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Ubiquitous heteroplasmy in *Mytilus* spp. resulting from disruption in doubly uniparental inheritance regulation

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ABSTRACT: Species within the marine mussel *Mytilus edulis* complex (*M. edulis*, *M. galloprovincialis*, and *M. trossulus*) have an unusual form of mitochondrial DNA (mtDNA) inheritance commonly referred to as doubly uniparental inheritance (DUI). With DUI, all progeny inherit mtDNA maternally (F), while male progeny also inherit mtDNA paternally (M) through their father's sperm. Therefore, females are normally homoplasmic for the F mtDNA, and males are heteroplasmic for the F and M mtDNA. In this study, we show that the regulation of DUI in populations of blue mussels in northern Japan is severely disrupted; consequently, the majority of individuals (89%) are heteroplasmic for M and F mtDNA. Disruption of DUI is primarily due to the failure of female embryos to exclude the father's M mtDNA from their somatic and germinal tissues. High proportions of heteroplasmic females have only been reported in Japanese mussel populations thus far. We show that this disruption occurs in both parental species (*M. trossulus* and *M. galloprovincialis*) as well as hybrids and is independent of geographic location around Hokkaido, Japan. This finding differs from many previous studies, which have shown that disruption in DUI is confined to hybrid zones. In addition, some individuals were heteroplasmic for M or F mtDNA, indicating that DUI disruption is multigenerational. Triplasmic individuals were 3 times more often female and were mostly confined within the hybrid zones; however, a few individuals were found within *M. galloprovincialis* parental populations.

KEY WORDS: *Mytilus galloprovincialis* · *Mytilus trossulus* · Doubly uniparental inheritance · Disruption · Heteroplasmy

INTRODUCTION

Mitochondrial DNA (mtDNA) has been an important tool for understanding phylogenetic relationships among organisms (Avise et al. 1987). In most animal species, mtDNA is normally inherited maternally, although cases of paternal leakage have been reported (Kondo et al. 1990, Avise 1991, Gyllensten et al. 1991, Kvist et al. 2003, Fontaine et al. 2007). In some groups of marine mussels, however, males inherit mtDNA from both their fathers and their mothers. Commonly referred to as either sex-limited inheritance (Skibinski et al. 1994) or doubly uniparental inheritance (DUI) (Zouros et al. 1994), this mode of inheritance has been found in bivalves from the families Donacidae (Theologidis et al. 2008), Hyriidae (Curole & Kocher 2005), Margaritiferidae (Curole & Kocher 2002, 2005), Mytilidae (Skibinski et al. 1994, Zouros et al. 1994, Hoeh et al. 1996, Rawson et al. 1996b, Passamonti 2007), Unionidae (Hoeh et al. 1996, Liu et al. 1996), and Veneridae (Passamonti & Scali 2001). Under DUI, all progeny receive mtDNA maternally (F), while male progeny also obtain mtDNA paternally (M) through their father's sperm. Therefore, females are normally homoplasmic for the F mtDNA and males are heteroplasmic for the F- and M-derived mtDNA.

DUI was first discovered in mussels of the *Mytilus edulis* species complex (*M. edulis*, *M. galloprovincialis*, *M. trossulus*), which continues to be the pri-

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mary system for studying the regulation of this mode of mtDNA inheritance (Fisher & Skibinski 1990, Hoeh et al. 1991). Phylogenetic analysis revealed that the 2 mtDNA lineages predate speciation in this group (Rawson & Hilbish 1995b); the M mtDNA is as much as 20% differentiated from the F mtDNA, depending on the region of the mitochondrial genome examined (Hoeh et al. 1991). Species within the *M. edulis* complex occur in the marine temperate zone throughout the world and hybridize in areas of sympatry (Gosling 1992). The disruption in mtDNA inheritance among natural and experimental hybrids has provided key insights into the normal regulation of DUI.

Departures from the normal DUI expectations have been reported in the *Mytilus edulis* complex since the early 1990s (Hoeh et al. 1991, Zouros et al. 1994, Rawson et al. 1996b). Early studies showed that hybridization frequently resulted in the failure of male hybrid mussels to inherit the M mtDNA from their fathers (Zouros et al. 1994, Rawson et al. 1996b). However, in individuals sampled from allopatric parental populations or progeny from experimental homospecific crosses, paternal transmission of M mtDNA occurred with high fidelity (Zouros et al. 1994, Rawson et al. 1996b). The failure to inherit the M mtDNA in hybrid males was observed in similar frequencies in *M. edulis*-*M. trossulus* (41%, Zouros et al. 1994) and *M. galloprovincialis*-*M. trossulus* crosses (35%, Rawson et al. 1996b); however, male hybrids formed from *M. edulis*-*M. galloprovincialis* crosses did not show this failure as frequently (11%, Rawson et al. 1996b).

In addition, Rawson et al. (1996b) found that in *Mytilus galloprovincialis*-*M. trossulus* hybrids, females may also display departures from normal DUI in the form of retention of the M mtDNA from their fathers. They found that among hybrid females, 41% contained M mtDNA in addition to the F mtDNA (Rawson et al. 1996b). This study demonstrated that a mechanism must exist in females to exclude paternally inherited mitochondria from cell lineages during early development. Failure to do so results in the occurrence of M mtDNA throughout the tissues of hybrid females. Rawson et al. (1996b) hypothesized that the failure to regulate the inheritance of F and M mtDNA was the result of incompatibilities between nuclear and mitochondrial DNA in hybrid individuals.

Studies of DUI in experimental and natural populations of mussels in the genus *Mytilus* indicate that genetically based mechanisms exist to ensure the inclusion of M mtDNA into the germ line of male mussels and to exclude it from the somatic cell lines of female mussels (Kenchington et al. 2002, Cao et al. 2004, Cogswell et al. 2006). Hybridization may result in the breakdown of these regulatory mechanisms, but the degree of disruption is related to the level of differentiation between the taxa involved in hybridization. In non-hybridizing populations of mussels, DUI is typically well regulated, resulting in high-fidelity maternal transmission of F mtDNA to daughters and sons and paternal transmission of M mtDNA to the germ line of males (Zouros et al. 1994, Rawson et al. 1996b).

More recent studies have added further insights to our understanding of DUI. Wood et al. (2003) found a large difference in the presence of the M haplotype when comparing 72 h larvae from *Mytilus galloprovincialis* and *M. edulis* homospecific (M haplotype presence: 47 to 68%) and heterospecific (M haplotype presence: 4 to 93%) crosses. The presence of the M haplotype in hybrid progeny appeared to be largely dependent on the species of the female parent (Wood et al. 2003). In heterospecific crosses where *M. galloprovincialis* was the female parent, the M haplotype was present in 4 to 14% of the progeny, while in crosses where *M. edulis* was the female parent, M haplotype presence ranged from 82 to 93% (Wood et al. 2003). These findings illustrate disruption in DUI in *M. galloprovincialis*-*M. edulis* hybrids at far greater frequencies than those reported in Rawson et al. (1996b). Kenchington et al. (2009) suggested that inheritance of the father's M mtDNA may not be related to gender of the individual but, rather, may be dependent on the female parent. Mothers that were biased to produce female offspring had progeny that did not contain the M genome, even if the offspring was male (Kenchington et al. 2009). On the other hand, male-biased mothers produced progeny that displayed normal DUI; sons were heteroplasmic for M and F mtDNA, and females were homoplasmic for only F mtDNA (Kenchington et al. 2009). Both of these findings held true for both heterospecific and homospecific crosses (Kenchington et al. 2009).

Brannock et al. (2009) described the geographic distribution of *Mytilus galloprovincialis, M. trossulus*, and their hybrids in northern Japan. They observed that mussel populations in this region had a highly heterogeneous pattern of DUI; up to 96% of sampled mussels were heteroplasmic for M and F mtDNA (P. M. Brannock, D. S. Wethey, T. J. Hilbish unpubl. data), but because the gender of individual mussels was not determined, examination of departures from DUI expectations could not be performed. However, these observations suggested the possibility that

there is a strong disruption of sex determination in these populations resulting in sex ratios highly skewed in favor of males. Alternatively, mussel populations in northern Japan may experience unusually elevated disruption of the regulation of DUI.

In this study, we show that exceptionally high levels of DUI disruption occurs in blue mussels from northern Japan and that this disruption is not caused by hybridization but rather occurs in allopatric populations of the parent species as well as among hybrids and non-hybrids within hybrid zones between *Mytilus galloprovincialis* and *M. trossulus*. We also demonstrate that disruption of DUI in this system is likely to occur almost exclusively through the failure of females to exclude the father's M mtDNA.

MATERIALS AND METHODS

A total of 1055 blue mussels (approximately 50 per site, ≥15 mm in shell length) were collected from each of 18 locations around the island of Hokkaido, Japan, in May and June 2008 (Fig. 1, Table 1). These locations are the same as those reported in Brannock & Hilbish (2010). Since the only way to successfully determine gender of these mussels is visualization of the gametic tissue, gender of individual mussels was assessed using gonad squashes and microscopic examination (100× and 400× magnification) of the mantle center, which serves as the principal site of gametogenesis (Lowe et al. 1982). Individuals for which gender could not be determined were not included in the present study; therefore, there may

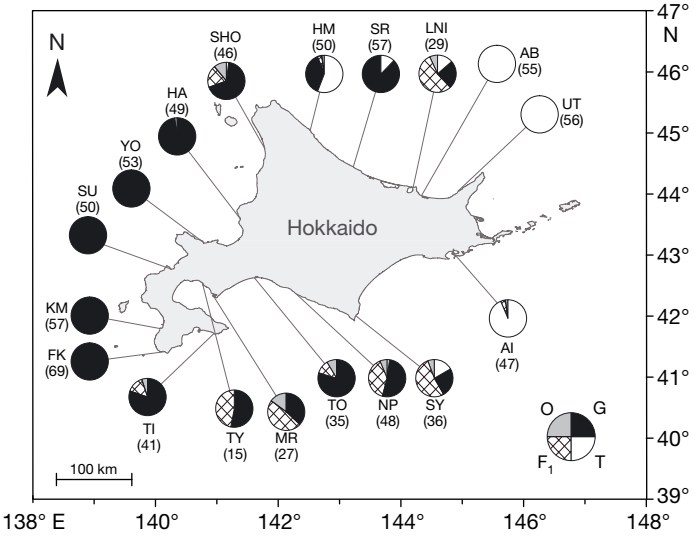

Fig. 1. *Mytilus galloprovincialis* and *M. trossulus*. Geographic distributions of *M. galloprovincialis* (black, G), *M. trossulus* (white, T), F_1 hybrids (cross-hatched, F_1), and other hybrids (gray, O). Figure is a modification of data reported in Brannock & Hilbish (2010) and only includes individuals where gender was determined in the field. Sample sizes are represented in parentheses. See Table 1 for full sampling site names and abbreviations

Table 1. Number of male and female individuals collected from each of the 18 locations around the island of Hokkaido in May and June 2008 and number of individuals that contained (M+) or lacked (M-) a paternal (M) mtDNA haplotype, separated by gender

	Site	Abbreviation	n	Gender		Gender/haplotype			
				Female	Male	Female/–M	Male/+M	Female/+M	Male/–M
West	Shosanbetsu	SHO	47	27	20	13	19	14	1
	Hamamasu	HA	49	29	20	6	19	23	1
	Yoichi Cape	YO	53	28	25	3	25	25	0
	Suttsu	SU	50	19	31	5	30	14	1
	Kaminokani	KM	57	18	39	6	39	12	0
	Fukushima	FK	69	31	38	2	38	29	0
North	Hamatonbetsu	HM	50	31	19	14	19	17	0
	Saruru	SR	57	32	25	5	25	27	0
	Lake Notoro Inner	LNI	29	13	16	4	14	9	2
	Abashiri	AB	55	27	28	1	28	26	0
	Utoro	UT	56	26	30	3	30	23	0
South	Toi	TI	41	25	16	10	15	15	1
	Toyako Town	TY	15	7	8	0	8	7	0
	Muroran	MR	27	11	16	1	15	10	1
	Tomakomai	TO	35	13	22	3	22	10	0
	Nikappu	NP	48	21	27	6	27	15	0
	Shoya	SY	36	14	22	3	22	11	0
	Akkeshi Intertidal	AI	47	20	27	1	27	19	0
Total			821	392	429	86	422	306	7

be discrepancies in population composition from what was reported in Brannock & Hilbish (2010). In addition, because Brannock & Hilbish (2010) reported high levels of disseminated neoplasia (Peters 1988, Elston et al. 1992) at Nemero, we excluded this location from our analysis to eliminate any potential influences of parasitism on the DUI results.

A piece of tissue from the center of the mantle, edge of the mantle, and adductor mussel was collected from each individual and preserved in 95% ethanol to be used for genetic analysis. These tissues were used to compare the presence of mtDNA in somatic (mantle edge and adductor) or gametic (mantle center) tissue. Total genomic DNA was extracted from all 3 tissues using the phenol chloroform protocol in Rawson et al. (1999). DNA extracted from the mantle edge was assayed at 3 single-locus nuclear markers: internal transcribed spacer region (*ITS*) (Heath et al. 1995), 5′ end of the polyphenolic adhesive protein gene (*Glu-5′*) (Inoue et al. 1995), and *Mytilus* anonymous locus-I (*MAL-I*) (Rawson et al. 2001). Amplification of all 3 of these markers was performed by PCR as previously described by Brannock et al. (2009); additionally, both *ITS* and *MAL-I* PCR products were subjected to a restriction enzyme digest (Brannock et al. 2009). All 3 of these markers completely differentiate between pure populations of *Mytilus trossulus* and *M. galloprovincialis* (Heath et al. 1995, Inoue et al. 1995, Rawson et al. 1996a, Rawson et al. 1996b, Rawson et al. 1999). Individuals were scored and genotyped at all 3 nuclear loci separately, and those that successfully amplified at all 3 nuclear markers were assigned to one of 4 genealogical classifications (*M. trossulus*, *M. galloprovincialis*, F_1 hybrid, and other hybrid) as described in Brannock et al. (2009).

Mitochondrial haplotypes were assayed for all 3 tissue samples from each individual by PCR amplification of a portion of the mitochondrial 16S rRNA gene as described by Rawson & Hilbish (1995b). All reactions were performed in 12.5 µl volume containing 1× PCR buffer (Promega), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 µM of each primer, 0.5 unit of *Taq* polymerase enzyme (Promega), and DNA. Previous reports have shown ~8.3% divergence between the F and M mtDNA lineages at this gene (Rawson & Hilbish 1995b). Maternal (F) and paternal (M) regions of the mitochondrial 16S rRNA gene were targeted by separate PCR reactions.

The maternally (F) inherited mitochondrial 16S rRNA region was amplified using F-mtDNA-specific primers JH48 (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Rawson & Hilbish 1995a) and PR19 (5′-CTG CCC AGT GCA ACT AGA GTA AT- 3′) (Rawson et al. 1996b). Thermal cycler conditions consisted of initial denaturation at 94°C for 1 min followed by an incubation of 30 cycles of 92°C for 30 s, 49°C for 60 s, and 75°C for 60 s with a final primer extension for 5 min at 75°C. Since the PCR product resulted in equal length fragments for all possible haplotypes, a double restriction enzyme digest using 1 U SpeI and 10 U EcoRV (New England BioLabs) (Rawson et al. 1996b) was used to produce restriction fragment length polymorphisms (RFLPs) that could reliably distinguish among the 3 different haplotypes (A, B, and D) (Rawson & Hilbish 1995b). Previous studies have shown that haplotype B only occurs in *Mytilus trossulus* individuals, haplotype D is associated with *M. galloprovincialis* individuals, and haplotype A is a shared haplotype between both *M. galloprovincialis* and *M. edulis* individuals (Rawson & Hilbish 1995a,b, 1998).

The paternally (M) inherited mitochondrial 16S rRNA was amplified using M-mtDNA-specific primers PR17 (5′-GCT TCT ACA CCT CTA GGA CAC-3′) and PR18 (5′-TGC CCW RTG CAA CTA AAT TAA C-3′) (Rawson & Hilbish 1995a). Thermal cycler conditions were the same as for the F mtDNA amplification, except the annealing temperature was 50°C. Since this reaction also resulted in equal length products for all haplotypes in the *Mytilus edulis* complex, a restriction enzyme digest using 5 U DdeI (New England BioLabs) was used to reliably distinguish between the 3 possible haplotypes for this species complex (C1, C2, C3) (Rawson & Hilbish 1995a,b). Haplotype C1 is solely found in *M. trossulus* individuals, C2 is only associated with *M. galloprovincialis* individuals, and C3 is a shared haplotype between both *M. galloprovincialis* and *M. edulis* (Rawson & Hilbish 1995b, 1998).

Several measures were implemented to ensure the results obtained were not the product of contamination or experimental artifacts. Throughout the study, 3 different controls were used: a negative control (no DNA), a known *Mytilus galloprovincialis*, and a known *M. trossulus*. In addition, for samples assayed as heteroplasmic at either the M or F mtDNA region or that exhibited inconsistent mtDNA results among tissue types, the DNA was re-extracted and the mtDNA haplotypes were re-analyzed.

Alternatively, it is possible that the PCR primers used here are amplifying some locus other than the one intended, leading to incorrect haplotype assignments. This could occur under at least 3 scenarios: the current primers are amplifying an entirely new locus elsewhere in the genome in addition to the tar-

get locus; priming sites in male or female haplotypes in Hokkaido are sufficiently different from those in previous studies that the gender-specific primers here also incorrectly amplify the alternate gender; or a Nuclear Mitochondrial Transposon (*numt*; a sequence of mtDNA that has been translocated from the mitochondrial genome to the nuclear genome) has retained the priming sites and is being amplified in addition to, or instead of, the intended locus. To explore these scenarios, 21 homozygous female individuals that were identified as heteroplasmic (10 *Mytilus galloprovincialis* and 11 *M. trossulus*) within parental populations were chosen at random, and both the M and F amplification products were sequenced bidirectionally using standard BigDye Terminator v3.1 Cycle Sequencing Kit protocols (Applied Biosystems) by Functional Biosciences (Madison, WI, USA) and subsequently compiled using Sequencher v4.1 (Gene Codes). Sixty additional homologous DNA sequences from *M. trossulus* (3 M haplotypes and 10 F haplotypes), *M. galloprovincialis* (15 M haplotypes and 17 F haplotypes), and *M. edulis* (6 M haplotypes and 9 F haplotypes) were downloaded from GenBank and included in the analyses as reference sequences. The accession numbers are U22864–U22885, AF023541–AF023571, AF023591 and AF023593–AF023599. These samples were reported as homozygous individuals in Table 1 of Rawson & Hilbish (1998). One homozygous individual, T-Ba2, was excluded from the analyses due to a discrepancy between the sequence deposited in GenBank (AF023592) and the haplotype assignment as reported in Rawson and Hilbish (1998). Individuals classified as hybrids in Rawson & Hilbish (1998) were not used in the present study. A phylogenetic tree was constructed using the neighbor-joining algorithm in MEGA v5 (Tamura et al. 2011); 5000 bootstrap replicates were performed using a Kimura 2-parameter substitution model, and unknown bases were treated alternatively with complete deletion and pairwise deletion methodologies.

Additionally, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was calculated using the software package Arlequin suite v3.5.1.3 (Excoffier & Lischer 2010), with collection locations (Hokkaido samples from this study versus Pacific North America samples from Rawson & Hilbish, 1995b, 1998) nested within parental haplotype origin (M versus F). Since the evolutionary split between the M and F haplotype lineages predates the split between the 2 species (Rawson & Hilbish 1995b), sequences were grouped as either M or F haplotypes, regardless of species.

RESULTS

Gender was successfully determined for 78.3% of individuals. There was no significant deviation from a 1:1 male (52%) to female (48%) sex ratio when examining all samples together (χ^2 = 0.705, p = 0.401), when individuals were separated by geographic region ($\chi^2 \leq 1.59$, $p \geq 0.207$), or by the specific collection site ($\chi^2 \leq 1.834$, $p \geq 0.226$) except at KM, where there were significantly more males (68%) than females (32%) collected (χ^2 = 4.032, p = 0.045); however, this difference was not significant when adjusted for multiple comparisons (Table 1). Almost all (99.5%) of the individuals for which gender was determined were successfully assayed at all 3 nuclear loci and assigned to one of the 4 genealogical classifications (*Mytilus trossulus*, *M. galloprovincialis*, F_1 hybrid, and other hybrid). A majority (60.6%) of individuals were classified as *M. galloprovincialis*, followed by *M. trossulus* (24.6%), F_1 hybrid (11.9%), and finally other hybrid (2.9%) (Fig. 1). These numbers differ slightly from Brannock & Hilbish (2010) because in this study, only individuals where gender was successfully determined were included. As previously reported in Brannock et al. (2009) and Brannock & Hilbish (2010), the current results show that mussel populations along the western and eastern coasts of Hokkaido are composed of predominantly parental populations of *M. galloprovincialis* and *M. trossulus*, respectively, while hybrid zones are found along both the northern and southern coastlines (Fig. 1).

All individuals were successfully assayed at the F 16S mtDNA locus in at least one of the 3 tissue types (mantle edge, mantle center, or adductor), and 99% were successfully assayed for all 3 tissues. Of these individuals, 99% showed a consistent F mtDNA haplotype among the 3 tissue types. The remaining 1% (8 individuals) had a different F mtDNA haplotype (Table 2) in one of the 3 tissues than in the other 2. A majority of these 8 individuals (n = 6, 75%) had a different F mtDNA in the mantle center tissue (gametic) in comparison to the mantle edge and adductor (somatic). The other 25% (n = 2) showed a different F haplotype in the adductor tissue. Gender of individual was not a factor in the F mtDNA heteroplasmy (Table 2). However, a majority of 8 individuals displaying F mtDNA heteroplasmy were classified as *Mytilus galloprovincialis* (n = 5, 62.5%), with lower percentages classified as other (n = 2, 12.5%) and F_1 hybrids (n = 1, 25%). However, when taking into consideration how many individuals were collected for each genotype, 1% of *M. galloprovincialis*, 1% of F_1

Table 2. Number of individuals at each sampled location around Hokkaido that displayed heteroplasmy at either the maternal (F) or paternal (M) mtDNA locus. Numbers in parentheses indicate how many males and females (males: females) displayed heteroplasmy at that locus. See Table 1 for full sampling site names and abbreviations

	Site	Heteroplasmy	
		F mtDNA	M mtDNA
West	SHO	2 (1:1)	5 (2:3)
	HA	1 (1:0)	6 (1:5)
	YO		1 (1:0)
	SU		2 (0:2)
	KM	1 (1:0)	1 (1:0)
North	HM		6 (2:4)
	SR		3 (2:1)
	LNI		2 (0:2)
South	TI	1 (0:1)	3 (0:3)
	TY		2 (0:2)
	MR		4 (1:3)
	TO	2 (1:1)	2 (1:1)
	NP		5 (1:4)
	SY		6 (2:4)

hybrids, and 7.4% of other hybrids displayed F mtDNA heteroplasmy. There was no F mtDNA heteroplasmy observed in individuals classified as *M. trossulus*.

A total of 88.8% of all individuals successfully amplified at the M mtDNA locus in at least one of the 3 tissue types (Table 1). Only 1.6% of male individuals of all genealogical categories did not contain an M mtDNA haplotype (Table 1). Of males lacking an M mtDNA haplotype, 4 were classified as *Mytilus galloprovincialis*, 2 as F_1 hybrids, and 1 as other hybrids. However, when taking into consideration how many male individuals were collected for each genotype, only 1.6% of *M. galloprovincialis*, 3.2% of F_1 hybrids, and 7.7% of other hybrids males failed to retain the M haplotype. None of the *M. trossulus* males lacked an M mtDNA haplotype. A total of 85% of individuals had the same M haplotype in all 3 tissue types. Of the 66 individuals that were not classified as having the same M haplotype across tissue types, a majority (n = 53, 80.3%) were the result of non-successful M mtDNA amplification in at least one of the 3 tissue types.

An M mtDNA haplotype was present in 78% of female individuals collected (Table 1). This high frequency of M and F heteroplasmy was observed in female mussels regardless of whether they originated from one of the parental populations or from within the hybrid zones and was independent of genealogical classification within the hybrid zones (Table 1). A majority of females from all 4 genealogical classifications contained an M mtDNA haplotype (93% of *Mytilus trossulus*, 75% of *M. galloprovincialis*, 64% of F_1 hybrids, and 70% of other hybrids). Of all female individuals containing an M mtDNA haplotype, 60% showed consistent results in all 3 tissues. Of the 123 individuals that displayed inconsistent M mtDNA results across tissues, a majority (n = 96, 78%) were due to amplification failures in at least one of the 3 tissue types.

In addition, 6% of all individuals were heteroplasmic for M mtDNA (Table 2). Overall, females contained more than 1 M mtDNA over 3 times more often than did males (11 versus 3%), and this difference was significant ($\chi^2 = 5.14$, $p = 0.023$) (Table 2). In pure *Mytilus galloprovincialis* populations from western Hokkaido, 1.4% of individuals were heteroplasmic for both possible *M. galloprovincialis* M haplotypes (C2 and C3) (Fig. 2). In the northern hybrid zone, 15.7, 10, and 7.7% of individuals classified as *M. galloprovincialis*, *M. trossulus*, and F_1 hybrids, respectively, were heteroplasmic for M mtDNA. In the northern hybrid zone, none of the other hybrids were heteroplasmic for M mtDNA. In the southern hybrid zone, 8.8, 14.3, 14.7, and 7.7% of mussels classified as *M. galloprovincialis*, *M. trossulus*, F_1 hybrids, and other hybrids, respectively, were heteroplasmic for M mtDNA. It is not possible to observe

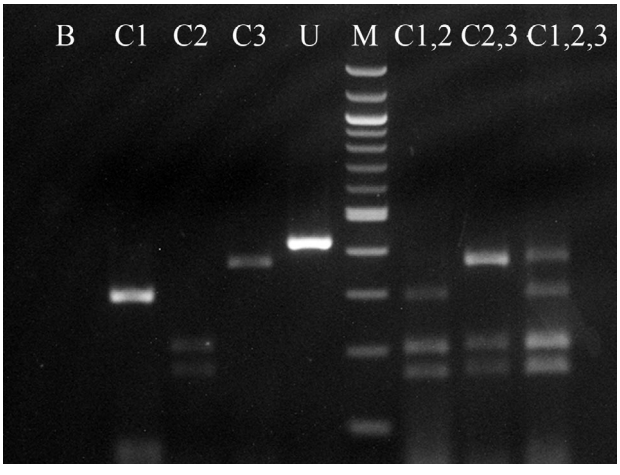

Fig. 2. *Mytilus* spp. Restriction fragment length polymorphism results when the paternal mtDNA locus is targeted and digested using DdeI: B, blank (no DNA); C1, male haplotype associated with *M. trossulus*; C2, male haplotype associated with *M. galloprovincialis*; C3, male haplotype associated with both *M. galloprovincialis* and *M. edulis*; U, uncut DNA; M, 100 bp DNA ladder; C1,2, heteroplasmic individual for C1 and C2 haplotypes; C2,3, heteroplasmic individual for C2 and C3 haplotypes; and C1,2,3, heteroplasmic individual for all 3 possible male haplotypes. Heteroplasmic individuals illustrated here had multiple haplotypes within at least one tissue type

heteroplasmy for M mtDNA in parental populations of *M. trossulus* because they contain only the C1 M haplotype. Of the individuals containing multiple M haplotypes, 18.4% contained all 3 possible M haplotypes (C1, C2, and C3) (Fig. 2). There was no relationship between gender and the presence of all 3 M haplotypes. Of the 9 individuals containing all 3 M haplotypes, 6 were classified as *M. galloprovincialis*, 2 as F_1 hybrids, and 1 as *M. trossulus*. However, when taking into account the total number of individuals sampled in each of these 3 genealogical classes, only 1.2% of *M. galloprovincialis*, 2% of F_1 hybrid, and 0.5% of *M. trossulus* individuals contained all 3 M haplotypes. A majority (n = 34, 70.8%) of individuals showing M mtDNA heteroplasmy had multiple M haplotypes in at least one of the 3 tissue types (Fig. 2). Individuals containing multiple M haplotypes in one tissue were restricted to locations where hybridization occurred, while M mtDNA heteroplasmy within pure *M. galloprovincialis* locations was the result of different M haplotypes between the tissue types.

None of the 3 controls (a negative control, a known *Mytilus galloprovincialis*, and a known *M. trossulus*) used in this study exhibited any evidence of contamination or deviated from expected results. In addition, in all DNA re-extraction cases of heteroplasmic individuals at either the M or F region, the re-analysis yielded the same result as the initial assay.

M and F haplotype sequences (424 bp) were obtained from 21 homozygous female individuals that were identified as heteroplasmic using RFLP analyses. All 42 aligned sequences were deposited in GenBank under accession numbers KC835210 through KC835251. The AMOVA revealed that 59.6% of the total variation in the sequences is due to differences between the M versus F groups, while only 10.3% of the total variation was attributable to differences between collection locations within the M and F groups. Variation within collection locations accounted for 30.1% of the total variation.

The phylogenetic trees revealed a deep split between the M and F haplotype lineages (Fig. 3). These

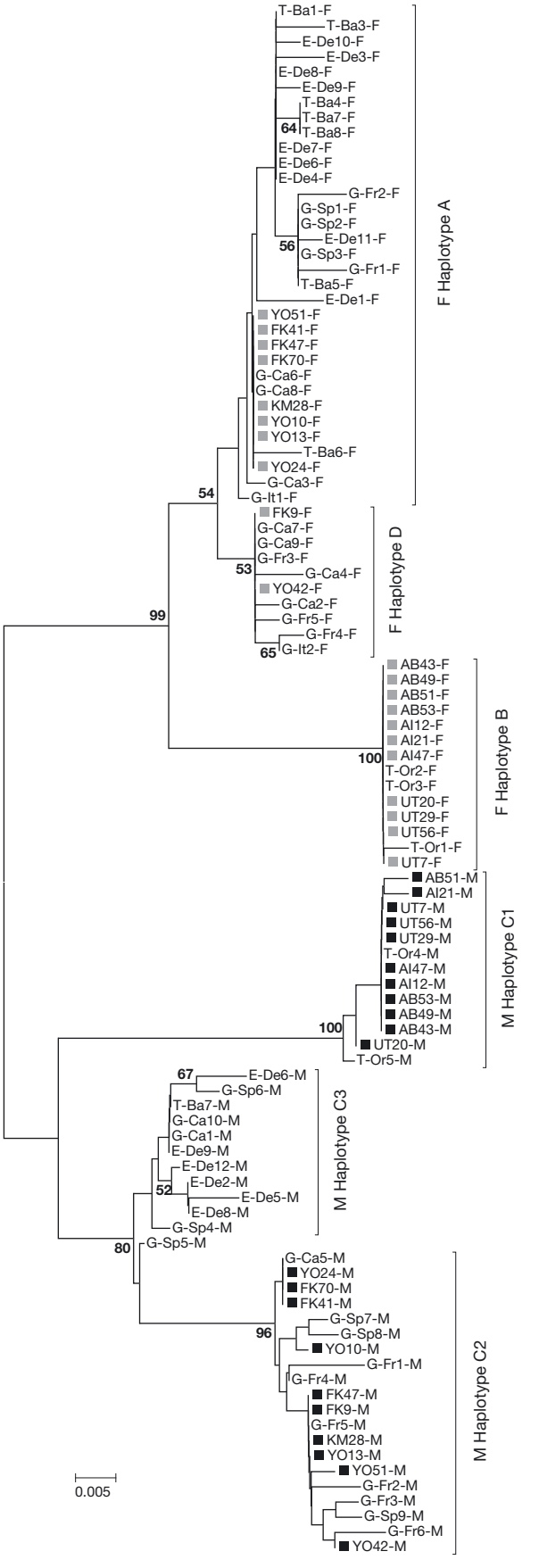

Fig. 3. *Mytilus* spp. Neighbor-joining dendrogram showing the relationships among *Mytilus* spp. 16S mtDNA haplotypes sequenced in this study and in Rawson & Hilbish (1998). Sequences from the current study are from female samples that were determined to be heterozygous for both M and F mtDNA haplotypes. The products of both the M-mtDNA-specific PCR (black squares) and the F-mtDNA-specific PCR (grey squares) were sequenced. Numbers at the nodes represent percent bootstrap support from 5000 replicates. Nodes with less than 50% support were not labeled. Scale bar represents the number of substitutions per site

lineages contained sequences from both *Mytilus trossulus* and *M. galloprovincialis* individuals. The overall topology of the trees was congruent regardless of how unknown bases were handled. Sequences of samples identified here as a particular RFLP haplotype (i.e. Rawson & Hilbish 1998; A, B, C1, C2, C3, or D haplotypes) clustered with known sequences of the same haplotype (Fig. 3). There was no consistent geographic structure within haplotype clades. None of the sequenced F haplotypes clustered with the alternate M haplotype (Fig. 3), as would occur if this study was accidentally amplifying M-specific haplotypes when attempting to amplify the F or vice versa. There were also no sequences that did not cluster with known haplotypes, as would occur if this study was accidentally amplifying a random locus as a result of PCR mispriming. Additionally, there were no new stop codons added to the sequences, as might be expected if this study was accidentally amplifying *numts*.

DISCUSSION

The high incidence of M mtDNA haplotypes in mussel populations around the island of Hokkaido is not a result of a skewed sex ratio, as previously suggested, but rather a product of a disruption in normal DUI of mitochondria. The sex ratio of mussel populations in Hokkaido is not skewed in favor of males; indeed, when considered as a whole or when considering each sample site separately, there is no evidence that mussel populations in northern Japan depart from a 1:1 sex ratio (Table 1). However, the majority of individuals contained an M mtDNA haplotype, including 78% of all female mussels (Table 1). Thus, the presence of M mtDNA haplotypes is largely independent of the sex of the mussels in this system.

There are 3 unusual features of the data reported here; most female mussels are heteroplasmic for M and F mtDNA, some individuals are heteroplasmic for F mtDNA, and some individuals are heteroplasmic for M mtDNA. Given the atypical nature of these results, it is important to consider whether they could be experimental artifacts or even the products of contamination. Due to the consistency and predictability of the results and their concordance with expectations for no contamination within both the data and the controls, we conclude that contamination cannot account for the high level of heteroplasmy observed in this study.

In addition, misamplification seems highly improbable. DNA sequencing of a subset of the samples clearly shows that haplotypes identified here as either M or F were appropriately classified in all cases (Fig. 3). The AMOVA provides additional evidence supporting these assessments since patterns of variance partitioning would be drastically different under the various mispriming scenarios, largely resulting in maximizing the differences between the collection locations and minimizing the differences between the M and F groups, exactly the opposite of the present analysis.

Also, it can be argued that the observation of multiple M or F haplotypes within one individual is possibly the result of incomplete cutting of the target DNA by the restriction enzyme. This is also highly unlikely for several reasons. First, incomplete cutting by the restriction enzyme does not result in fragment patterns consistent with the diagnostic F and M haplotypes found in this study (Rawson & Hilbish 1995a,b). Second, if there was a fault within the restriction enzyme digest assay, we would expect M haplotype heteroplasmy to be observed equally at all study locations. This was not the case; 80% of M haplotype heteroplasmy was observed in hybrid zone locations and 20% in pure *Mytilus galloprovincialis* populations. Third, in all cases, the DNA re-analysis of M or F heteroplasmic individuals yielded the same result as the initial assay. Thus, we conclude that the unusual results found in this study cannot be the product of either contamination or artifacts associated with the assays but, instead, are the result of a pervasive disruption in the regulation of normal DUI.

Unlike previous studies (Fisher & Skibinski 1990, Zouros et al. 1994, Stewart et al. 1995, Rawson et al. 1996b, Wood et al. 2003), DUI disruption found in the current study is not restricted to hybrid individuals. Heteroplasmy for M and F haplotypes observed in Hokkaido is unrelated to whether the sample was from a population of one of the 2 parent species or from a population within one of the hybrid zones. It is also independent of the nuclear genotype of the individual and has no apparent association with gender. Rawson et al. (1996b) found a highly significant association between the gender and the presence of the M haplotype in homozygous *Mytilus galloprovincialis* and *M. trossulus* individuals, but this association dissipated in hybrid individuals. If the disruption in DUI observed in Hokkaido was solely caused by hybridization, we would expect a majority of female individuals from the parental populations to be homoplasmic for F mtDNA lineages. In strong contrast to this expectation, 93 and 82% of female individuals within *M. trossulus* and *M. galloprovincialis* parental zones, respectively, are heteroplasmic for F and M mtDNA. Thus, the level of heteroplasmy among female mussels within the parental popula-

tions was comparable to that observed for the 4 genealogical classes within each hybrid zone (94% of *M. trossulus*, 88% of *M. galloprovincialis*, 86% of F_1 hybrids, and 83% of other hybrids).

Since we only used 3 genetic markers to classify individual mussels into genealogical categories, it is possible that individuals classified as homozygous *Mytilus trossulus* or *M. galloprovincialis* may actually be the result of extensive hybridization and backcrossing. If so, the observed disruption of DUI, especially within putative pure populations, may be a consequence of hybridization and not due to other factor(s). We find this possibility highly unlikely for several reasons. First, there is no evidence that the populations that border the hybrid zones are anything other than pure populations of *M. trossulus* or *M. galloprovincialis*; all 3 nuclear markers confirm that the pure population assignments and heteroplasmic females within each population had M and F mtDNA haplotypes exclusive to that species. Second, the majority of individuals classified as hybrids in the northern and southern hybrid zones had nuclear genotypes consistent with F_1 hybrids. It is rare to observe individuals with multilocus genotypes indicative of being the result of backcrossing to either parent species (Brannock et al. 2009, Brannock & Hilbish 2010), which suggests that backcrossing is uncommon. Third, Brannock & Hilbish (2010) found hybrids in Hokkaido, of any type, to be highly sterile compared to mussels classified as *M. trossulus* or *M. galloprovincialis* from the pure populations. Hybrid sterility will strongly restrict backcrossing in these populations and likely explain the rarity of individuals with multilocus genotypes indicative of backcrossing. Finally, Brannock & Hilbish (2010) found mussels from the hybrid zones classified as *M. galloprovincialis* or *M. trossulus* that were highly fertile, comparable to mussels from the putative pure populations outside of the hybrid zones. These results all indicate that mussels in Hokkaido have been successfully classified into genealogical categories and, more importantly, that it is improbable that the populations classified as containing the pure species actually contain hybrid individuals resulting from repeated backcrossing. Thus, we conclude that the cause of the observed disruption of DUI in Hokkaido is not likely to be linked to hybridization.

Kenchington et al. (2009) described a new model for the regulation of DUI in mussels; they suggest that a factor expressed in the mother determines the sex of her offspring and that this factor is tightly coupled with another factor that determines whether the mitochondria inherited from the father will be incorporated into the germ line of male offspring. They suggest that the female-determining factor (S_1 in their model) is typically tightly coupled with the factor for the exclusion of male mitochondria (z) and that the male-determining factor (S_2) is tightly coupled with the inclusion of male mitochondria into the germ line (Z). Thus, under typical conditions, daughters (S_1z) exclude mitochondria inherited from their father, and sons (S_2Z) incorporate those mitochondria into their germ line. In their model, both the S and Z factors are only expressed in females. Kenchington et al. (2009) show that under some circumstances, the loci for these factors may be uncoupled. While their model does not necessarily require the 2 factors to be genetically encoded, it may well be the case that these are determined by 2 genetic loci that are tightly linked (Kenchington et al. 2009).

This model suggests 2 hypotheses that would explain our observation of high levels of M and F mtDNA heteroplasmy in female mussels in Japan. In the first, hybridization may promote uncoupling of these factors, perhaps by frequent recombination, which should result in females that fail to exclude their father's mitochondria and males that fail to incorporate their father's mitochondria into their germ line. If the 2 factors were completely uncoupled, we would expect to see equal proportions of females and males with and without M mtDNA. This is not what we observed; males without M mtDNA were rare (1.6%), and the frequency of heteroplasmic females was 78%, which is much greater than 50%. In addition, one would also predict that this uncoupling would decline with distance from the hybrid zones, but this also does not appear to be true; Obata et al. (2006) observed that populations of *Mytilus galloprovincialis* in central Honshu, over 1000 km from the hybrid zones in Hokkaido, also produced heteroplasmic females at a high frequency. These results, combined with our observation that female heteroplasmy is unrelated to the hybrid origin of the mussel, weigh against this hypothesis.

Alternatively, it is possible that infrequent recombination and genetic drift account for the pattern of DUI misregulation observed in Japan. Recombination between the S and Z factors may create a new combination, S_1Z, that would produce daughters unable to exclude their father's mitochondria, which is what we observe in Japan. This combination may have drifted to a high frequency as a result of a founder event during the initial invasion of Japan by *Mytilus galloprovincialis*. On the other hand, the S_2z combination, which would produce males without M mtDNA, would not necessarily have drifted to high frequency and may not be present in the population

at all. If this hypothesis were true, it predicts that female heteroplasmy for M and F mtDNA occurs in other populations of *M. galloprovincialis* in Asia and that the Hokkaido populations should show considerable evidence of a population bottleneck in the past. As noted above, Obata et al. (2006) found that female *M. galloprovincialis* were heteroplasmic for F and M mtDNA at sites in Honshu >450 km and >1000 km from the hybrid zones in Hokkaido. This hypothesis also suggests that the disruption of DUI in populations of *M. trossulus*, observed in Hokkaido, is due to the leakage of this recombinant allele through the hybrid zones and, thus, will be confined to this region. This hypothesis is not without precedent; populations of *M. galloprovincialis* from the Black Sea contain high frequencies of males without M mtDNA (Ladoukakis et al. 2002), which may mean that they were derived from populations with a high frequency of the alternate combination of alleles, S_2z.

The disruption of DUI observed around the island of Hokkaido is predominantly female biased and is therefore likely a failure of female individuals to eliminate the M mtDNA transmitted during fertilization. A total of 78% of all females collected in 2008 were heteroplasmic for F and M mtDNA, while only 1.6% of males were homoplasmic for F mtDNA, indicating that they had excluded the M mtDNA inherited from their fathers. Obata et al. (2006) also found high levels of F and M heteroplasmy in female *Mytilus galloprovincialis* from Honshu. A high proportion of heteroplasmic females is a feature that has only been reported in Japanese mussel populations thus far (the current study and Obata et al. 2006). Previous reports of disruption in DUI have mainly shown males failing to retain the M mtDNA (Fisher & Skibinski 1990, Zouros et al. 1994, Kenchington et al. 2009) or females retaining the M mtDNA along with males concomitantly failing to retain the M mtDNA (Rawson et al. 1996b). The finding that DUI disruption in Hokkaido is almost exclusively due to DUI disruption in females, most likely by failing to exclude M mtDNA, supports previous speculation that there are separate mechanisms controlling the retention (in males) or elimination (in females) of M mtDNA (Rawson et al. 1996b). If the same mechanism was responsible for both the retention and elimination in the separate sexes, we would expect that a breakdown in DUI would afflict both males and females. The current study suggests that the mechanism for female mussels in Hokkaido to eliminate the M mtDNA from their cells is either not working or highly flawed, while the mechanism for males to retain both the F the M mtDNA remains intact.

Another possible explanation for the unexpected level of heteroplasmy in the current study is recombination between the M and F genomes. Recombination in the mtDNA region has been reported in *Mytilus trossulus* in the Baltic (Burzynski et al. 2003), *M. galloprovincialis* in the Black Sea (Ladoukakis & Zouros 2001), and *M. trossulus* along the east and west North American coasts (Rawson 2005), as well as among European *Mytilus* populations (Filipowicz et al. 2008). In the current study, if recombination occurred within the F genome that resulted in a new F type that included both F and M loci, and then the new F type was transmitted under normal DUI (through the maternal lineage), this could explain the high frequency of female heteroplasmy observed. However, if this were to occur, we would expect that males would continue to transmit an M genome to their sons through the sperm, consequently producing a high frequency of male individuals that were triplasmic. We would expect to see triplasmic individuals in both the *M. galloprovincialis* parental populations and hybrid zones in relatively equal frequencies. This scenario is not supported with the current results. Only about 6% of the current samples assayed were classified as triplasmic, and a majority of those individuals were found within the hybrid zone and were female. In addition, for the successful amplification and further haplotype assignment, the entire M 16S region assayed in the current study would have to be replaced during recombination with the F 16S region to obtain the observed results. We believe this is unlikely. For these reasons, we do not believe recombination of the mtDNA haplotypes can explain the patterns we observed in this study.

Approximately 6% of the mussels examined in this study contained an F haplotype and were also heteroplasmic for 2 M haplotypes. The occurrence of M heteroplasmy is illuminating in 2 ways. First, it is a strong indication that misregulation of DUI has been occurring for multiple generations in mussel populations around Hokkaido. It is likely that such individuals are formed through the fertilization of an egg that is already heteroplasmic for F and M haplotypes, implying that the female that produced the egg was herself a product of misregulation of DUI by her mother. It is not likely that heteroplasmic females are produced through the fertilization of a homoplasmic egg by a heteroplasmic sperm because sperm contain only 5 mitochondria (Longo & Dornfeld 1967, Cao et al. 2004, Cogswell et al. 2006) and Venetis et al. (2006) found no evidence of F mtDNA in sperm. If an egg was heteroplasmic for F and M mtDNA and

was fertilized by a sperm containing a different M haplotype, the resulting embryo would initially be triplasmic for 1 F and 2 M haplotypes. Moreover, in this study, ~71% of triplasmic individuals were female, which means that for them to be heteroplasmic for M haplotypes, they would have to have also failed to exclude their father's mtDNA during early development. Second, the inference that these individuals are derived from heteroplasmic eggs indicates that the failure to exclude paternal mtDNA by female embryos does not just lead to the inclusion of M haplotypes into the soma but, at least occasionally, into the germ line as well. Obata et al. (2006, 2007) and Sano et al. (2007) have both previously shown heteroplasmic (the presence of both F and M haplotypes) unfertilized *Mytilus galloprovincialis*-spawned eggs. When fertilizing heteroplasmic eggs in the laboratory, Obata et al. (2007) found that the M haplotype in the resulting progeny was consistent with the M type of the sperm. They also found 30% (n = 6) of the adult mussels that they used contained 2 M haplotypes and an F haplotype (Obata et al. 2007). They did not find any individuals that contained 3 M haplotypes (Obata et al. 2007). In the current study, out of the individuals that contained multiple M haplotypes (n = 49), 18.4% (n = 9) contained all 3 possible M haplotypes, indicating that females are perpetuating the transmission of M mtDNA for several generations.

Finally, it was unexpected that females were about 3 times more likely to be triplasmic than were males. Even if there was complete failure of female embryos to exclude their father's M mtDNA, we would expect males and females to both be descended from heteroplasmic eggs and thus initially exhibit equal frequencies of triplasmy. If some fraction of female progeny subsequently exclude their father's mitochondria, they will become either heteroplasmic for the F haplotype and the M haplotype originally contained in the egg or perhaps homoplasmic if they exclude both their father's mitochondria and the M types originally contained in the egg. The fraction of female progeny that fail to exclude their father's mitochondria should be triplasmic, having the F and M haplotypes of the egg and the M haplotype of their father. Thus, triplasmy is expected to occur less often in females than in males. Our observation, however, contrasts strongly with this expectation. Triplasmy occurs significantly more often in females than in males. This suggests to us that males possess a cellular mechanism that allows for the exclusion of one of the M haplotypes during early development. Mussels have deterministic cleavage patterns such that beginning with the first division, cell lines are established that are fated to become specific tissues, including the germ line. Thus, under normal regulation of DUI, male embryos must segregate mtDNA derived from the sperm into cell lines that are destined to become germinal tissue (Rawson et al. 1996b, Cao et al. 2004, Cogswell et al. 2006). If M mtDNA from a heteroplasmic egg can still be recognized by this segregation machinery, then it is possible that M mtDNA from the cytoplasm of the egg is segregated into the germ line and excluded from somatic tissues. At the time of fertilization, M mtDNA in the egg likely outnumbers the M mtDNA from the sperm; thus, we predict that this mechanism would tend to drop sperm M mtDNA from the germ line in favor of M mtDNA that is derived from the egg. This prediction could be tested by determining whether developing male mussels retain their father's M mtDNA.

The overwhelming observation of female heteroplasmy and the occurrence of triplasmic individuals may not be independent of each other. It is possible that the Hokkaido populations have undergone a genetic change that has caused females to retain the M mtDNA more often than populations in other geographical locations. The retention of the M genome in females could lead to a greater likelihood of heteroplasmic eggs (containing an M and F haplotype) which, upon fertilization, could lead to triplasmy (1 F genome and 2 M haplotypes). This scenario would provide an explanation of why heteroplasmy of the M genome is more common than heteroplasmy of the F genome. If there were separate mechanisms in males and females that determined the fate of the M genome and only the female mechanism was flawed, this could explain why triplasmy occurs more commonly in females than in males.

The results of the current study illustrate a widespread disruption in DUI within species of the *Mytilus edulis* complex around Hokkaido, Japan. This disruption is independent of multilocus genotype and geographic location of the sample site and provides support of the disruption being multigenerational. The disruption in DUI occurring around Hokkaido is almost exclusively a result of the failure of female individuals to exclude their father's M mtDNA. Once thought to be associated with maleness (Skibinski et al. 1994, Zouros et al. 1994), the transmission of the M mtDNA in the *Mytilus edulis* complex is poorly understood, as the current study further demonstrates. Before the population and ecological impacts of hybridization can be fully explored in this system, the mechanism(s) of mtDNA transmission need to be more clearly understood.

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