosobranch gastropods, high concentrations of exogenous tributyltin chemicals (used in anti-fouling paints) are also known to impose anatomical sex changes (Gibbs *et al.* 1988; Power & Keegan 2001): Females become 'imposex' specimens displaying a vas deferens and penis (although it is doubtful that these organs are fully functional; Power & Keegan (2001)). This direction of sex change is the opposite of that predicted for whelks under protandrous hermaphroditism. Indeed, any ontogenetic switches between sex, if both functionally effective and common in wild knobbed whelks, would be hard to reconcile with our current findings implying that this species is genetically dioecious.

Jenner (1978, 1979) used the term pseudohermaphroditism to refer to environment-induced switches she observed between male and female phenotypes in other neogastropod species. This and other evidence led her to question previous assertions of functional genetic hermaphroditism: 'Reports of protandry based on size differences between the sexes or on the occurrence of penial organs in females cannot be considered valid.' Similarly, our data cast doubt on earlier notions that melongenid whelks are sequential hermaphrodites in a genetic or functional sense.

(c) *Sex ratios*

In many taxa, and especially at early life-history stages, an individual's gender is not evident from morphological inspection. This has prompted efforts to identify sex-specific molecular markers, and several such systems are now available, for example for birds (Griffiths *et al.* 1998) and mammals (Fernando & Melnick 2001). We extend this approach to another taxonomic group.

Field observations have indicated highly variable sex ratios in natural populations of adult knobbed whelks (e.g. Magalhaes 1948). For example, in intertidal collections in Georgia (but not necessarily in the sub-tidal zone; A. J. Power, unpublished data), mature females often outnumber mature males by more than 10 to 1 (Walker 1988). Perhaps males tend to occupy sub-tidal habitats less accessible to human collectors, or otherwise are less subject to harvest. Perhaps males suffer higher mortality during life. Or perhaps females outnumber males because of a biased primary sex ratio (i.e. at conception). Our data allowed the first tests in whelks of this last possibility.

In the two whelk broods assayed, 49.9% out of the 768 surveyed embryos proved to be genetic females. If the sex ratio we observed in these embryonic knobbed whelks (1 : 1) generally holds true for this species, then the huge sex-ratio bias (i.e. greater than 10 : 1) often observed in adults is not simply a consequence of an unbalanced sex ratio at conception. In other words, if whelk broods have a nearly equal proportion of male and female zygotes (as both the genetic model and our initial genetic surveys imply), then any lopsided sex ratios reported in adult populations must be due to other considerations such as habitat or collection biases, or differential post-embryonic mortality.

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