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LISTE DES ABRÉVIATIONS

ADN / DNA	Acide désoxyribonucléique / Deoxyribonucleic acid
ADNmt / mtDNA	ADN mitochondrial / Mitochondrial DNA
ADNnu / nuDNA	ADN nucléaire / Nuclear DNA
ADP	Adénosine 5'diphosphate / Adenosine 5'diphosphate
ARN / RNA	Acide ribonuléique / Ribonucleic acid
ARNr / rRNA	ARN ribosomal / ribosomal RNA
ARNt / tRNA	ARN de transfert / transfer RNA
ATP	Adénosine 5'triphosphate / Adenosine 5'triphosphate
COX	Cytochrome *c* oxydase / Cytochrome *c* oxidase
CS	Citrate synthase
CYC	Cytochrome *c*
dNTP	Deoxyribonucleotide triphosphate / Désoxyribonucléotide triphosphate
DUI	Doubly uniparentale inheritance / Double transmission uniparentale
EDTA	Ethylenediaminetetraacetic acid / acide éthylène-diamine-tétraacétique
ETS	Electron transport system / système de transport des électrons
$FADH_2$	Reduced flavin adenine dinucleotide / Flavine adénine dinucléotide réduite
KCN	Potassium cyanide / cyanure de potassium

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LE NUMERO 1 MONDIAL DU MÉMOIRES

$MgCl_2$	Magnesium chloride / chlorure de magnésium
NADH	Nicotiamide adenine dinucleotide (reduced form) / Nicotiamide adénine dinucléotide (forme réduite)
OXPHOS	Oxidative phosphorylation / phosphorylation oxidative
PCR	Polymerase chain reaction / réaction de polymérisation en chaîne
RFLP	Restriction fragment lenght polymorphism / polymorphisme de longueur des fragments de restriction
SDH	Succinate dehydrogenase / succinate déshydrogénase
SMI	Strict maternal inheritance / transmission strictement maternelle
trnI	tRNA for isoleucine / ARNt pour l'isoleucine
trnM	tRNA for methionine / ARNt pour la méthionine
trnN	tRNA for asparagine / ARNt pour l'asparagine
trnQ	tRNA for glycine / ARNt pour la glycine
trnS	tRNA for serine / ARNt pour la sérine
trnY	tRNA for tyrosine / ARNt pour la tyrosine

RÉSUMÉ

Les mitochondries sont les organites qui produisent la quasi-totalité de l'énergie consommée par les cellules animales. Une de leurs particularités est de posséder leur propre génome (ADN mitochondrial ou ADNmt) qui, avec la collaboration indispensable du génome du noyau (ADNnu), code pour les enzymes responsables de la production d'énergie. Pour que ces organites fonctionnent adéquatement, la co-adaptation des génomes nucléaire et mitochondrial a donc été fortement sélectionnée au cours de l'évolution. Chez les animaux, l'ADNmt est, contrairement à l'ADNnu, transmis uniquement par la mère. L'hypothèse principale pour expliquer cette transmission maternelle est qu'en ayant un seul type d'ADNmt, la cellule évite un conflit génomique dans l'association intime entre les protéines codées par l'ADNnu et l'ADNmt. Autrement dit, la sélection naturelle doit nécessairement prévenir toute modification du dialogue entre les mitochondries et le noyau, ce qui risquerait de provoquer l'apparition de phénotypes indésirables. Exceptionnellement, un seul système mitochondrial «défie» les lois naturelles de transmission des mitochondries chez les animaux, soit celui observé chez les moules marines et d'eau douce. Les espèces possédant ce système atypique, qui est désigné sous le nom de double transmission uniparentale (doubly uniparental inheritance ou DUI), sont caractérisées par la présence de 2 ADNmt distincts qui sont hérités de façon maternelle (ADNmt F) ou paternelle (ADNmt M). Généralement, les femelles ne contiennent que le génome F, tandis que les mâles contiennent le génome F dans leurs tissus somatiques et le génome M dans leur gonade. Les divergences observées entre les ADNmt F et M chez les moules peuvent atteindre plus de 20%. Les produits des deux différents ADN mitochondriaux peuvent-ils interagir normalement avec les protéines d'un seul système génétique nucléaire (en raison de la co-adaptation intergénomique), ou certaines fonctions mitochondriales ont-elles été compromises dans une ou l'autre des lignées (c'est-à-dire, lignée paternelle M ou maternelle F)?

Le grand attrait du système de double transmission uniparentale est qu'il est le seul modèle animal où deux variantes mitochondriales fortement divergentes coexistent et s'adaptent simultanément à un génome nucléaire. Cette situation se traduit probablement par la conservation d'une intégrité relative de certaines portions du génome mitochondrial afin de maintenir une cohésion fonctionnelle dans les interactions structurales entre les peptides mitochondriaux et nucléaires. L'objectif principal de mon doctorat était d'étudier l'évolution moléculaire des génomes mitochondriaux F et M chez les espèces du genre *Mytilus* et de vérifier si les gènes nucléaires et les gènes F et M mitochondriaux qui interagissent pour former le système de la phosphorylation oxydative co-évoluent. Spécifiquement, l'objectif premier du chapitre 1 a été d'analyser les ADNmt M et F complets chez trois espèces

de moules marines (*Mytilus edulis*, *M. trossulus*, et *M. galloprovincialis*) afin de vérifier si leur évolution dans un environnement nucléaire commun (ou différent) se traduit par la conservation (ou non) de certaines de leurs portions (c'est-à-dire, évolution congruente des génomes M et F). Pour ce faire, les ADNmt ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique. Les principaux résultats ont effectivement démontré que le fait de co-exister dans un même environnement nucléaire contraint les ADNmt F et M à expérimenter des pressions sélectives similaires, ce qui se traduit par une évolution congruente des différentes portions des ADNmt M et F (c'est-à-dire la corrélation positive des patrons de taux de substitution protéiniques).

L'objectif du chapitre 2 a été de déterminer, chez les spermatozoïdes, si le remplacement d'un génome M par un génome F avait un effet sur les capacités métaboliques des mitochondries. L'étude visait à déterminer si la co-évolution du génome nucléaire avec deux types d'ADNmt différents avait été favorisée dans une où l'autre des lignées. Pour ce faire, des dosages enzymatiques (activité des complexes mitochondriaux I+III, II et IV et de la citrate synthase) ont été réalisés par spectrophotométrie. Les résultats ont démontré que la combinaison «ADNmt récemment-masculinisé – ADNnu» (qui est essentiellement une combinaison ADNmtF – ADNnu) performe mieux au niveau enzymatique que la combinaison «ADNmt M – ADNnu».

Le troisième volet a été consacré à l'étude de l'évolution moléculaire des protéines interactives du système énergétique (c'est-à-dire, produits nucléaires et mitochondriaux en contact physique) afin de vérifier si les portions génétiques qui interagissent co-évoluent. Pour ce faire, le gène nucléaire codant pour le transporteur mobile d'électron cytochrome *c* (C*YC*) et des gènes mitochondriaux et nucléaires codant pour des sous-unités protéiques des complexes III (ADNmt = *CYTB*; ADNnu = *C3S6*) et IV (ADNmt = *COX1* et *COX2*; ADNnu = *COX4*) du système de la phosphorylation oxydative ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique. Les données moléculaires semblent démontrer que la co-évolution entre le génome F et le génome nucléaire est mieux couplée, ce qui vient appuyer les résultats enzymatiques du chapitre 2.

Mots clés : ADN mitochondrial, génomique comparative, ADN nucléaire, *Mytilus*, co-évolution intergénomique

INTRODUCTION GÉNÉRALE

Fonctions mitochondriales

Les mitochondries sont des organites à double membrane retrouvées dans le cytoplasme de la quasi-totalité des cellules eucaryotes. Bien qu'elles soient impliquées dans divers mécanismes cellulaires, tel le maintien de l'homéostasie ionique et la mort cellulaire programmée ou apoptose, leur fonction principale est de convertir l'énergie libérée par la dégradation des nutriments en adénosine triphosphate (ATP), forme d'énergie utilisée dans la plupart des réactions cellulaires. C'est pourquoi les mitochondries sont généralement décrites comme les centrales énergétiques des cellules. Un processus clé par lequel les mitochondries répondent aux besoins énergétiques cellulaires se nomme la phosphorylation oxydative ou oxydation phosphorylante (OXPHOS). Cette dernière est accomplie par la chaîne respiratoire et le complexe de l'ATP synthase (Saraste, 1999).

Le système de la phosphorylation oxydative est composé de cinq complexes enzymatiques localisés dans la membrane mitochondriale interne : le complexe de la NADH : ubiquinone oxydoréductase (complexe I), le complexe de la succinate : ubiquinone oxydoréductase (complexe II), le complexe de l'ubiquinone : cytochrome *c* oxydoréductase (complexe III), le complexe de la cytochrome *c* oxydase (complexe IV) et le complexe de l'ATP synthase (complexe V). Les complexes I à IV constituent la chaîne respiratoire mitochondriale et le complexe V ou ATP synthase constitue un tunnel à protons qui s'étend sur l'épaisseur de la membrane interne couplé à un élément catalytique synthétiseur d'ATP. Chaque complexe est formé de plusieurs sous-unités protéiques, le plus gros étant le complexe I avec plus de 40 peptides (Saraste, 1999).

La figure 0.1 schématise le système de la phosphorylation oxydative. Spécifiquement, la fonction des complexes I à IV de la chaîne respiratoire est de transférer les électrons fournis par les coenzymes réduits NADH (nicotinamide adénine dinucléotide) et $FADH_2$ (flavine adénine dinucléotide) qui proviennent de l'oxydation des nutriments jusqu`à l'accepteur final d'électron, l'oxygène moléculaire. La respiration mitochondriale se définit donc comme la mesure de la consommation d'oxygène permettant de quantifier le transfert des électrons dans la chaîne respiratoire. Des transporteurs mobiles d'électrons situés entre les complexes I et II (l'ubiquinone ou coenzyme Q) et les complexes III et IV (le cytochrome *c* ou *CYC*) participent également au transfert des électrons le long de la chaîne respiratoire. Cette migration d'électrons est couplée à une translocation de protons par les complexes I, III et IV depuis la matrice mitochondriale vers l'espace intermembranaire. Il se forme ainsi un gradient de concentration en protons de part et d'autre de la membrane mitochondriale interne, ce qui génère un potentiel électrique (négatif sur la face mitochondriale interne ou matricielle) et un potentiel chimique (basique sur la face matricielle) responsables d'une force proton motrice. La membrane interne mitochondriale étant imperméable aux protons, le retour de ceux-ci dans la matrice mitochondriale se fait grâce au complexe V ou ATP synthase qui utilise l'énergie contenue dans la force proton motrice pour la synthèse d'ATP à partir d'ADP et de phosphate inorganique (Saraste, 1999; May-Panloup *et al.*, 2006). En résumé, la phosphorylation oxydative consiste en deux processus étroitement couplés : (1) l'oxydation de substrats par la chaîne respiratoire et le transfert d'électrons à travers la chaîne respiratoire qui entraînent une translocation de protons à travers la membrane mitochondriale interne, générant ainsi un gradient de concentration de protons constituant une réserve d'énergie et (2) la dissipation de ce gradient de concentration de protons couplée à la synthèse d'ATP, au moment où les protons repassent la membrane interne en traversant l'ATP synthase.

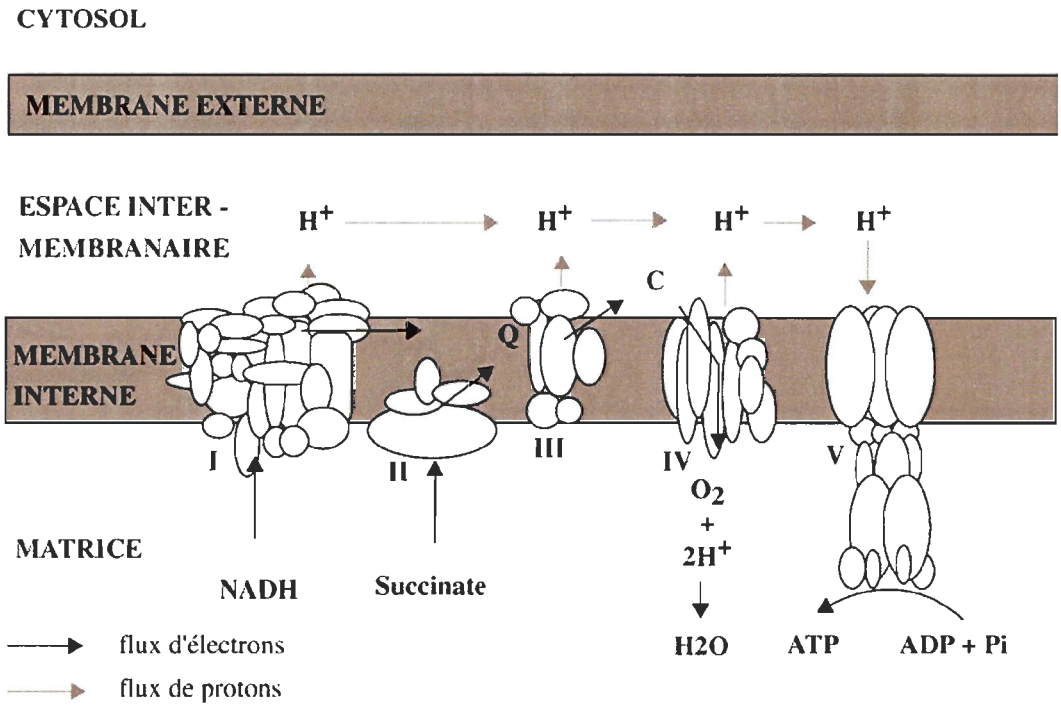

Figure 0.1 Chaîne respiratoire mitochondriale (complexes I à IV) et ATP synthase (complexe V). Le passage des électrons dans les complexes I, III et IV libère des protons dans l'espace intermembranaire pour créer un gradient de protons entre la matrice et cet espace. L'ATP synthase transporte les protons le long du gradient électrochimique (ou force proton motrice) et récupère cette énergie potentielle pour fabriquer de l'ATP à partir d'ADP et de phosphate inorganique. (H^+ : protons; NADH : nicotinamide adénine dinucléotide réduit; Q : coenzyme Q; C : cytochrome *c*; ATP : adénosine 5'triphosphate; ADP : adénosine 5'diphosphate; Pi : phosphate inorganique). (Tirée de May-Panloup *et al.*, 2006.)

L'ADN mitochondrial chez les métazoaires

Avec le noyau, les mitochondries sont les seules organites des cellules animales qui possèdent leur propre matériel génétique : le génome mitochondrial (ADN mitochondrial ou ADNmt). Chez les métazoaires, chaque cellule contient plusieurs mitochondries qui elles-mêmes contiennent plusieurs copies d'ADNmt. À l'exception de quelques espèces de cnidaires qui détiennent des ADNmt linéaires, tous les

métazoaires possèdent des molécules d'ADNmt circulaires bicaténaires (deux brins d'ADN) d'environ 15 000 à 20 000 paires de bases (pb) (Boore, 1999). Chaque molécule d'ADNmt est généralement composée de 37 gènes dépourvus d'intron et d'une région non-codante d'environ 1 kb qui contient les principales séquences de régulation de la réplication et de la transcription de la molécule d'ADNmt (fig. 0.2). Les 37 gènes mitochondriaux codent pour 22 ARN de transfert (ARNt) et 2 ARN ribosomaux (ARNr *16S* et *12S*) qui sont nécessaires à l'expression de 13 autres gènes mitochondriaux codant pour des sous-unités protéiques des complexes enzymatiques du système de la phosphorylation oxydative. Chez les mammifères par exemple, l'ADN mitochondrial est responsable de la production de 7 des 45 sous-unités protéiques du complexe de la NADH : ubiquinone oxydoréductase (complexe I : *ND1* à *ND6* et *ND4L*), une des 11 sous-unités protéiques du complexe de l'ubiquinone : cytochrome *c* oxydoréductase (complexe III : *CYTB*), 3 des 13 sous-unités protéiques du complexe de la cytochrome *c* oxydase (complexe IV : *COXI* à *COXIII*) et 2 des 16 sous-unités protéiques du complexe de l'ATP synthase (complexe V : *ATP6* et *ATP8*) (Boore, 1999; May-Panloup *et al.*, 2006). L'ensemble des autres sous-unités de la chaîne respiratoire et de l'ATP synthase [c'est-à-dire les 38 autres sous-unités du complexe I, toutes les sous-unités (4) du complexe II, et les 34 sous-unités restantes des complexes III, IV et V] sont codées par le génome nucléaire (ADN nucléaire ou ADNnu). Celui-ci est également responsable de la production de la totalité des enzymes de la matrice mitochondriale (par exemple, les enzymes du cycle de Krebs) et de tous les facteurs impliqués dans les autres fonctions mitochondriales (par exemple, les transporteurs de métabolites et les facteurs responsables de la réplication de l'ADNmt et son expression).

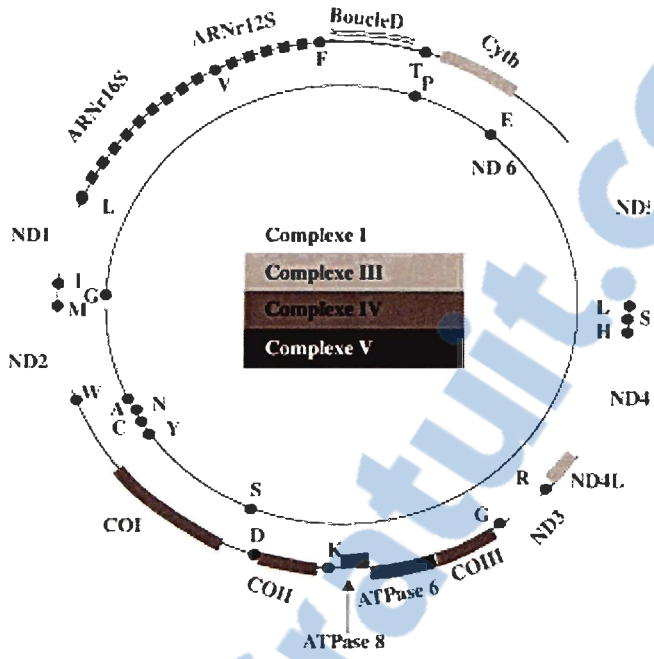

Figure 0.2 Structure du génome mitochondrial d'un mammifère. (ND : NADH ubiquinone oxydoréductase; Cyt : cytochrome *c* oxydoréductase; CO : cytochrome *c* oxydase; ATPase : ATP synthase; BoucleD : origine de réplication; ARNr : ARN ribosomal; les gènes d'ARN de transfert sont schématisés par les points-et-lettres). (Tirée de May-Panloup *et al.*, 2006.)

L'activité des mitochondries dépend donc de l'expression coordonnée de deux systèmes génétiques physiquement séparés (ADNmt et ADNnu). Par conséquent, les génomes nucléaire et mitochondrial sont contraints à co-évoluer pour maintenir une fonction mitochondriale optimale (c'est-à-dire pour un assemblage et un fonctionnement adéquats du système de la phosphorylation oxydative, pour la réplication, la transcription et la traduction de l'ADNmt et pour toutes les autres fonctions mitochondriales) (Blier *et al.*, 2006). Une des approches les plus fructueuses pour l'étude de la communication nucléo-mitochondriale a été l'utilisation d'organismes simples tels que la levure *Saccharomyces cerevisiae*. La

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disponibilité des séquences complètes des génomes nucléaire et mitochondrial de la levure a permis le développement d'une perspective globale des phénomènes de la communication directe du noyau vers les mitochondries et de la communication rétrograde des mitochondries vers le noyau. En exploitant les propriétés remarquables que possède cet organisme pour la recombinaison homologue, c'est-à-dire le processus par lequel deux régions d'ADN portant des séquences nucléotidiques similaires se les échangent par un jeu moléculaire de «coupures-soudures», un consortium international de laboratoires a généré une importante collection de souches de levure ayant chacune un gène nucléaire différent de supprimé. C'est ainsi que 5943 «mutants délétés» ont été créés, soit une collection presque complète (~97%) de tous les gènes nucléaires codant pour des protéines (Winzeler *et al.*, 1999; Kumar *et al.*, 2002). Chaque gène a été remplacé de façon à ce que si sa délétion cause un dysfonctionnement mitochondrial, la levure ne va croître que sur un milieu de culture contenant un substrat fermentable comme le glucose tandis qu'elle ne croîtra pas sur un milieu contenant un substrat non-fermentable comme le lactate. Steinmetz *et al.* (2002) ont employé cette approche pour identifier 466 gènes nucléaires dont les suppressions ont altéré la fonction mitochondriale chez *S. cerevisiae*. Selon ces auteurs, il est probable que beaucoup d'autres gènes nucléaires encore inconnus aient une fonction importante dans le maintien de l'homéostasie mitochondriale chez les levures.

Puisque les gènes assurant les fonctions de base de la cellule (par exemple, le métabolisme) ont été conservés au cours de l'évolution chez l'ensemble des eucaryotes, la levure s'est avérée utile dans l'identification des gènes nucléaires impliqués dans les fonctions mitochondriales chez les organismes supérieurs. Chez l'homme par exemple, 1040 protéines différentes ont été retrouvées dans les mitochondries dont 98% d'entre elles se sont avérées être codées par le génome nucléaire (Ryan et Hoogenraad, 2007). Jusqu'à maintenant, les protéines nucléaires retrouvées dans les mitochondries animales ont été associées à la structure des

mitochondries, la fission et la fusion mitochondriale, l'importation des protéines dans les mitochondries, l'apoptose, la réplication, la transcription, la traduction, la recombinaison et la réparation de l'ADNmt, et l'assemblage et la fonction du système de la phosphorylation oxydative (Scheffler, 2001; Chinnery, 2003).

Un autre domaine pour lequel la levure s'est avérée fort utile est la communication rétrograde ou l'influence des fonctions mitochondriales sur l'expression des gènes nucléaires. Par exemple, il a été démontré que l'expression de plusieurs gènes nucléaires [c'est-à-dire les gènes codant pour la citrate synthase (*CIT2*), la lactate déshydrogénase (*DLD3*), une protéine ribosomale destinée aux mitochondries (*MRP13*) et les sous-unités *COX5A* et *COX6* du complexe IV du système de la phosphorylation oxydative] était significativement augmentée chez des levures dont le génome mitochondrial avait été supprimé (Traven *et al.*, 2001). Ces résultats suggèrent que l'activité métabolique et biosynthétique cellulaire peuvent être ajustées lors d'une situation de déficit énergétique. Autrement dit, compte tenu de la fonction vitale des mitochondries pour la survie des cellules, celles-ci ont développé des mécanismes de régulation de l'expression génique permettant de maintenir une fonction mitochondriale optimale face à une variété de stress mitochondriaux potentiels et le signalement des mitochondries vers le noyau est une composante clé de ces mécanismes.

Manifestement, l'évolution concertée des génomes nucléaire et mitochondrial semble obligatoire pour le maintien de l'homéostasie mitochondriale. Advenant qu'un des deux génomes évoluerait de façon indépendante, la nature des interactions, entre les deux, n'en serait que perturbée. Par exemple, une simple prédiction est que le génome mitochondrial d'une espèce ayant une valeur adaptative élevée dans son propre environnement nucléaire aura une valeur adaptative réduite s'il se retrouve dans l'environnement nucléaire d'une espèce ou d'une population différente. En outre, plus la divergence nucléotidique entre un «ADNmt natif» et un «ADNmt

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étranger» sera élevée, plus l'incompatibilité entre les génomes nucléaire et mitochondrial sera importante (Hutter et Rand, 1995).

La transmission de l'ADNmt chez les métazoaires : une règle universelle ?

La caractéristique la plus remarquable du génome mitochondrial animal est qu'il est transmis exclusivement de façon maternelle (strict maternal inheritance ou SMI) (Birky, 2001). Toutes les copies d'ADNmt dans chacune des cellules d'un organisme possèdent une séquence nucléotidique identique, une situation nommée homoplasmie. La transmission maternelle et l'homoplasmie permettent d'éviter toute compétition inter-mitochondriale (en empêchant la coexistence de différents ADNmt dans les cellules ou l'hétéroplasmie) et assurent le maintien d'une interaction fonctionnelle entre les protéines codées par l'ADNnu et l'ADNmt. La SMI prévient ainsi toute modification du dialogue entre les mitochondries et le noyau, ce qui risquerait de provoquer l'apparition de phénotypes indésirables (par exemple, un déficit de production d'énergie sous forme d'ATP) (Rand, 2001; Ballard et Whitlock, 2004). Une centaine de maladies mitochondriales sont actuellement recensées chez l'humain (Zeviani et Carelli, 2007). Dans un grand nombre de pathologies, la présence d'un ADN mitochondrial muté a été observée. Spécifiquement, des dysfonctionnements mitochondriaux liés à des mutations de l'ADNmt peuvent être rencontrés dans des pathologies humaines telles que les maladies neurodégénératives, les myopathies, les ischémies cardiaques et cérébrales, l'obésité et le diabète. Des dysfonctionnements mitochondriaux semblent également être impliqués dans le vieillissement.

Exceptionnellement, un seul système mitochondrial «défie» les lois de transmission des mitochondries chez les animaux, soit celui observé chez les moules marines (Bivalvia : Mytilidae), les palourdes marines (Bivalvia : Veneridae) et les moules d'eau douce (Bivalvia : Unionidae) (fig. 0.3; voir Breton *et al.*, 2007 pour une revue de littérature). Les espèces possédant ce système atypique, désigné sous le nom

de double transmission uniparentale ou hérédité doublement uniparentale (doubly uniparental inheritance ou DUI), sont caractérisées par la présence de deux ADNmt distincts qui sont hérités de façon maternelle (ADNmt F) ou paternelle (ADNmt M). Typiquement, les femelles sont homoplasmiques et contiennent uniquement l'ADNmt F tandis que les mâles sont hétéroplasmiques. Chez ces derniers, l'ADNmt F domine dans les tissus somatiques alors que la gonade contient presque exclusivement l'ADNmt M.

Figure 0.3 De gauche à droite: la moule marine *Mytilus edulis* (Bivalvia : Mytilidae), la palourde marine *Venerupis philippinarum* (Bivalvia : Veneridae) et la moule d'eau douce *Pyganodon grandis* (Bivalvia : Unionidae).

Les premières études génétiques effectuées sur des séquences partielles des gènes mitochondriaux codant pour les sous-unités I et III du complexe IV (*COXI* et *COXIII*) ont démontré que les divergences nucléotidiques entre les ADNmt F et M chez les moules peuvent atteindre plus de 20% à 30% (tableau 0.1) (Stewart *et al.*, 1995; Hoeh *et al.*, 1996; Hoeh *et al.*, 1997). Cette situation particulière confronte notre conception classique du système universel de l'héritabilité maternelle de l'ADNmt chez les animaux. Une différence fondamentale entre les systèmes SMI et DUI est que ce dernier est le seul mode de transmission de l'ADNmt animal qui pourrait permettre à un génome mitochondrial d'origine paternelle de s'adapter spécifiquement à des

fonctions mâles. Autrement dit, chez les moules, la sélection peut agir directement sur l'ADNmt paternel puisqu'il ne représente plus un cul-de-sac évolutif.

Tableau 0.1
Divergences nucléotidiques observées entre les ADNmt F et M chez les espèces de moules marines du genre *Mytilus*

Espèce	Divergence nucléotidique	Référence
Mytilus edulis	> 20 % (*COXI*, *COXIII*)	Stewart *et al.*, 1995
	2 % (*COXI*, *COXIII*, entre un ADNmt F et un ADNmt de type récemment-masculinisé M'; voir plus bas)	Stewart *et al.*, 1995; Résultats de GenBank
M. trossulus	15-30 % (*COXI*)	Hoeh *et al.*, 1997; Résultats de Genbank
M. galloprovincialis	18-25 % (*COXIII*)	Résultats de Genbank

La double transmission uniparentale de l'ADNmt chez les bivalves

Le système de la DUI a été initialement découvert chez les moules marines du genre *Mytilus* (Fisher et Skibinski, 1990; Hoeh, Blakley et Brown, 1991; Zouros *et al.*, 1992). Comme mentionné ci-haut, des études subséquentes ont démontré que le même système se retrouvait également chez les palourdes marines de la famille des Veneridae et chez les moules d'eau douce de la famille des Unionidae (fig. 0.4). Considérant l'étendue de la distribution taxonomique de la DUI, il a été proposé que ce système était apparu dans une seule lignée ancestrale de bivalves pour ensuite être «perdu» chez certains descendants (par exemple, les pétoncles et les huîtres) (Zouros, 2000).

La transition d'un système de transmission strictement maternel vers un système de double transmission uniparentale a vraisemblablement nécessité une modification des mécanismes de reconnaissance des mitochondries des spermatozoïdes par les œufs. Chez les mammifères, la diminution drastique du nombre de copies d'ADNmt au cours de la spermatogenèse, l'absence de réplication de l'ADNmt au sein de l'ovocyte et l'élimination spécifique des mitochondries paternelles dans l'ovocyte sont des mécanismes assurant l'absence de transmission de l'ADNmt paternel (May-Panloup *et al.*, 2006). Le mécanisme de reconnaissance et de destruction des mitochondries paternelles est contrôlé par le système ubiquitine; l'ubiquitinylation étant un processus de «marquage» des protéines condamnées à être éliminées par les protéasomes. Des études récentes ont suggéré qu'un système similaire, mais légèrement modifié, opérerait chez les moules (Cao, Kenchington et Zouros, 2004; Cogswell, Kenchington et Zouros, 2006; Breton et Doucet Beaupré, 2007). Un facteur génétique d'origine nucléaire présent dans l'œuf serait responsable du «blocage» du système ubiquitine et donc de la rétention des mitochondries paternelles chez les futures mâles. Plus précisément, chez les embryons destinés à devenir des femelles, les mitochondries paternelles se dispersent aléatoirement dans les cellules et/ou sont détruites par un processus semblable à l'ubiquitinylation, tandis que chez les embryons destinés à devenir des mâles, les mitochondries du sperme sont préférentiellement canalisées vers les cellules primordiales qui formeront la gonade, permettant ainsi à l'ADNmt paternel de demeurer la composante prédominante chez la lignée germinale mâle (fig. 0.5).

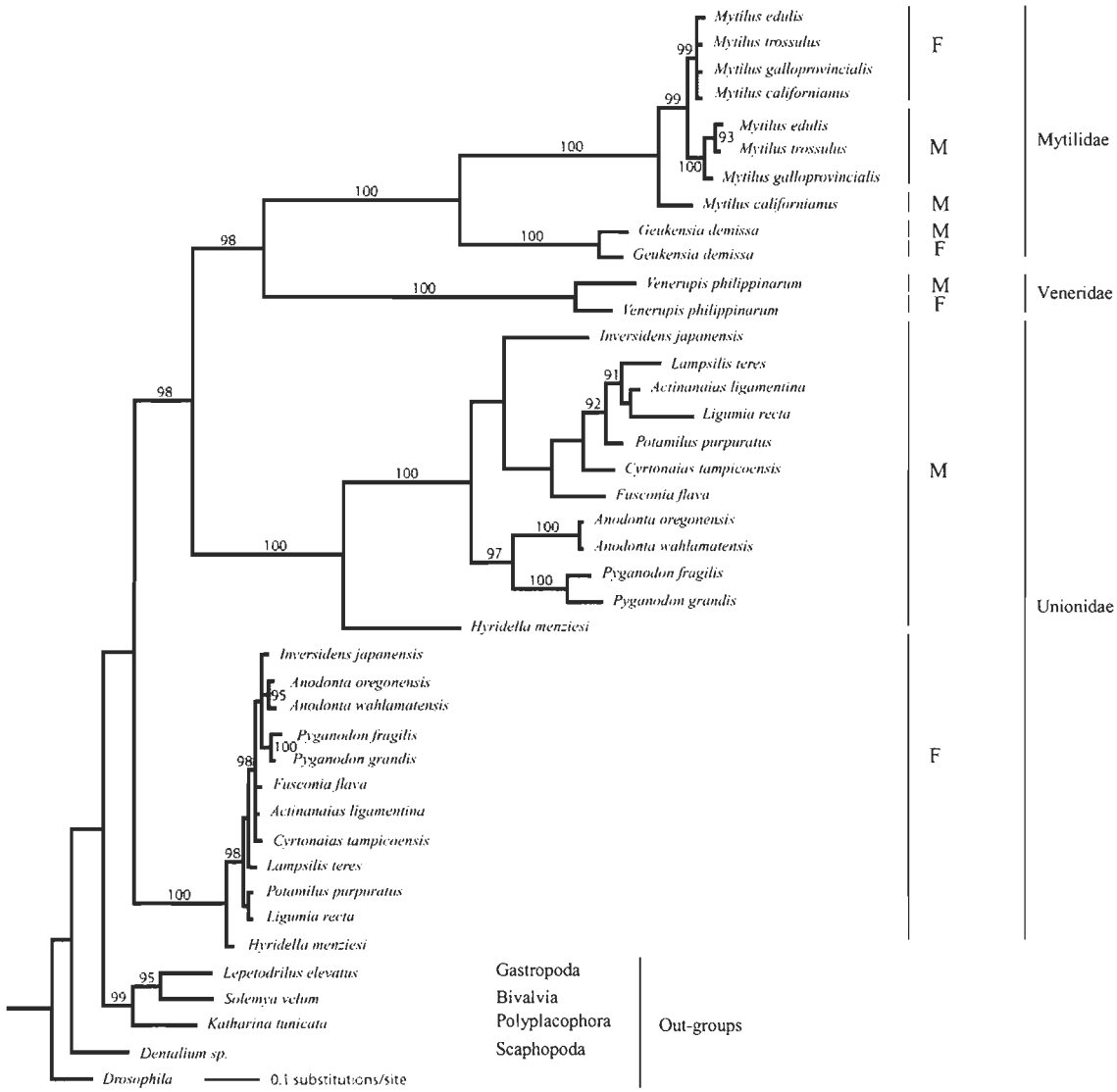

Figure 0.4 Phylogénie des espèces possédant le système DUI. La phylogénie est basée sur l'analyse de 199 acides aminés du gène mitochondrial codant pour la sous-unité I (*COXI*) du complexe IV de la chaîne respiratoire. Le nom de l'espèce et le genre de l'ADNmt de chacun des individus sont indiqués à droite. L'analyse Bayesienne a été réalisée en utilisant le modèle de substitution d'acide aminé de Jones, Taylor et Thornton (JTT) sur 10 millions de générations avec 8 chaînes Markov. Au total, 9000 arbres ont été utilisés pour construire l'arbre consensus. (F : génome mitochondrial maternel; M : génome mitochondrial paternel). (Tirée de Breton *et al.*, 2007.)

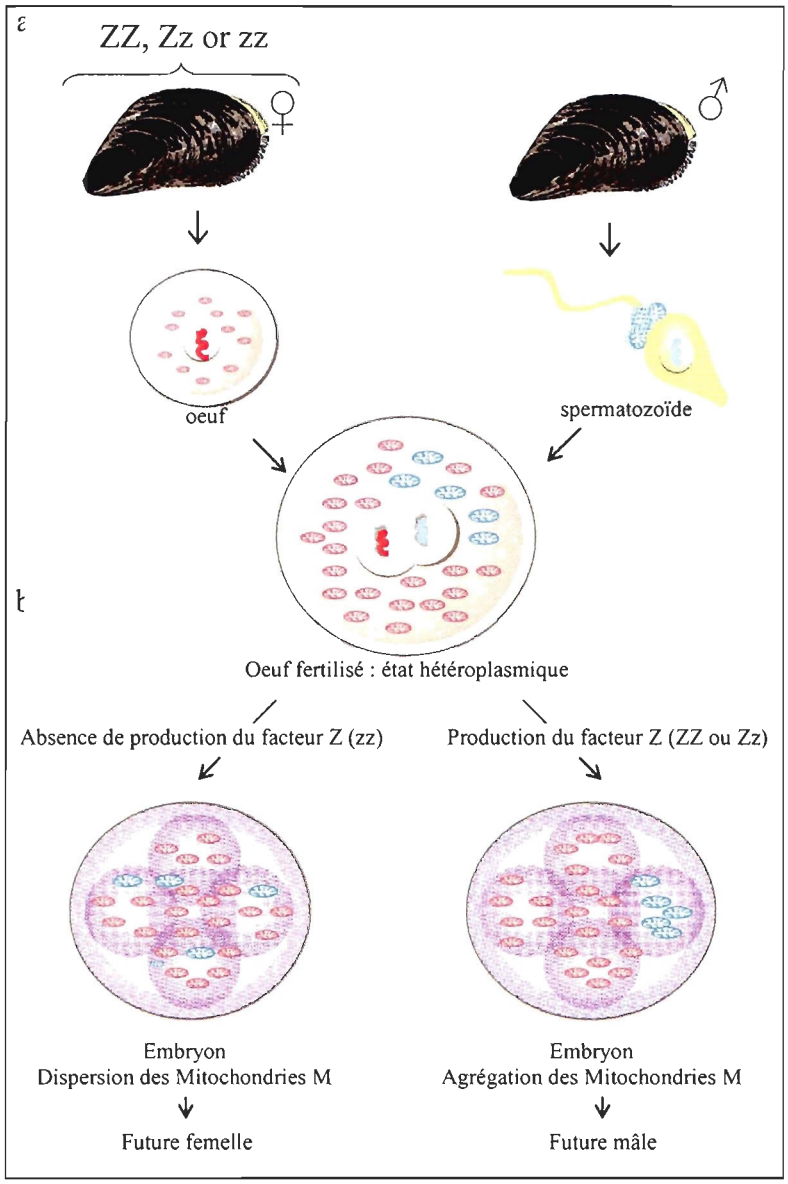

Figure 0.5 Modèle expliquant la double transmission uniparentale chez les moules. (a) Les mitochondries des gamètes mâles et femelles forment la population mitochondriale dans l'œuf fécondé (b) le modèle prédit que le génotype de l'oeuf est responsable du comportement des mitochondries : les oeufs possédant l'allèle dominant Z vont produire le facteur Z. Ce facteur bloque un système d'élimination des mitochondries paternelles similaire à l'ubiquitine et permet la rétention et l'agrégation des mitochondries paternelles dans la future gonade mâle. Les oeufs récessifs zz ne produisent pas de facteur Z et les mitochondries paternelles sont dispersées ou éliminées dans les embryons destinés à devenir des femelles. (Tirée de Breton *et al.*, 2007.)

L'évolution moléculaire des ADNmt F et M chez les Mytilidae

Actuellement, le système de la double transmission uniparentale des espèces du genre *Mytilus* est celui qui est le mieux décrit. Plusieurs études ont démontré des taux d'évolution différents pour les ADNmt du système DUI (Rawson et Hilbish, 1995; Stewart *et al.*, 1995; Stewart *et al.*, 1996). En comparant les séquences des gènes mitochondriaux codant pour les sous-unités I et III du complexe IV (*COXI* et *COXIII*), il a été constaté que l'ADNmt transmis via les mâles (ADNmt M) évolue plus rapidement que l'ADNmt transmis via les femelles (ADNmt F). Un relâchement de la sélection a été proposé comme explication à ce phénomène (Stewart *et al.*, 1996; Zouros, 2000). En effet, contrairement à l'ADNmt F qui doit performer dans la gonade femelle et toutes les cellules somatiques, l'ADNmt M ne fonctionne que dans les spermatozoïdes, ce qui expliquerait la relaxation des contraintes sélectives agissant sur ce génome.

Au début des années 1990, le séquençage du génome mitochondrial F complet de l'espèce *Mytilus edulis* a permis, outre l'identification et la caractérisation des différents gènes mitochondriaux, de démontrer un contenu génique considérablement différent de celui retrouvé chez la majorité des espèces animales (Hoffmann, Boore et Brown, 1992). En effet, l'ADNmt F de *M. edulis* possède un ARN de transfert supplémentaire pour la méthionine, augmentant ainsi son nombre à 23 au lieu des 22 ARNt habituellement présents dans les génomes mitochondriaux d'animaux. De plus, parmi les 13 gènes usuels codant pour des protéines, le gène *atp8* est absent. Cependant, il est à noter que d'autres espèces de bivalves telles que le pétoncle *Placopecten magellanicus* contiennent aussi plusieurs ARNt pour la méthionine et ont également perdu le gène codant pour la protéine *ATP8* (Smith et Snyder, 2007).

Dans les années qui ont suivi, d'autres ADNmt F mais aussi des ADNmt M complets ont été séquencés chez des espèces à DUI (Serb et Lydeard, 2003; Mizi *et al.*, 2005; Okazaki et Ueshima, données non publiées). Généralement, le contenu

génique et l'arrangement des gènes des ADNmt F et M d'une même espèce sont similaires. Chez la moule *Mytilus galloprovincialis*, les deux génomes possèdent un ARNt supplémentaire pour la méthionine et le gène de l'*ATP8* est manquant (Mizi *et al.*, 2005). Un point important de cette étude est que la divergence en acides aminés observée entre les peptides codés par les ADNmt F et M chez *M. galloprovincialis* (~15%) est beaucoup moins importante que celles observées chez la palourde marine *Venerupis philippinarum* (~35%) et la moule d'eau douce *Inversidens japanensis* (~50%), ce qui suggère que le génome M a récemment été remplacé par un génome F dans la lignée des *Mytilus* (Mizi *et al.*, 2005). À présent, ce phénomène de renversement des rôles a été observé uniquement chez les Mytilidae (Stewart *et al.*, 1995; Saavedra, Reyero et Zouros, 1997). Spécifiquement, certains mâles, au lieu de posséder un ADNmt M standard, possèdent un ADN de type F dans leur gonade. On parle de phénomène de renversement des rôles ou encore de masculinisation parce qu'un ADNmt de type F a «envahi» la lignée de transmission paternelle et assume maintenant le rôle d'un génome M dans les spermatozoïdes (fig. 0.6)

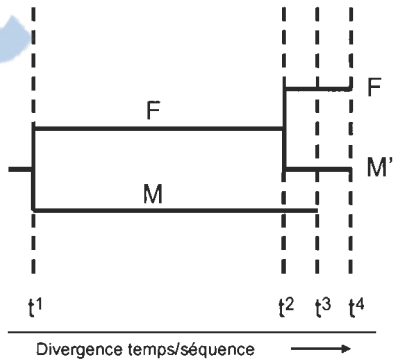

Figure 0.6 Phénomène de masculinisation chez les Mytilidae. Au temp t^1, les ADNmt F et M hautement divergents sont retrouvés dans la population. Au temps t^2, un ADNmt F se retrouve dans la gonade mâle et prend le rôle d'un ADNmt M standard pour rétablir la divergence entre les ADNmt F et M à 0%. Au temps t^3, l'ADNmt M standard disparaît de la population. Au temps t^4, les mâles de la population semblent posséder deux ADNmt de type F, mais en fait ils possèdent un ADNmt F standard dans leur tissus somatiques et un ADNmt ressemblant à un type F dans la gonade qui assure désormais le rôle d'un ADNmt M standard et qui a déjà commencé à diverger de l'ADNmt F pour devenir M'.

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« Ménage à trois» chez les moules : un génome nucléaire et deux génomes mitochondriaux

Les systèmes naturels permettant d'étudier les interactions fonctionnelles entre les génomes nucléaire et mitochondrial sont limités. La co-évolution intergénomique a surtout été mise en évidence en laboratoire par la manipulation directe de cultures cellulaires ou encore d'embryons et par l'analyse comparée de séquences nucléotidiques mitochondriales et nucléaires. De tous les modèles de cultures cellulaires, les cybrides (hybrides cytoplasmiques) permettent le meilleur contrôle de la composition génétique à tester. La technique consiste en la fusion d'une cellule énucléée, donneuse de mitochondries, avec une cellule contenant un noyau mais dépourvue de mitochondrie. Ainsi, en utilisant des cellules donneuses de mitochondries issues de différentes populations ou espèces animales, on obtient une collection de cybrides possédant des ADNmt différents avec un environnement nucléaire identique. Ce type de cellules est généralement utilisé pour étudier le phénotype associé à une «rupture» des complexes ADNmt/ADNnu co-adaptés en mesurant l'activité des enzymes du système de la phosphorylation oxydative. Par exemple, en introduisant des mitochondries d'espèces de plus en plus divergentes dans des cellules de la souris *Mus musculus domesticus*, McKenzie *et al.* (2003) ont observé une diminution drastique de l'activité enzymatique des complexes I, III et IV chez les combinaisons ADNmt/ADNnu les plus divergentes, en l'occurrence la combinaison souris-rat (fig. 0.7). N'étant formé que de sous-unités protéiques codées par le génome nucléaire, le complexe II ne présentait pas de tel dysfonctionnement, démontrant clairement la co-évolution concertée obligatoire des génomes nucléaire et mitochondrial pour le maintien des fonctions mitochondriales (McKenzie *et al.*, 2003).

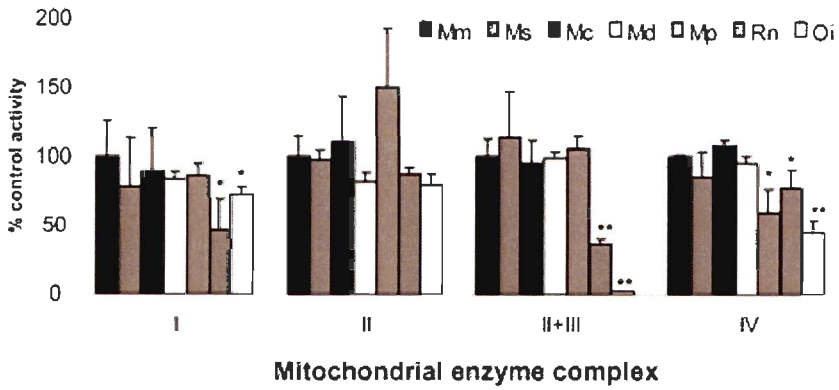

Figure 0.7 Activité des complexes du système de transport des électrons dans des cellules cybrides de muridae. L'activité des enzymes de la phosphorylation oxydative a été mesurée par spectrophotométrie et normalisée avec l'activité du groupe contrôle «ADNmt/ADNnu *Mus musculus-M. musculus*» (Mm, 100%). Les activités enzymatiques chez les cybrides *M. musculus-M. spretus* (MS), *M. musculus-M. carolis* (Mc) et *M. musculus-M. dunni* (Md) étaient normales. Le cybride *M. musculus-M. pahari* (Mp) montrait des activités enzymatiques normales pour tous les complexes, excepté le complexe IV qui présentait une activité réduite par rapport au groupe contrôle. Les cybrides *M. musculus-Rattus norvegicus* (Rn) et *M. musculus-Otomys irroratus* (Oi) montraient une réduction de l'activité de tous les complexes excepté le complexe II. $*P < 0.05$; $**P < 0.005$. (Tirée de McKenzie *et al.*, 2003.)

Un modèle animal fréquemment utilisé pour l'étude de la co-évolution intergénomique chez les organismes complets est le copépode *Tigriopus californicus*. Ce crustacé constitue un excellent modèle puisque les divergences nucléotidiques mitochondriales observées entre les populations sont très importantes (>15%) (Willett et Burton, 2004). Il a été démontré chez cette espèce que l'hybridation d'individus provenant de différentes populations induit non seulement une baisse de la valeur adaptative, mais aussi une baisse significative de l'activité du système de transport des électrons. Tout comme les résultats obtenus chez les cybrides, seuls les complexes formés de sous-unités protéiques provenant des génomes nucléaire et mitochondrial montrent une diminution d'activité (fig. 0.8; Ellison et Burton, 2006).

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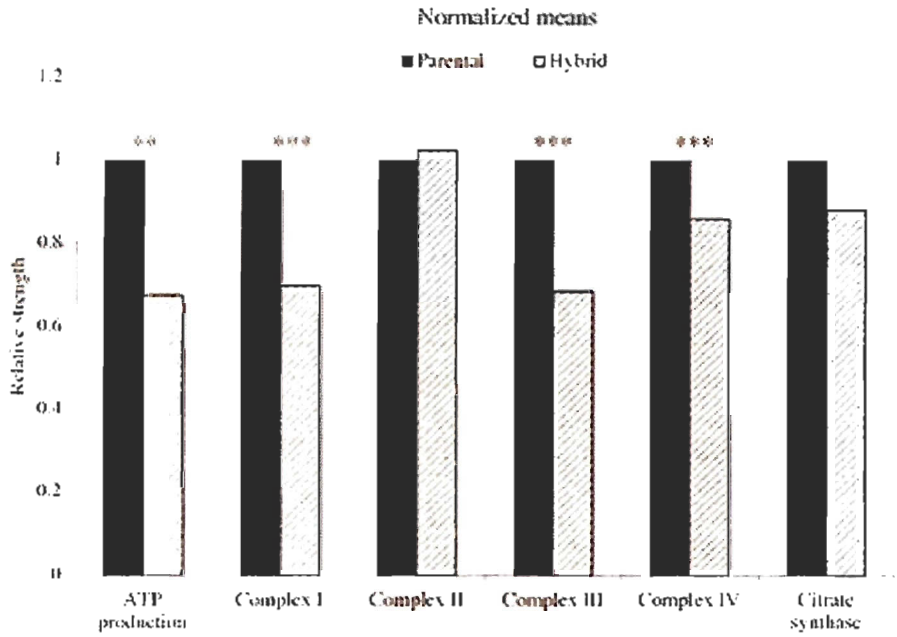

Figure 0.8 Activité d'enzymes clé mitochondriales chez des individus hybrides et non-hybrides de copépodes *Tigriopus californicus*. L'activité des enzymes de la phosphorylation oxydative a été mesurée par spectrophotométrie. Les valeurs moyennes du taux de production d'ATP et l'activité des complexes I, II, III et IV et de la citrate synthase pour les groupes contrôles (« parental» ou non-hybrides) et les hybrides sont normalisées avec les valeurs du groupe contrôle. ** $P < 0.01$, *** $P < 0.0001$. (Tirée de Ellison et Burton, 2006.)

Au niveau moléculaire, plusieurs études ont mis en évidence la co-évolution des génomes du noyau et des mitochondries (Wu *et al.*, 2000; Schmidt *et al.*, 2001; Schmidt, Goodman et Grossman, 2002 ; Willett et Burton, 2004). Chez les primates par exemple, l'analyse comparée des séquences mitochondriales et nucléaires a montré une accélération des taux de substitution d'acides aminés des sous-unités protéiques qui interagissent pour former les complexes du système de la phosphorylation oxydative (Wu *et al.*, 2000). Ces taux de substitution élevés coïncident avec la période évolutive de l'expansion du cerveau et donc de son besoin énergétique croissant durant l'émergence des primates (Schmidt *et al.*, 2001). L'accélération parallèle des taux de substitutions a donc permis de maintenir une

cohésion fonctionnelle dans les interactions structurales entre les peptides mitochondriaux et nucléaires.

Depuis les quarante dernières années, de gros efforts de recherche ont été consentis à l'étude des questions suivantes : comment les deux informations génétiques (nucléaire et mitochondriale) qui coexistent dans une cellule eucaryote sont structurées, comment elles fonctionnent et communiquent entre elles, comment elles évoluent, comment la synthèse des enzymes du système de la phosphorylation oxydative est régulée et enfin pourquoi y a-t-il des gènes dans la mitochondrie? Le grand attrait du système de double transmission uniparentale est qu'il est le seul modèle animal où deux variantes mitochondriales fortement divergentes coexistent et s'adaptent simultanément à un génome nucléaire commun. Les résultats obtenus jusqu'à maintenant pour des séquences mitochondriales partielles montrent que les divergences observées entre les ADNmt F et M présents chez les mâles des espèces du genre *Mytilus* (mais cela demeure également vrai pour les palourdes marines et les moules d'eau douce) sont plus importantes que les divergences mesurées entre les espèces ou populations chez les modèles classiques utilisés pour l'étude de la co-évolution des génomes nucléaire et mitochondrial et pour lesquels une disruption de l'activité des mitochondries a été observée. D'un point de vue «co-évolutif», une question importante se pose : Les produits des deux différents ADNmt F et M peuvent-ils interagir normalement avec les protéines d'un seul système génétique nucléaire (en raison de la co-évolution intergénomique), ou certaines fonctions mitochondriales ont-elles été compromises dans une ou l'autre des lignées (la lignée paternelle M ou maternelle F)?

Objectif général du projet de recherche

L'objectif principal du présent projet de recherche est d'étudier l'évolution moléculaire des génomes mitochondriaux F et M chez les espèces du genre *Mytilus* et de vérifier si les gènes nucléaires et les gènes F et M mitochondriaux qui interagissent pour former le système de la phosphorylation oxydative évoluent de façon concertée (co-évolution).

Objectifs spécifiques du projet de recherche

Le premier chapitre se concentre sur l'analyse des ADN mitochondriaux F et M et récemment masculinisé complets chez trois espèces de moules marines (*Mytilus edulis*, *M. trossulus*, et *M. galloprovincialis*). **L'objectif premier** de l'étude est de vérifier si l'évolution des ADNmt dans un environnement nucléaire commun (ou différent) se traduit par la conservation (ou non) de certaines de leurs portions (c'est-à-dire une corrélation positive des patrons évolutifs ou évolution congruente des ADNmt F et M). **Le second objectif** du chapitre 1 est d'analyser la séquence complète d'un génome de type récemment-masculinisé afin d'identifier les régions nucléotidiques mitochondriales anciennement de type F qui seraient les premières à présenter des adaptations spécifiques de type M suite à une masculinisation (renversement des rôles). **Le dernier objectif** est de cibler des régions nucléotidiques mitochondriales qui pourraient être responsables des différentes transmissions de l'ADNmt chez les Mytilidae (c'est-à-dire transmission maternelle ou paternelle). Pour ce faire, deux ADNmt M complets de l'espèce *M. edulis* et un ADNmt de type récemment-masculinisé de l'espèce *M. trossulus* ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique. Leurs séquences nucléotidiques et protéiniques sont comparées avec celles de l'ADNmt F complet de l'espèce *M. edulis* (Hoffmann, Boore et Brown, 1992) et des ADNmt F et M complets

de l'espèce *M. galloprovincialis* (Mizi *et al.*, 2005) déjà disponibles dans la base de données moléculaires «GenBank».

Le deuxième chapitre teste l'hypothèse suggérant que le système de la double transmission uniparentale a été maintenu chez certains bivalves parce qu'il permet une adaptation de l'ADNmt M pour des fonctions mâles (par exemple, pour la motilité des spermatozoïdes). **L'objectif** de ce chapitre est de déterminer, chez les spermatozoïdes de la moule *M. edulis*, si le remplacement d'un ADNmt M par un ADNmt F (le phénomène de renversement des rôles) a un effet sur les capacités métaboliques des mitochondries. L'étude permet donc de vérifier si la co-évolution du génome nucléaire avec deux types d'ADNmt différents a été favorisée dans une ou l'autre des lignées. Elle fait suite à une première expérience (Jha *et al.*, 2007) comparant les vitesses de nage des spermatozoïdes contenant un ADNmt M standard versus ceux contenant un ADNmt de type récemment-masculinisé M'. Pour les mêmes individus, nous avons mesuré en spectrophotométrie et comparé les activités enzymatiques mitochondriales des complexes I+III, II et IV et de la citrate synthase.

Le troisième chapitre est consacré à l'étude de l'évolution moléculaire des protéines interactives du système de la phosphorylation oxydative (c'est-à-dire les produits nucléaires et mitochondriaux en contact physique). Cette étude permet également de déterminer si la co-évolution du génome nucléaire avec deux types d'ADNmt différents a été favorisée dans une où l'autre des lignées. Le gène nucléaire de l'espèce *M. edulis* codant pour le transporteur mobile d'électron cytochrome *c* et des gènes mitochondriaux et nucléaires codant pour des sous-unités protéiques des complexes III et IV du système de la phosphorylation oxydative ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique. Leurs séquences nucléotidiques et protéiniques sont comparées avec celles de l'espèce *M. galloprovincialis* (Mizi *et al.*, 2005 ; Venier *et al.*, données non publiées) déjà disponibles dans la base de données moléculaires «GenBank».

Dans l'ensemble, cette recherche contribue à l'acquisition de nouvelles connaissances sur les interactions et la co-évolution des génomes nucléaire et mitochondrial. En outre, l'étude du système de la double transmission uniparentale pourrait permettre de démystifier les mécanismes de la transmission strictement maternelle de l'ADNmt chez les animaux. Les résultats du projet seront donc des éléments clés de l'amélioration de notre compréhension de l'évolution et du fonctionnement du système énergétique mitochondrial.

CHAPITRE I

COMPARATIVE ANALYSIS OF GENDER-ASSOCIATED, COMPLETE MITOCHONDRIAL GENOMES IN MARINE MUSSELS (MYTILUS SPP.)

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Résumé

Dans ce chapitre, nous analysons les ADNmt M et F et récemment masculinisé complets chez trois espèces de moules marines (*Mytilus edulis*, *M. trossulus*, et *M. galloprovincialis*) afin de vérifier si leur évolution dans un environnement nucléaire commun (ou différent) se traduit par la conservation (ou non) de certaines de leurs portions (c'est-à-dire, évolution congruente des génomes M et F). Pour ce faire, les ADNmt ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique. Les principaux résultats démontrent que le fait de co-exister dans un même environnement nucléaire contraint les ADNmt F et M à expérimenter des pressions sélectives similaires, ce qui se traduit par une évolution congruente des différentes portions des ADNmt M et F (c'est-à-dire la corrélation positive des patrons de taux de substitution protéiniques).

1.1 Abstract

Marine mussels of the genus *Mytilus* have an unusual mode of mitochondrial DNA (mtDNA) transmission termed doubly uniparental inheritance (DUI). Female mussels are homoplasmic for the F mitotype, which is inherited maternally, while males are usually heteroplasmic, carrying a mixture of the maternal F mitotype and the paternally inherited M genome. Two classes of M genomes have been observed; "standard" M genomes and "recently-masculinized" M genomes. The latter are more similar to F genomes at the sequence level but are transmitted paternally like standard M genomes. In the present study we report the complete sequences of two standard male *M. edulis* and one recently-masculinized male *M. trossulus* mitochondrial genomes. A comparative analysis, including the previously sequenced *M. edulis* F and *M. galloprovincialis* F and M mtDNAs, reveals that these genomes are identical in gene order, but highly divergent in nucleotide and amino acid sequence. The large amount (>20%) of nucleotide substitutions that fall in coding regions imply several amino acid replacements between the F and M genomes, which likely have an impact on the structural and functional properties of the mitochondrial proteome. Correlation of divergence rate of different protein coding genes indicates that mtDNA-encoded proteins of the M genome are still under selective constraints, although less highly than genes of the F genome. The mosaic F/M control region of the masculinized F genome provides evidence for lineage-specific sequences that may be responsible for the different mode of transmission genetics. This analysis shows the value of comparative genomics to better understand the mechanisms of maintenance and segregation of mtDNA sequence variants in mytilid mussels.

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1.2 Introduction

Several species of mollusk bivalves in the families Mytilidae, Unionidae, and Veneridae have an unusual mode of mitochondrial DNA (mtDNA) transmission termed doubly uniparental inheritance (DUI) (Skibinski, Gallagher & Neynon, 1994a,b; Zouros *et al.*, 1992; Zouros *et al.*, 1994; Hoeh *et al.*, 1996; Passamonti & Scali, 2001; Serb & Lydeard, 2003). Instead of transmitting mtDNA exclusively through the female line as seen in most animals (uniparental inheritance), these species are characterized by the presence of two highly divergent mtDNAs: one that is inherited maternally (the F genome), and a second that is inherited paternally (the M genome). In the best studied family Mytilidae, typical females are homoplasmic for the F mitotype throughout the entire animal, whereas males are heteroplasmic with varying mixtures of the maternal and paternal mtDNA in all tissues except the gonad, which contains almost exclusively the M-type mtDNA (Hoeh, Blakley & Brown, 1991; Zouros *et al.*, 1994; Stewart *et al.*, 1995; Garrido-Ramos *et al.*, 1998). The factors that segregate M and F mtDNAs in different tissues of heteroplasmic mussels as well as the physiological significance of this segregation are not yet understood. It was proposed that this tissue-specific segregation is a complex interaction between maternal and paternal factors of eggs and sperm (Saavedra, Reyero & Zouros, 1997; Garrido-Ramos *et al.*, 1998). A recent report demonstrates sex-specific behavior of sperm mitochondria following fertilization (Cao, Kenchington & Zouros, 2004). In embryos from females that produce mostly sons, the sperm mitochondria tended to aggregate in a single blastomere, which is thought to go on to produce the male germ line. In contrast, in embryos from females that produce only daughters, sperm mitochondria were randomly dispersed among blastomeres. This mechanism might explain the predominance but not the exclusive presence of the paternal genome in male gonads (Cao, Kenchington & Zouros, 2004).

The non-random segregation of F and M variants in heteroplasmic male mussels could also be modulated at the genome level (e.g., replication of the M-type being

favored in the male gonad and that of the F-type favored elsewhere). This view draws support from the observed masculinization of F-type mtDNA. Specifically, F mtDNA seems to occasionally invade the male route of inheritance, thereby resetting the degree of sequence divergence between M and F genomes to zero (Hoeh *et al.*, 1997; Zouros, 2000). Male mussels that lack a typical M molecule thus appear to have inherited mtDNA only from their mother, and they appear to carry two F-type molecules. Such males are referred to as "recently-masculinized males". These "ancient F" or "newly masculinized" molecules behave as M mtDNA and are transmitted from generation to generation only through sperm (Hoeh *et al.*, 1997; Saavedra, Reyero & Zouros, 1997; Zouros, 2000). It has been suggested that before an F molecule can become "masculinized" (i.e., reverse its role and become transmitted paternally), it must first acquire one or more key genes from a standard M mtDNA genome (Zouros, 2000). This hypothesis is consistent with the observation of mtDNA recombination between F and M molecules in mytilid mussels (Burzyński *et al.*, 2003).

Little was known about the mtDNA genome of bivalves before the discovery of DUI. The first sequence of a nearly complete *Mytilus edulis* mt genome was published in 1992 (Hoffmann, Boore & Brown, 1992), which was later identified as an F-type mtDNA (Skibinski, Gallagher & Beynon, 1994b). (Note: the sequence of this genome was recently completed and reported by Boore, Medina & Rosenberg [2004]). Interestingly, the gene arrangement of the *M. edulis* F mtDNA is considerably different from that found in any other known metazoan mtDNA. It contains an extra tRNA (*trnM*), and lacks the *atp8* gene, a condition that is known to occur in other bivalves, including *Venerupis* (M. Okazaki and R. Ueshima, personal communication) and *Crassostrea* (S.-H. Kim, E.-Y. Je, and D.-W. Park, personal communication). Recently, the complete maternal and paternal mitochondrial genomes of the Mediterranean mussel *Mytilus galloprovincialis* have been reported (Mizi *et al.*, 2005). Despite a high degree of divergence in nucleotide sequence (about

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20%), both genomes retained identical gene content, gene arrangement, nucleotide composition, and codon usage bias (Mizi *et al.*, 2005). Still, these data do not provide much insight into the role of recombination in the process of masculinization. In fact, to gain insight into DUI, we also require sequence information from recently-masculinized mtDNA.

More generally, Mytilus mussels provide an excellent model for studying the evolutionary forces that operate on the mitochondrial genome, given that different levels of divergence can be studied (i.e., within and between intra- and interspecific F and M genomes). Because of the intimate interactions of nuclear and organelle genomes (Blier, Dufresne & Burton, 2001), one can predict significant correlations in the rates of amino acid substitutions between M and F mtDNAs at positions of structural or catalytic importance to enzyme function. At the same time, because the M and F mitochondrial genomes must function in different cellular environments with different physiological demands, distinct selective pressures may be operating on these molecules (Ballard & Dean, 2001; Dalziel & Stewart, 2002). One could argue that more similar mtDNA regions in the standard/recently-masculinized male comparisons versus female/recently-masculinized male comparisons might suggest molecular adaptations over time to the functional role of male transmitted mitochondria.

In this study, whole mtDNA genome sequencing has been undertaken on two *M. edulis* standard male mitotypes, and one recently-masculinized *M. trossulus* male mitotype, which have been compared with that of the published complete mtDNA sequences from other mytilid species. By a comparative genomic approach we have identified (i) the distribution of mutational differences across these genomes, (ii) evolutionary patterns from M and F genomes having similar or different nuclear backgrounds, (iii) regions that may have undergone specific adaptations as a consequence of masculinization, and (iv) gender-specific regions that may be

responsible for the different transmission genetics of mtDNA. On the basis of these comparisons, we discuss mitochondrial genome diversity and evolution within mytilid mussels.

1.3 Materials and Methods

1.3.1 Collection, genotyping, and mitotyping of specimens

Mytilus mussels were collected from Chester Basin, Nova Scotia throughout the summer of 2002. Mussels were transported on ice and housed in flowing, filtered saltwater tanks at Acadia University's Animal Care Facility. Sex was determined by microscopic examination of the gonads and only males with mature gonads were utilized for further study.

Total DNA was extracted from male mussel gonads using a modified saturated-salt extraction procedure (Miller, Dykes & Polesky, 1988; Sutherland *et al.*, 1998). Gonad tissue was used because it consistently contains and expresses the male mitochondrial genome, whereas its expression is detected in only 50% of somatic tissues of males (Garrido-Ramos *et al.*, 1998; Dalziel & Stewart 2002). An Ultrospec 3100 pro UV/visible spectrophotometer (Amersham Pharmacia Biotech) was used to quantify the extracted DNA which was then stored at 4 °C.

Males were classified as *Mytilus edulis*, *M. trossulus*, or hybrid, using a PCR-based assay which targets a section of the internal transcribed spacer (ITS) region between the 18S and 28S nuclear rRNA coding regions (Heath, Rawson & Hilbish, 1995). After digestion with the restriction enzyme *HhaI*, the amplification products from *M. edulis* produced bands of 450 and 180 bp in length along with many smaller fragments, whereas that from *M. trossulus* produced bands of 280 and 180 bp as well as several smaller fragments. Hybrids possessed both bands at 450 and 280 bp. This assay appears to be highly diagnostic for these species in Nova Scotia (Garrido-

Ramos *et al.*, 1998). Only male individuals identified as "pure" species were used for subsequent analyses.

The detection of a standard male mitochondrial genome (M) in *M. edulis* samples was done using two levels of testing. The first involved PCR amplification of a region of the mitochondrial cytochrome oxidase III (*cox3*) gene using primers designed to be specific to the M mtDNA (Stewart *et al.*, 1995; Sutherland *et al.*, 1998). These primers correspond to the nucleotide positions 207-234 and 710-737 of the *cox3* gene, and amplify a 530 bp fragment only from the main M mitotype of *M. edulis* (M-ed 1; Stewart *et al.*, 1995). The second level of testing was done using the primers designed by Zouros *et al.* (1994). This pair of primers, which correspond to nucleotide positions 460-482 and 1326-1301 of segment 5 of the *M. edulis* female genome in Hoffmann, Boore & Brown (1992), amplify an 860 pb fragment of the *cox3* gene from both female and male mitochondrial genomes (Zouros *et al.*, 1994; Saavedra, Reyero & Zouros, 1997). The PCR products were then digested using *Hinf*I: standard males produced bands of 600 and 250 bp in length, whereas that from recently-masculinized males (M-A) produced bands of 375 and 275 bp with some smaller fragments. Female types (F) could also be distinguished as they produced bands of 450 and 275 bp as well as some smaller fragments. Two standard males were used for further study.

For *M. trossulus* samples, gender identification was performed by PCR amplification of a 700 bp fragment of the mitochondrial cytochrome oxidase subunit I (*cox1*) gene using the primers LCO-22MF 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO-700ME 5'-TCA GGG TGA CCA AAA AAT CA-3' (J.M. Archibald and D.T. Stewart, unpublished data). PCR products were subsequently digested with *Alu*I: standard males produced a pattern with a large fragment (~450 bp) and several smaller fragments, whereas recently-masculinized males (M-O) produced several fragments smaller than 250 bp. Female types showed different

patterns from both of the M types (J.M. Archibald and D.T. Stewart, unpublished data). One recently-masculinized male was used for further study.

1.3.2 MtDNA amplification and sequencing protocols

The entire male *M. edulis* and recently-masculinized *M. trossulus* mitochondrial genomes were amplified using species- and gender-specific primers pairs listed in Table 1.1. As previously reported (Garrido-Ramos *et al.*, 1998; Dalziel & Stewart 2002), male and female mitochondrial genomes can both be present and expressed in male gonads, making difficult the direct sequencing of PCR products. To overcome this difficulty, a DNA extraction from a *M. edulis* female gonad, which is known to contain only the F genome, served as a control for the primers designed to amplify the standard male genome from the same species. For *M. trossulus* samples, DNA extractions were tested with male and female primers (designed from F, M, and M-O GenBank sequences), and individuals that were indicative of homoplasmy for either male type or female type were used for further study. Samples that tested positive for F and negative for M mtDNA were classified as recently-masculinized types because they were isolated from male gonads (Saavedra, Reyero & Zouros, 1997).

PCR amplifications were performed in 50 µl of a solution containing approximately 20 ng of total DNA, 0.4 µM of each primer, 1X Expand ™ high fidelity DNA polymerase buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 2.5 U Expand ™ high fidelity DNA polymerase (Roche Applied Science). The thermal cycling parameters were as follows: 1 min at 94 °C, 2 min at 88 °C, afterwhich the Taq DNA polymerase was added, then 35 cycles of: (94 °C, 15 sec; 55 °C, 20 sec; 72 °C, 2.30 to 6.30 min depending on fragment size), followed by a termination step of 10 min at 72 °C. All PCR reactions (except one which yielded two bands, one stronger and of the expected size, and the other weaker and of smaller size) produced a single band when visualized under UV light following ethidium bromide staining

on a 0.8% agarose gel. The resulting fragments were purified either with Microcon concentrators (Millipore) or with QIAquick Gel Extraction Kit (QIAGEN) in the case of the two-bands product. These purified DNAs were used as template in BigDye terminator cycle sequencing reactions according to manufacturer's protocols (Applied Biosystem). Unincorporated dye terminator and nucleotides were removed by gel filtration (Millipore) according to the manufacture's instructions. Readings were obtained by automated sequencing on a BaseStation ™ DNA fragment analyzer (MJ Research, CA, U.S.A.). Sequencing of the three mtDNA molecules were completed by using the primer walking method with specific primers designed for the sequences obtained. One strand was sequenced, with several overlaps of sequences from both directions. Two to fourteen measures were taken to assess the reliability of the sequence data, except for a few non ambiguous regions (<250 bp).

1.3.3 Data analysis

Sequence readings were assembled using the XGAP package V3.6 1996 (Bonfield, Smith & Staden, 1995). Sequence analyses were performed on SUN workstations using software developed by Lang & Burger (1986), as well as with tools included in the Staden (1996) sequence analysis package. The BLAST network service (Altschul *et al.*, 1990) was employed for similarity searches in GenBank at the National Center for Biotechnology Information. The genome organization, total length, and features of all protein-coding and rRNA genes of the three mtDNA molecules were identified by comparison with *M. edulis* and *M. galloprovincialis* mitochondrial genomes previously published (Table 1.2). Candidate tRNA genes were identified either using tRNAScan-SE version 1.21 (Lowe & Eddy, 1997) or by comparison with similar position of *M. edulis* and *M. galloprovincialis* tRNAs previously published. We also evaluate their potential to form tRNA-like secondary structure, and looked for the presence of the anticipated anticodon sequence.

Table 1.1
Primers used in the amplification of the entire mitochondrial genomes of Mytilid mussels

Region		Oligonucleotide Pair (5' to 3')	Source Used for Primer Design
16S-cob	em16SF	GAAGCCTCTAAGGGTAGGTCTGTTC	(AY350784-92)[a]
2.5 kb	emUR	CTTATCAGCTCACTTGCGTACACC	(U50214)[b]
u-cox3	emUF	GGTGTACGCAAGTGAGCTGATAAG	(U50214)[b]
4.5 kb	emCOIIIR	CCTCGAAACCAAAATGGCGTTGGC	(AY130186-99)[c]
Cox3-cox1	emCOIIIF	GACACTTTGTGGATGTAGTATGAG	(AY130186-99)[c]
3 kb	emCOIR	CCTACTAGAGACCTTACTCCAGCTA	(AY101397-1429)[c]
Cox1-16S	emCOIF	CTGGATGAACTGTCTACCCTCCACT	(AY101397-1429)[c]
6.8 kb	em16SR	GAACAGACCTACCCTTAGAGGCTT	(AY350784-92)[a]
16S-cob	trm16SF	GAAGGACGACAAGACCCTATGAAG	(AF023591-99)[d]
2.5 kb	trmUR	CATTTCAGGTCTACAGGACGACTCC	(U50213)[b]
u-cox3	trmUF	GGAGTCGTCCTGTAGACCTGAAATG	(U50213)[b]
4.5 kb	trmCOIIIR	CGACTACGTGCATTCCATGAAACCC	(AY130177-85)[c]
cox3-cox1	trmCOIIIF	GTGTTCTTCCTAGTGCAACTTCGAG	(AY130177-85)[c]
3 kb	trmCOIR	GGTCCTAGCAAAGTTAATAGCCCC	(U68773-77)[e]
cox1-16S	trmCOIF	GAAAGGAGAACGAGCGGAGCTTTA	(U68773-77)[e]
8.7 kb	trm16SR	CCAACATCGAGGTCGCAATCTTCC	(AF023591-99)[d]

NOTE.—em, *M. edulis* standard male mitochondrial genome; trm, *M. trossulus* recently-masculinized mitochondrial genome; *u*, segment of the mtDNA genome (located between the *cytochrome b* and *cytochrome c oxidase* subunit II genes) for which no function has yet been assigned (Hoffmann, Boore & Brown, 1992).

[a]Cao *et al.* (2004); [b]Stewart *et al.* (1996); [c]Riginos *et al.* (2004); [d]Rawson & Hilbish (1995); [e]Hoeh *et al.* (1997).

Table 1.2
Gender-associated mitochondrial genomes in Mytilid mussels

Species	Gender	Genome Size	A+T Content	GenBank Accession Number	Reference	Abbreviation
Mytilus trossulus	Recently-masculinized	18,651 bp	61.5%	AY823625	Present study	*Mtr*_RM
M. edulis	Male	16,622 bp 16,624 bp	62.9%	AY823623- AY823624	Present study	*Med*_M
M. edulis	Female	16,740 bp	61.8%	AY484747	Hoffmann, Boore & Brown, 1992; Boore, Medina & Rosenberg, 2004	*Med*_F
M. galloprovincialis	Male	17,671 bp*	63%	AB065374	Mizi *et al.*, 2005	*Mga*_M
M. galloprovincialis	Female	16,744 bp	61.8%	AB065375	Mizi *et al.*, 2005	*Mga*_F

NOTE.—*This *M. galloprovincialis* male genome is 16,626 bp in length with a presence of a 1045 bp insertion.

The ClustalW program (Thompson, Higgins & Gibson, 1994) was used for multiple DNA and protein alignments. Sequence comparisons (nucleotide differences, synonymous and nonsynonymous substitutions, total amino acid differences and distances, and number of synonymous substitutions per synonymous site (K_s) and number of nonsynonymous substitutions per nonsynonymous site (K_a) (Nei & Gojobori, 1986) were carried out with MEGA, version 2.1 (Kumar *et al.*, 2001) and DNAsp 4.0 (Rozas & Rozas, 2000). Unrooted trees were constructed for the six *Mytilus* mitotypes from the amino acid sequence data using the following methods: equal-weighted parsimony using a branch-and-bound search (PAUP 4.0 version b2; Swofford, 1998) and neighbor-joining using a poisson correction as the model of amino acid evolution (Saitou & Nei, 1987). For both the parsimony and neighbor-joining, bootstrap support was assessed with 1000 bootstrap replicates. Sliding window analyses were performed in PROSEQ version 2.9 (Filatov, 2002).

1.4 Results and discussion

1.4.1 Sequence, Gene Content and Organization of Bivalve Mitochondrial Genomes

The complete mtDNA sequences of two *Mytilus edulis* male and one *M. trossulus* recently-masculinized genomes have been sequenced and deposited in GenBank (accession numbers AY823623, AY823624, and AY823625, respectively). In the following, we will characterize these genomes and compare them with available data from other mytilids (Table 1.2). Standard male mitochondrial genomes of *Mytilus edulis* are 16,622 and 16,624 bp in length with an overall A+T of 62.9%. Indels of one nt each in the large subunit ribosomal RNA and in the large noncoding region account for the length difference between mtDNA of the two individuals. The length and nucleotide composition of these mtDNAs is comparable to that of other mytilids (Table 1.2). All *Mytilus* mtDNA genomes share an identical gene complement, consisting of two rRNAs, 23 tRNAs, 12 protein-coding genes, arranged in the same

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order; compared to non-bivalve invertebrates, they contain an extra *trnM*, and *atp*8 is missing (Hoffmann, Boore & Brown, 1992; Boore, Medina & Rosenberg, 2004).

The identity of the recently-masculinized *M. trossulus* was confirmed on the basis of a portion of the *cox1* gene, whose standard M- and recently-masculinized-type sequences are known from previous studies (Hoeh *et al.*, 1997). The mitochondrial genome of the recently-masculinized mtDNA is significantly longer than that of the standard male counterparts (Table 1.2). This gain in length is due to the presence of four additional putative tRNA genes (*trnS*, *trnN*, *trnI*, and *trnQ*) and two large noncoding regions instead of one (see below). Figure 1 compares the gene map of a typical male or female Mytilus mtDNA with that of the recently-masculinized *M. trossulus*.

Mytilid mitochondria use the arthropod genetic code (Hoffmann, Boore & Brown, 1992): AGR codons specify serine, ATA and AAA specify methionine and lysine respectively, and TGA is a tryptophan codon. As the presence of multiple potential start codons and often incomplete stop codons in animal mtDNAs complicate the precise inference of the coding region, we have used the same start and end points described previously for the *M. edulis* female mt genome (Hoffmann, Boore & Brown, 1992; Boore, Medina & Rosenberg, 2004). Notably, some genes start with nonstandard initiation codons and some incomplete termination codons are found (Table 1.3), which are assumed to be completed by polyadenylation of their mRNAs (Ojala, Montoya & Attardi, 1981).

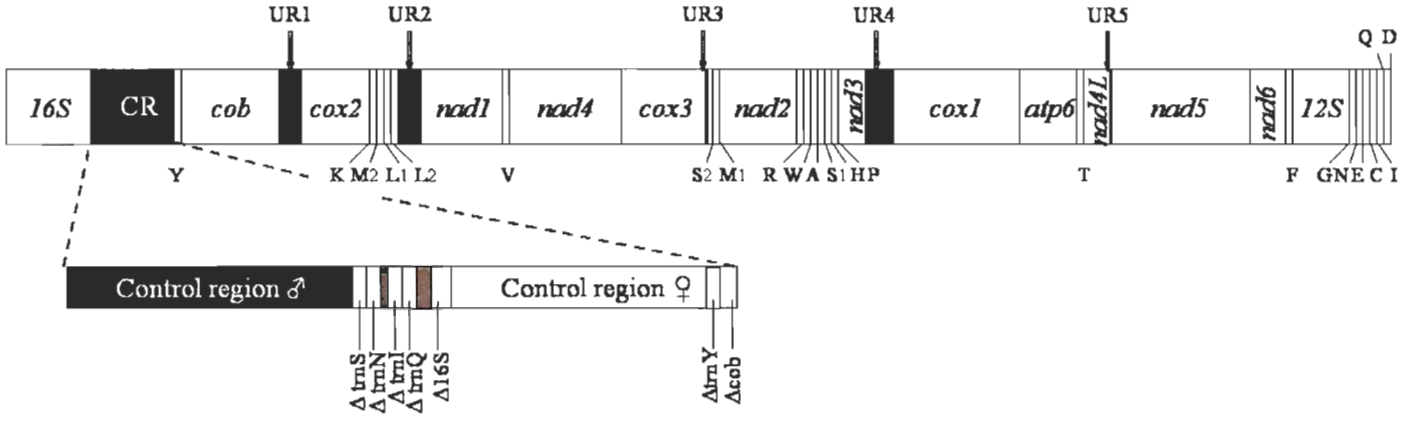

Figure 1.1 Gene maps of a mitochondrial genome of *Mytilus*. All genes are transcribed clockwise. Names of the tRNA genes are indicated by the amino acid (1-letter code) they specify. Black areas indicate non-coding regions; UR1 to UR5, unassigned regions #1 to 6. Non-coding sequences of less than 30 bp are not shown. *16S* and *12S*, large and small subunits of ribosomal RNA. The recombinant region in the recently-masculinized mtDNA is shown by the dotted line. Control Region ♂, corresponds to the 1265 bp male part; ΔtrnS-ΔtrnQ, are full copies of the *trnS*, *trnN*, *trnI*, *trnQ*; Δ16S, corresponds to a portion sharing 89% nt identity with the normal *16S*; Control Region ♂, corresponds to the 1340 bp female part; ΔtrnY is a full copie of *trnY*; Δcob corresponds to the 5' end of the cytochrome *b* gene.

Table 1.3
Comparisons of initiation and termination codon usage in Mytilid mitochondrial genomes

	Initiation and Termination Codons				
Protein	*Med*_M	*Mga*_M	*Med*_F	*Mga*_F	*Mtr*_RM
cox1	ATA/TAG	ATA/TAA	ATG/TAA	ATA/TAA	ATG/TAA
cox2	ATG/TAA	ATG/TAA	ATG/TAG	ATG/TAG	ATG/TAA
cox3	ATG/T **	ATG/T**	ATG/T**	ATG/T**	ATG/T**
Cob	ATG/TAG[a]	ATG/TAG[a]	ATG/TAA	ATG/TAA	ATG/TAA[a]
nad1	GTG/TA*	GTG/T*	GTG/TA*	GTG/T*	GTG/TA*
nad2	ATG/TAA	ATG/TAA	ATG/TAG	ATG/TAG	ATG/TAG
nad3	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA
nad4	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA
nad4L	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA
nad5	ATA/TA*	ATA/T*	ATA/TA*	ATA/T*	ATA/TA*
nad6	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA
atp6	ATG/TAA	ATG/TAA	ATG/TAG	ATG/TAG	ATG/TAG

NOTE.—See Table 1.2 for mitochondrial genome descriptions. *An asterisk indicates that the predicted stop codon is incomplete.

[a]As interpreted, it is possible that *cob* of standard male and recently-masculinized genomes ends on an incomplete stop codon given that there is no similarity between the extension observed and other mtDNA sequences.

1.4.2 Transfer RNA and Ribosomal RNA Genes

All 23 tRNA genes of the newly sequenced mtDNAs of *M. edulis* standard males can be folded into standard cloverleaf structures. The only exception is *trnS2* (located between *cox3* and *trnM1*), which lacks a dihydro-uridine arm (DHU-arm). Remarkably, the *M. trossulus* recently-masculinized mt genome contains four additional tRNA-like structures (△*trnS*, △*trnN*, △*trnI*, and △*trnQ*), inserted between the genes coding for the large subunit ribosomal RNA (*16S* rRNA) and *trnY*.

This segment normally contains the large noncoding region of the mussel mtDNA. Some of the tRNA-like structures possess several mismatched nucleotide pairs, and it remains to be determined if they are properly processed and produce functional tRNAs (fig. 1.2).

The boundaries of the genes coding for the large (*16S*) and small (*12S*) subunit ribosomal RNAs (the genes are designated *rnl* and *rns*) have been inferred by secondary structure modelling for the genomes reported here. Length variations among mussel genes range from 1243 bp± 2 bp to 938 bp ± ~10 bp for *rnl* and *rns*, respectively.

1.4.3 Noncoding Sequences

In the newly sequenced mtDNAs as well, the largest noncoding region is ~1000 bp long and located between *rnl* and *trnY* (see fig. 1.1). This region likely harbours the control region of the leading strand, as inferred from the comparison with mammamlian mtDNAs, where replication and transcription initiation have been studied experimentally in great detail (Clayton, 1991; Cao *et al.*, 2004).

Five other significant noncoding regions, designated UR1-UR5, are ~20 bp to 500 bp long and occur in across all six sequenced mytilid mtDNAs. These regions contain potential secondary structures which have been implicated in replication, transcription, or RNA processing (Clayton, 1991; Mizi *et al.*, 2005).

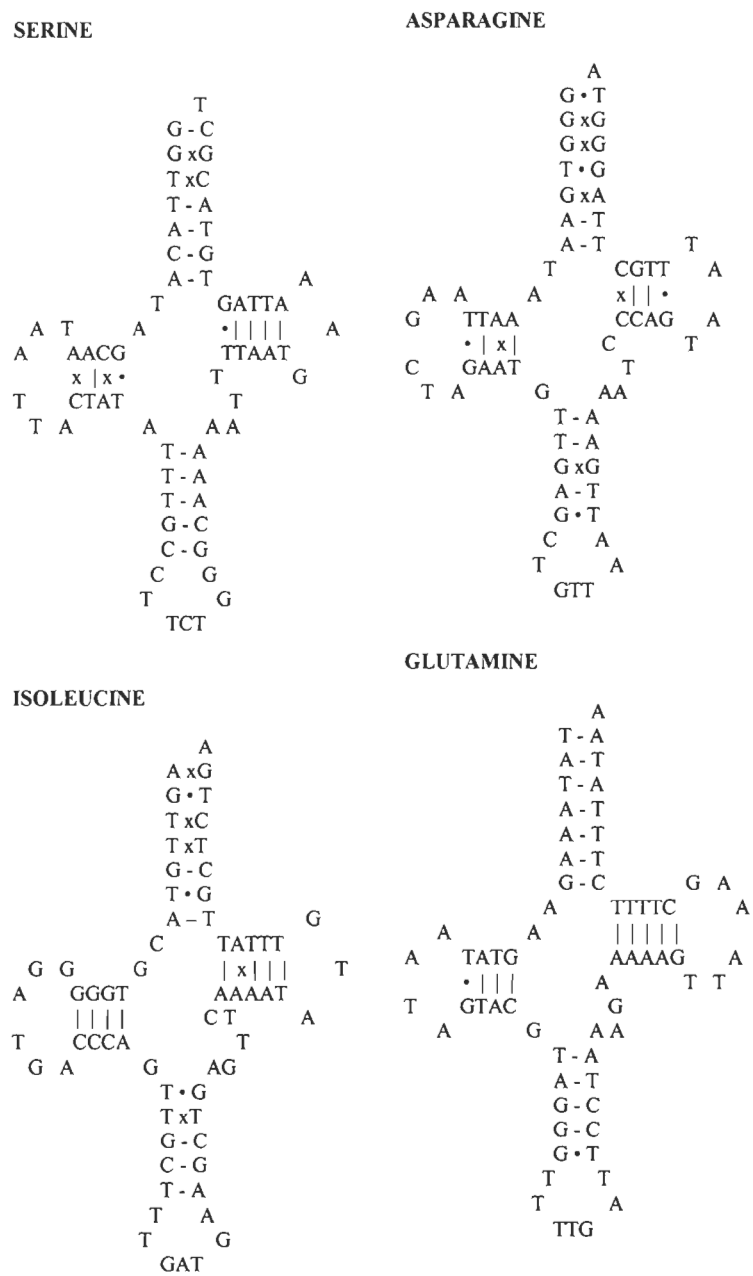

Figure 1.2 Additional tRNA-like structures only found in *M. trossulus* recently-masculinized mtDNA.

1.4.4 Comparative Analysis of Gender-Associated Mitochondrial Genomes in Mytilid Mussels

Based on the newly determined sequences, we conducted intra- and interspecific comparisons of gene sequences from standard male, recently-masculinized male and female mytilid (Table 1.4). Interestingly, the lowest number of nucleotide and amino acid differences in protein-coding genes are observed between the two females from different species, *M. edulis* and *M. galloprovincialis* (21 amino acid and 117 nucleotide substitutions), which are even less than the differences observed between the two standard males of *M. edulis*.

The number of nonsynonymous substitutions per nonsynonymous site (K_a) and the number of synonymous substitutions per synonymous site (K_s) (Nei & Gojobori, 1986) have been calculated and their ratios have been plotted for all pairwise comparisons. Figure 1.3 shows that Ka to Ks ratios are lowest for *cox1* (most conserved), and highest for *nad6* (least conserved). Several studies have reported significant variation in the nonsynonymous to synonymous substitution rate ratios for different mitochondrial genes across animals (Rand, Dorfsman & Kann, 1994; Pesole *et al.*, 1999; Ballard, 2000a,b; Doiron, Bernatchez & Blier, 2002). In general, animal mitochondrial genes coding for subunits of the cytochrome *c* oxidase complex are slowly evolving (Pesole *et al.*, 1999; Doiron, Bernatchez & Blier, 2002). Similarly, the *nad6* gene appears to be weakly conserved in two other pairs of conspecific genomes (the clam *Venerupis philippinarum* and the freshwater mussel *Inversidens japanensis*) (Mizi *et al.*, 2005), suggesting that the high rate of evolution of this gene is not a characteristic property of the *M. edulis* species complex.

Table 1.4
Intra- and interspecies comparisons for all mitochondrial genes

			Protein-Coding Genes					
Pairs	rRNAs	tRNAs	I	III	IV	V	N_a	D
*Med*_M – *Med*_M	12 (0.5%)	3 (0.2%)	17	4	3	5	29 (0.8%)	0.008
*Med*_M – *Mga*_M	130 (5.9%)	96 (6.3%)	179	38	34	13	264 (6.5%)	0.072
*Med*_F – *Mga*_F	12 (0.5%)	3 (0.2%)	16	2	3	0	21 (0.6%)	0.006
*Med*_M – *Med*_F	288 (13.1%)	150 (9.8%)	366	65	72	37	540 (14.7%)	0.157
*Mga*_M – *Mga*_F	316 (14.4%)	176 (11.5%)	357	52	85	32	526 (14.3%)	0.151
*Med*_M – *Mga*_F	283 (12.9%)	151 (9.9%)	382	64	71	37	554 (15%)	0.155
*Mga*_M – *Med*_F	319 (14.5%)	177 (11.6%)	362	53	79	32	526 (14.3%)	0.154
*Mtr*_RM – *Med*_F	166 (7.5%)	96 (6.3%)	190	39	9	7	245 (6.7%)	0.067
*Mtr*_RM – *Mga*_F	166 (7.5%)	95 (6.2%)	188	38	8	7	241 (6.6%)	0.065
*Mtr*_RM – *Med*_M	275 (12.5%)	166 (10.9%)	341	42	69	35	487 (13.2%)	0.142
*Mtr*_RM – *Mga*_M	312 (14.2%)	181 (11.9%)	385	62	76	31	554 (15%)	0.161

NOTE.—Sequence divergences for rRNAs and tRNAs are given in total number of nucleotide differences (percent in parenthesis); I-V, different subunits of the same oxidative phosphorylation complex were pooled for amino acid substitutions; N_a, total number of amino acid differences; D, poisson-corrected amino acid distances for all protein-coding genes.

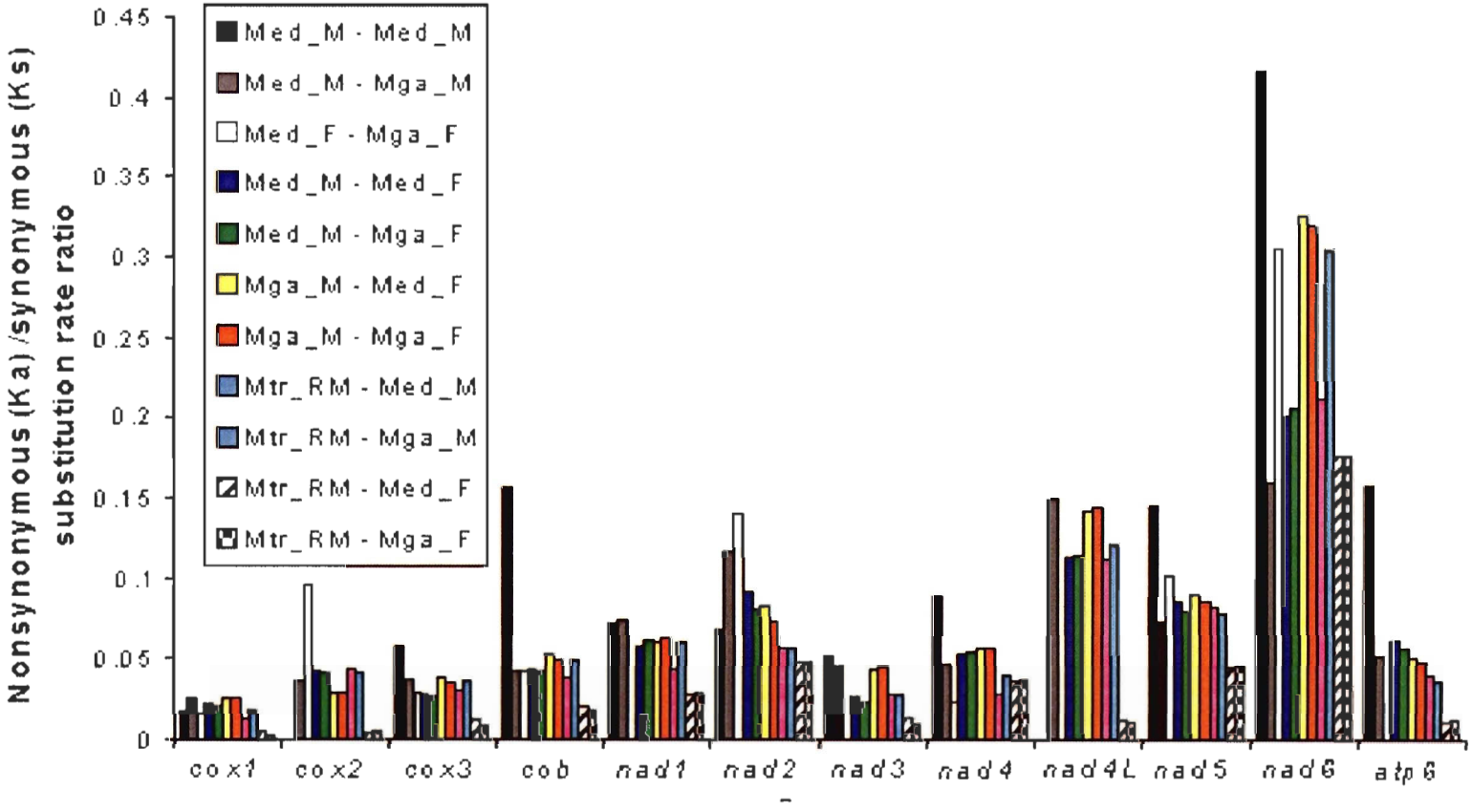

Figure 1.3 Comparisons between the number of nonsynonymous substitutions per nonsynonymous site (K_a) and the number of synonymous substitutions per synonymous site (K_s) among mytilid mussel mtDNA-encoded protein genes.

1.4.5 Analysis of expanded *cox1* data set

A broad survey of mytilid cytochrome *c* oxidase subunit one (Table 1.5) confirms earlier observations that the level of nucleotide variability is higher within the M than the F lineage (Rawson & Hilbish, 1995; Stewart *et al.*, 1995, Stewart *et al.*, 1996). Several mechanisms have been suggested to explain these differences (Skibinski, Gallagher & Beynon, 1994b; Stewart *et al.*, 1996; Schmidt *et al.*, 1997; Hasegawa, Cao & Yang, 1998; Ballard, 2000a,b), one of which proposing a lower selective constraints for the M genome relative to its F-type (Stewart *et al.*, 1996). Indeed, contrary to the F genome, which has to function fully in all somatic tissues and the female gonad, the male genome is expressed and serves primarily in the male gonad and only partially in somatic tissues, where it periodically occurs in conjunction with the more abundant F type (Stewart *et al.*, 1996; Dalziel & Stewart, 2002). However, because evolutionary rates may depend on multiple overlapping variable constraints, more data are needed to draw conclusions about the factors that are affecting the levels of mtDNA polymorphism in *Mytilus*.

Table 1.5
Intraspecies comparisons for *cox1* mitochondrial gene

Taxon	Nucleotide divergence (standard error)
Mytilus edulis (F genome, n = 5)[a, b]	0.005 (0.002)
M. galloprovincialis (F genome, n = 5)[b, c]	0.015 (0.005)
M. trossulus (F genome, n = 4)[b, present study]	0.009 (0.004)
M. edulis (M genome, n = 5)[b, present study]	0.080 (0.015)
M. galloprovincialis (M genome, n = 4)[b, c]	0.093 (0.017)
M. trossulus (M genome, n = 5)[b]	0.026 (0.007)
M. edulis (F/M genomes, n = 5)	0.239 (0.034)
M. galloprovincialis (F/M genomes, n = 5)	0.248 (0.037)
M. trossulus (F/M genomes, n = 5)	0.219 (0.032)

NOTE.—Nucleotide distances have been calculated with MEGA, version 2.1 (Kumar *et al.*, 2001) using the kimura-2 parameter model.

[a]Hoffmann, Boore & Brown (1992); [b]Riginos *et al.* (2004); [c]Mizi *et al.* (2005).

1.4.6 Evidence for Non-Homologous Recombination Between Gender-Associated mtDNA Lineages in *Mytilus trossulus*

Intriguingly, the major noncoding region (3061 bp) of *M. trossulus* recently-masculinized genome is a chimera that consists of a 1265-bp segment with high identity (93%) to the "D-loop" of the *M. trossulus* M lineage (P.D. Rawson; GenBank accession numbers DQ013366-67), followed by a ~400-bp segment having sequence similarity with several tRNA genes and the adjacent 5' end portion of the *rnl* gene in the F-type *M. edulis* (see fig. 1.1). The last 1434 bp-long segment of the *M. trossulus* recently-masculinized noncoding region contains three elements: (i) another "D-loop" sequence with potential control segment having high sequence similarity (98% identity) to that of the *M. trossulus* F lineage (T. Barna & R. Showman; GenBank accession number AF188281); (ii) a repeat copy of *trnY*; and (iii) a 97-bp fragment of the 5' end of F-type *cob*. Blast searches in GenBank indicate that similar sequences have been found recently in the F lineage noncoding region of North American *M. trossulus*, but the corresponding mtDNAs have only been sequenced partially (P. D. Rawson, personal communication; GenBank accession numbers AY636148-53). These tandem arrangement of F and M control region sequences have been fully described and designated as follows (based on size): F2 (~3060 bp; TFCR5' for the M-type part and TFCR3' for the F-type part of the noncoding region; without an extra *trnY* and 5' *cob*) and F3 (~3370 bp; with an extra *trnY* and 5' *cob*). The F2 haplotype is nearly always present in a homoplasmic state in eggs and in a heteroplasmic state together with the standard M-type mtDNA in males. In contrast, the F3 haplotype has been found to be relatively rare and was nearly always detected in a state of heteroplasmy with the F2 haplotype. Moreover, the F2 haplotype is transmitted maternally, whereas the F3 haplotype appeared to be transmitted both maternally and paternally. Apparently, the presence of a male control region in the F-type mitochondrial genome of *M. trossulus* is the result of non-homologous recombination between the *M. trossulus* M and F genomes.

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1.4.7 The presence of two potential control regions and their implication in the transmission dynamics of the mitochondrial genomes in blue mussels

The recombinant haplotype identified in our study differs from those observed previously. The product generated by PCR is 3061 bp in length, corresponding with the size of the F2 haplotype described above, but the presence of a repeat of the *trnY* gene and a portion from the 5' end of *cob* is similar to the F3 haplotype. As we did not find any trace of standard M-type mtDNA or control region size variation in the DNA extraction from the male gonadal tissue (see Material and Methods), it is reasonable to speculate that this recombinant mitotype is paternally transmitted.

Other studies also provide evidence for mitochondrial recombination in mytilid mussels (Ladouzakis & Zouros, 2001; Burzyński *et al.*, 2003). For example, some PCR-amplified main control regions in male gonads of *M. trossulus* individuals from the Baltic Sea are mosaic for F- and M-like sequences (Burzyński *et al.*, 2003). Because these recombinant variants are transmitted to sperm like the M genome, and because RFLP studies of these whole mtDNA genomes and amplified PCR products have shown high similarity to the *M. edulis* F genome, Burzyński *et al.* (2003) speculated that the M-type part of the control region confers a paternal role on mtDNA that otherwise resemble the F genome in sequence. Phylogenetic analysis confirms the high degree of similarity between the two putative M and F control regions of the recently-masculinized recombinant mitotypes (TFCR5'and TFCR3' from the F2 and F3 haplotypes, P.D. Rawson, personal communication; male and female "D-loop" from the *Mtr*_RM mtDNA, present study), and their respective M and F control region homologs in other mytilid mussels (fig. 1.4). If the control region plays a role in the transmission of the mitochondrial genomes in blue mussels, then the regulation of DUI would imply at least a two-level selection model: a segregation of sperm mitochondria in the male gonad in developing embryos (Cao, Kenchington & Zouros, 2004), and a replication advantage of mtDNA containing an M-type control region in male gonads.

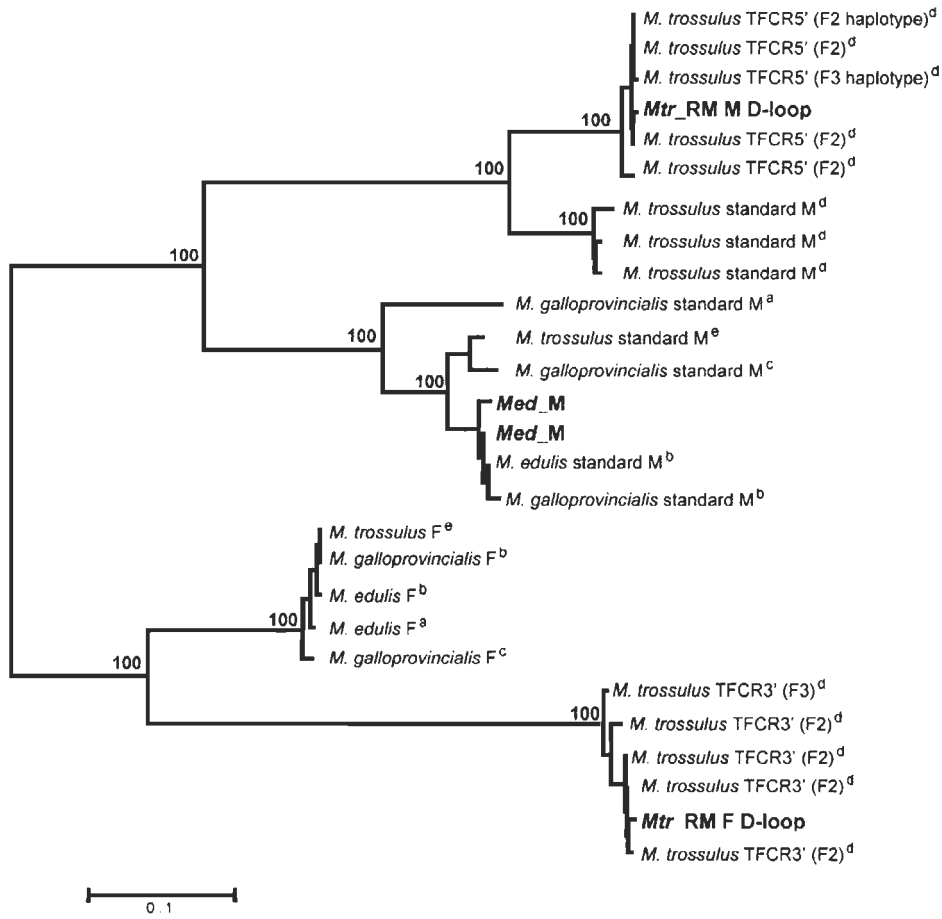

Figure 1.4 Unrooted neighbor-joining tree for the male and female "D-loop" portions of the recently-masculinized *M. trossulus* mt genome (*Mtr*_RM, presented in bold), the male and female control region portions (TFCR5' and TFCR3', respectively) of the *M. trossulus* F2 and F3 haplotypes (P.D. Rawson, personal communication), and the M and F-type control regions for *M. edulis*, *M. galloprovincialis*, and *M. trossulus* ([a]Hoffmann *et al.*, 1992; [b]Cao *et al.*, 2004; [c]Mizi *et al.*, 2005; [d]P.D. Rawson, personal communication; [e]T. Barna & R. Showman, personal communication). Numbers indicate bootstrap support from 1000 replicates. Parsimony analysis yielded the same topology.

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Interestingly, examination of three other *M. trossulus* recently-masculinized genomes, two extracted from the same male gonad sample and one from a different male gonad, indicated two size classes of PCR products after using primers trm16SF and trmUR (data not shown). Both male gonads succeeded to identify the presence of a segment that corresponded well with the size of the product from the recently-masculinized sequenced genome. The second PCR product obtained from the male gonad that generated two haplotypes was approximately 2 kb shorter than the product from the sequenced genome. The presence of a male and/or a female control region was determined using the RFLP assay described in Rawson (personal communication): the largest PCR products contained both male and female control regions, while the smallest contained an F-type control region sequence. This suggests that "extra motifs" are not necessarily a typical feature of recently-masculinized mtDNAs. However, one cannot rule out the possibility that an M-type control region is required for the transcription of a mt genome in the male gonad. At present, it is not known whether the two potential control regions are functional, whether they act in a tissue-specific manner, or what their role may be in the transmission of the mitotypes. It is premature to draw any firm conclusions about the relationship between recombination and masculinization from the available *M. trossulus* control region sequences available. The difficulty of discerning closely related recently-masculinized types and their sister F types makes it equally difficult to definitively identify the gender and tissue associations of these types. The similarity of the TFCR elements within *M. trossulus* also makes it difficult to reconstruct the phylogenetic relationship of these fragments (fig. 1.4), particularly when the nearest outgroup (e.g., the F control region segment from *M. edulis*), is considerably diverged from the sequences of the ingroup. A population level study of control region sequences will be necessary to answer these questions and to determine how dynamic and frequent recombinational events are within this complex of *M. trossulus* genomes.

1.4.8 The DUI System: A Network of Nuclear/Cytoplasmic Interactions

The high number of amino acid replacements observed between F and M genomes of a species could affect interactions with nuclear-encoded respiratory chain subunits and lead to an impairment of mitochondrial function (see Schon, 2000 and Blier, Dufresne & Burton, 2001 for reviews). Indeed, as the majority of the polypeptides in the respiratory chain complexes are encoded by nuclear genes, these could function more efficiently with one or the other mtDNA genotype (Jenuth, Peterson & Shoubridge, 1997). If the M type has experienced a relaxation of selection compared to the F type, it may have accumulated more slightly deleterious mutations, and some of these mutations could alter oxidative phosphorylation in male gonad or in somatic tissues (Stewart *et al.*, 1996). Alternatively, standard male types might have been subject to selection for particular amino acid substitutions that could improve the functional properties of mitochondrial genes in male gonad. In this context, it is interesting to note that no significant differences have been observed in sperm swimming speeds between standard male and recently-masculinized mitotypes in *Mytilus edulis*, suggesting that the selective advantage of one mitotype in sperm mitochondria is not related to motility capacity (Everett *et al.*, 2004). Swimming speed is, however, only one of the key parameters of sperm motility and fitness, and we can not exclude the possibility of more subtle effects on mitochondrial respiratory chain function. Additional comparative analyses (e.g., measuring OXPHOS complex activities) of masculinized and standard M-type bearing-sperm are needed to clarify the potential impact associated with the amino acid substitutions observed between these mtDNAs. Such studies are currently underway in our laboratory.

The frequent hybridization between pairs of species within the *M. edulis*, *M. trossulus* and *M. galloprovincialis* species complex, and the retention of both species-specific M and F mtDNA in hybrid individuals (Saavedra *et al.*, 1996; Comesaña *et al.*, 1999), suggest a high degree of compatibility between sequence variants in the nuclear- and mtDNA-encoded subunits of the respiratory chain complexes in mytilid mussels. It was hypothesized that cytonuclear co-adaptation

should be evident from coordinated amino acid changes of M and F mtDNAs from the same nuclear background. Our data demonstrate a highly significant level of correlation in amino acid substitution rates for the different F and M genes irrespective of the nuclear background (fig. 1.5). This suggests that strong structural and/or functional constraints are gene specific rather than species- or gender- specific. Comparative studies of OXPHOS complex activities in natural mytilid hybrids could help to evaluate the limit of compatibility between sequence variants of nuclear- and mtDNA-encoded subunits of the respiratory chain complexes in mytilid mussels.

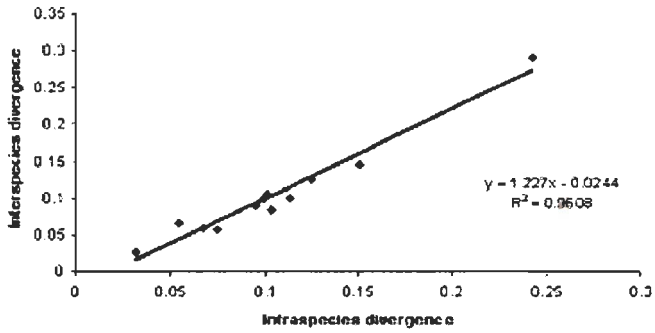

Figure 1.5 Relationships between intraspecies divergence in amino acid substitutions for protein-coding genes relative to interspecies divergences. Each dot denotes an individual gene.

A primary motivation of this study was to present and describe the first complete sequence of a recently-masculinized mitochondrial genome of a mytilid mussel. It aimed to determine: (1) candidate mitochondrial DNA regions that may demonstrate rapid adaptation associated with specific male functions, and (2) distinct regions of mitochondrial DNA that may be responsible for the different transmission genetics of the gender-specific genomes. To address the first issue, a sliding window analysis has been performed to parallel the differences in amino acid substitution for all protein genes in three categories of comparisons: among

standard males, between standard male and recently-masculinized genomes, and between female and recently-masculinized genomes (fig. 1.6). These pairwise comparisons showed that (i) the variability within mtDNA regions of the M lineage is highly correlated with the variability within the F lineage, and (ii) the regions of mtDNA-encoded subunits that are more similar in the standard/recently-masculinized males pair than in the standard female/recently-masculinized male pair are situated in the NADH oxidoreductase complex (complex I) (Table 1.6). The degree of correlation of patterns of variability in the M and F genomes suggests that they are experiencing similar selective pressures. A comparison of mtDNA-encoded and nuclear-encoded proteins from mytilid mussels could help to determine if the subunits that directly interact with one another evolve in concert. If so, nucleotide substitutions in either an M-type or F-type genome should constitute a selective pressure for compensatory substitutions on the opposite gender-associated genome.

In conclusion, data on North American *M. trossulus* mussels may give some insights into the mechanism of germline mitochondrial segregation and mtDNA maintenance. The observation that a recently-masculinized mitotype has been found to possess a standard F control region, is not consistent with the hypothesis that portions of the control region may be responsible for the different mode of transmission of the mitochondrial genomes in blue mussels (Burzyński *et al.*, 2003; Cao *et al.*, 2004; Mizi *et al.*, 2005, P.D. Rawson, pers. communication). Nevertheless, our observations do not directly confirm the complete absence of DUI regulation by the mtDNA itself. It is clear that gender-specific sequences can be acquired through recombination (Ladoukakis & Zouros, 2001; Burzyński *et al.*, 2003; present study). Furthermore, the requirement for recombination to introduce a critical control region or other sequence into an otherwise female type could explain why the masculinization events are absent in unionids (Mizi *et al.*, 2005), which apparently do not possess recombinant F-M genomes (Hoeh, Stewart & Guttman, 2002).

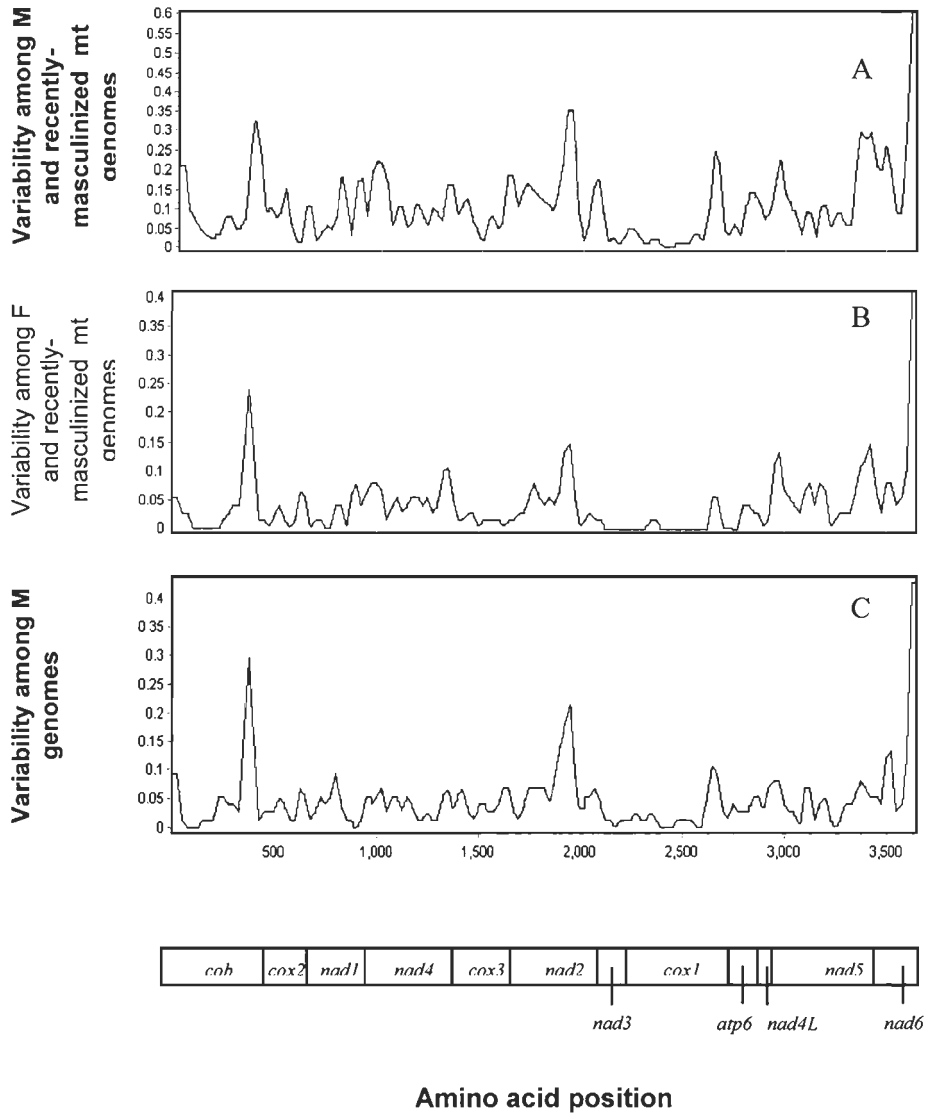

Figure 1.6 Variability of amino acids across the mitochondrial genome detected in sliding windows of 50 amino acid with an increment of 25 a.a. Protein sequences have been concatenated as shown in the bar at the bottom of the figure. Sliding-window analyses comparing the variability among A Male mitochondrial genomes (considering the masculinized molecule). B Female mitochondrial genomes (considering the masculinized molecule). C Male mitochondrial genomes.

Table 1.6
Gene position of amino acid substitutions that are more similar in the male/recently-masculinized pairs than in the female/recently-masculinized pairs

Gene	Position	*Med*_M	*Med*_M	*Mga*_M	*Mtr*_RM	*Med*_F	*Mga*_F
Cob	322….	S	S	S	S	T	T
	325….	S	S	S	S	T	T
	336….	F	F	F	F	L	L
Cox3	50….	S	S	S	G	V	V
	97….	S	S	S	S	T	T
	170….	L	L	L	L	V	V
Nad1	5….	S	S	S	G	A	A
	304….	S	S	S	S	G	G
nad2	79….	F	F	F	A	S	S
	93….	S	S	S	S	T	T
	140….	M	M	M	M	L	L
	151….	L	L	L	L	S	S
	220….	G	G	G	G	S	S
	295….	M	M	M	M	I	I
nad4	20….	M	M	M	M	V	V
	36….	T	T	T	T	A	A
	40….	M	M	M	M	T	T
	76….	I	I	I	I	V	V
	230….	M	M	M	M	L	L
	246….	M	M	M	V	S	S
	289….	V	V	V	V	L	L
	300….	I	I	I	I	V	V
	337….	S	S	S	S	V	V
	414….	V	V	V	V	L	L
	422….	V	V	V	A	S	S
Nad4L	10….	F	F	F	F	L	L
Nad5	9….	V	V	V	I	T	T
	22….	K	K	K	K	S	S
	166….	T	T	T	T	M	M
	230….	F	F	F	F	Y	Y
	450….	L	L	L	L	F	F
	454….	V	V	V	V	A	A
	460….	W	W	W	W	R	R
	463….	L	L	L	L	I	I
nad6	41….	V	V	V	V	I	I
	42….	T	T	T	T	A	A
	97….	M	M	M	M	V	V
	105….	T	T	T	S	V	A
	123….	G	G	G	S	K	K

NOTE.—Amino acid substitutions that are non-conservative are underlined.

Our data reveal individual patterns of evolution of the different mitochondrial genes: these patterns seem independant of the nuclear or tissue background. Despite a higher evolutionary rate possibly associated with relaxed or positive selection, our results suggest that the M genome is functional and under selective constraints. As proposed earlier, selective constraints on the M genome may be required to meet the specific needs of sperm (Skibinski, Gallagher & Beynon, 1994b). Qualitative or quantitative differences in respiratory chain activity between the male gonad and somatic tissues may cause the biased distribution of M mtDNA in heteroplasmic males. However, cytonuclear interactions, interaction between different F and M mtDNAs, and/or differences in their replication abilities should also be important cellular and molecular mechanisms that underlie the phenomenon of doubly uniparental inheritance.

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CHAPITRE II

ROLE-REVERSAL OF GENDER-ASSOCIATED MITOCHONDRIAL DNA AFFECTS MITOCHONDRIAL FUNCTION IN MYTILUS EDULIS (BIVALVIA: MYTILIDAE)

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Résumé

Dans le chapitre précédent, nous avons démontré que le fait de co-exister dans un même environnement nucléaire contraint les ADNmt F et M à expérimenter des pressions sélectives similaires, ce qui se traduit par une évolution congruente des différentes portions des ADNmt M et F (c'est-à-dire la corrélation positive des patrons de taux de substitution protéiniques). Dans le présent chapitre, nous essayons de déterminer si les produits des deux différents ADN mitochondriaux peuvent interagir normalement avec les protéines d'un seul système génétique nucléaire (en raison de la co-adaptation intergénomique). Pour ce faire, nous avons vérifié, chez les spermatozoïdes de l'espèce *M. edulis*, si le remplacement d'un génome M par un génome F (phénomène de renversement des rôles) avait un effet sur les capacités métaboliques des mitochondries. Pour ce faire, des dosages enzymatiques (activité des complexes mitochondriaux I+III, II et IV et de la citrate synthase) ont été réalisés par spectrophotométrie. Les résultats démontrent que la combinaison « ADNmt récemment-masculinisé – ADNnu » (qui est essentiellement une combinaison ADNmtF – ADNnu) performe mieux au niveau enzymatique que la combinaison « ADNmt M – ADNnu ».

2.1 Abstract

Mussel species of the genus *Mytilus* possess an unusual system of mtDNA transmission termed doubly uniparental inheritance (DUI). They are characterized by the presence of two highly divergent gender-associated mtDNAs that are inherited either maternally (F mtDNA) or paternally (M mtDNA). Female are typically homoplasmic for the F mtDNA and males are heteroplasmic with the F mtDNA in all tissues except the gonad, which is dominated by the M mtDNA. Males also contain two classes of M mtDNAs known as the "standard" and "recently-masculinized" M types. The recently-masculinized M-types are recombinant mtDNAs that possess a standard M-type control region but F-type coding sequences. These recombinant molecules have invaded the male route of inheritance and are transmitted paternally from generation to generation as new M mtDNAs. Because the standard and recently-masculinized mtDNAs exhibit important amino acid sequence divergence at the protein coding level, we hypothesized that these differences could affect mitochondrial respiratory chain complex enzyme activities. The activity of the major mitochondrial respiratory chain complexes (complexes II, I + III, and IV) as well as the activity of citrate synthase were thus measured in gonad samples of standard and recently-masculinized individuals. Our data strongly suggest that the mitochondrial subunits encoded by the recently-masculinized mtDNA are associated with higher enzymatic activities than the gene products of the standard M-type mtDNA.

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2.2 Introduction

Animal mitochondrial DNA (mtDNA) is an exclusively maternally inherited circular DNA molecule about 16.5-kilobase long (but see Burger, Gray & Lang, 2003 and Xu, 2005 for exceptions) that encodes a small number of genes, usually 37 (Boore, 1999). Among them, 24 mitochondrial genes encode components that are involved in the mitochondrial translational machinery (22 tRNAs and two rRNAs). The additional 13 genes encode protein subunits of the respiratory chain complexes and ATP synthase: seven subunits of the NADH: ubiquinone oxidoreductase (complex I; *ND1* to *ND6*, and *ND4L*), one subunit of the ubiquinone: cytochrome *c* oxidoreductase (complex III; *CYTB*), three subunits of the cytochrome *c* oxidase (complex IV; *COX1* to *COX3*) and two subunits of the ATP synthase (complex V; *ATP6* and *ATP8*) (Anderson *et al.*, 1981; Garesse & Vallejo, 2001). Other subunits of respiratory chain complexes I, III, IV and V, all the components of complex II (succinate: ubiquinone oxidoreductase), the enzymes of the mitochondrial matrix, as well as the factors involved in other mitochondrial functions (e.g., substrates and ions transport, mtDNA replication and mtDNA expression) are all nuclear-encoded in animals.

Mitochondrial homeostasis thus depends on the coordinated expression of two physically separated genetic systems (mtDNA and nuDNA), and on complex processes that require a variety of well orchestrated mechanisms. To maintain efficient mitochondrial function (i.e., for correct assembly and function of the oxidative phosphorylation system, for mtDNA replication, transcription and translation, and for all other mitochondrial functions), natural selection should continually favor co-adaptation of interacting components encoded by the nuclear and mitochondrial genomes (see Blier, Dufresne & Burton, 2001 and Rand, Haney & Fry, 2004 for reviews). Given this degree of co-evolution, one might predict that a mitochondrial genome having high fitness in its own nuclear genetic background would have reduced fitness in the nuclear genetic environment of a different species or population. Moreover, the degree of functional incompatibility

would presumably be a function of the amount of sequence divergence between the native and the foreign mtDNAs (Hutter & Rand, 1995).

Genetic compatibility between the nuclear and mitochondrial genomes has been studied by: 1) directly manipulating genome combinations of cells or embryos in culture, 2) repeatedly back-crossing animals in the laboratory to obtain specific genetic combinations and 3), studying natural hybrid animals where unusual mtDNA-nuDNA combinations occur (for reviews see Blier, Dufresne & Burton, 2001 and Rand, Haney & Fry, 2004). In whole organism experiments, repeated back-crosses between genetically isolated populations of the marine copepod *Tigriopus californicus* have shown a decrease in the activity of the mitochondrial enzyme cytochrome *c* oxidase (complex IV or *COX*) as mitochondria from one population were moved to a purer and purer nuclear background from the other (Burton, Rawson & Edmands, 1999). In another study of inbred recombinant hybrid lines derived from a series of interpopulation crosses of *T. californicus*, Ellison & Burton (2006) demonstrated a disruption of respiratory chain function for all complexes except complex II, which lacks mtDNA-encoded peptides. Congruent with mitonuclear co-adaptation, the disrupted phenotypes are only seen in those nuclear-mitochondrial complexes in which the encoded subunits interact.

The discovery of an unusual mtDNA system in marine mussels (Mytiloida), freshwater mussels (Unionoida), and marine clams (Veneroida) has challenged several paradigms of mitochondrial genomics. Species possessing this unusual system, which is referred to as doubly uniparental inheritance (DUI) (Zouros *et al.*, 1994), are characterized by the presence of distinct gender-associated mtDNAs (often with nucleotide divergences >20%) that are inherited either maternally (F mtDNA) or paternally (M mtDNA). Specifically, females pass on their F mtDNA to both sons and daughters while males pass on their germ line M mtDNA only to their sons. Females are typically homoplasmic and thus contain and express only the F mtDNA. Males are typically heteroplasmic and express predominantly the F mtDNA in all tissues except the gonad, which is dominated

by the M mtDNA, and the spermatozoa, which express exclusively the M mtDNA (see Zouros, 2000 and Breton *et al.*, 2007 for reviews). As a further complication of this unusual system, in the family Mytilidae, male mussels contain two classes of M mtDNAs known as the "standard" and "recently-masculinized" M types (e.g., Hoeh *et al.*, 1997). The recently-masculinized M-types are recombinant mtDNAs that possess a standard M-type control region but F-type coding sequences. These recombinant molecules have invaded the male route of inheritance and are transmitted paternally from generation to generation as new M mtDNAs (i.e., as a result of "role-reversal" event or "gender-switching") (Burzyński *et al.*, 2003; Breton *et al.*, 2006; Burzyński *et al.*, 2006; Venetis *et al.*, 2007).

An attractive feature of DUI is that it allows selection to act directly on the male mitochondrial DNA. Unlike animals with strict maternal inheritance, male mussels do not represent an evolutionary dead-end for mitochondrial genomes; they transmit their M mtDNA (Zeh, 2004; Zeh & Zeh, 2005). Thus, the paternally-transmitted mtDNA may evolve adaptively for male function, such as sperm quality (e.g., motility) and fertilizing efficiency. Previous studies have already shown that sperm motility is one male trait that may be negatively affected by mtDNA mutations (Kao, Chao & Wei, 1995; Ruiz-Pesini *et al.*, 1998, Ruiz-Pesini *et al.*, 2000). An interesting approach to address the issue of "M genome-specific adaptation for sperm performance" in mussels employs comparison of functional properties of mitochondria between recently-masculinized (RM) and standard M (SM) mtDNA bearing-sperm in the species *Mytilus edulis*. The flagellum of *M. edulis* sperm is powered by five large spheroid mitochondria located within the mid-piece (Longo & Anderson, 1969). Sperm of standard and recently-masculinized *M. edulis* are thus differentiated by their mtDNAs, which exhibit almost 9% amino acid sequence divergence with many non-conservative a.a. substitutions (Stewart *et al.*, 1996). One intriguing consequence of this sperm polymorphism is that if there has effectively been positive selection on M mtDNA for its function in males, the invasion of the male

gonad by the more female-optimized coding sequence of a recently-masculinized mtDNA can theoretically affect ATP production. This could, in turn, affect sperm motility (Ruiz-Pesini *et al.*, 1998; Everett *et al.*, 2004). The initial test of this hypothesis indicated no significant differences in sperm swimming speeds between standard M and recently-masculinized mitotypes in *Mytilus edulis* (Everett *et al.*, 2004). In contrast, a recent analysis, using larger samples and technical enhancements (Jha *et al.*, 2007), has demonstrated that the recently-masculinized males were significantly faster than the standard males, suggesting that the recently-masculinized mtDNA/nuDNA (which is essentially an F mtDNA/nuDNA combination) results in faster spermatozoa than the standard M mtDNA/nuDNA combination.

The aim of this study was to determine if the differences in sperm motility previously observed between recently-masculinized and standard M mtDNA bearing-sperm in *Mytilus edulis* were associated with differences in mitochondrial respiratory chain enzyme activities. We thus measured the activity of the major mitochondrial respiratory chain complexes (complexes II, I + III, and IV) as well as the activity of citrate synthase in gonad samples of the same individuals. Our data strongly suggest that the mitochondrial subunits encoded by the recently-masculinized mtDNA are associated with higher enzymatic activities than the gene products of the standard M-type mtDNA.

2.3 Materials and Methods

2.3.1 Experimental animals

Mytilus mussels were obtained from an aquaculture operation located near Halifax, Nova Scotia in June 2006. These animals were then transported on ice to the Université du Québec à Rimouski. Spawning was induced for the previous experiment exploring swimming speeds of standard vs. recently-masculinized sperm (Jha *et al.*, 2007) by transferring individuals into plastic containers containing ~200 ml sea water at 20 °C after having rinsed them in room

temperature sea water three times and agitated them in a tub for approximately one minute. Sex was determined after spawning. The male spawn is white and cloudy in appearance and the female spawn consists of yellow- to orange-colored clumps of eggs. For mitotyping, genotyping and enzymatic assays, all spawned mussels were dissected and samples of gonad tissue were frozen at -80 °C until used. Shell sizes (width and length) were measured for each individual used for enzymatic assays.

2.3.2 Mitotyping and genotyping

Total DNA was extracted from ~20 mg gonad tissue per individual using a Qiagen DNA Extraction Kit (QIAGEN Inc., Mississauga, Canada) following the manufacturer's protocol. An Ultrospec 3100 Pro UV/visible light spectrophotometer (Amersham Pharmacia Biotech) was used to quantify the extracted DNA, which was then stored at -20 °C.

Mussels were classified as *M. edulis*, *M. trossulus* or hybrid using a PCR-based assay which targets a ribosomal DNA internal transcribed spacer (ITS) region (see Heath, Rawson & Hilbish, 1995; Dalziel & Stewart, 2002). After digestion with the restriction enzyme *HhaI*, the amplified product from *M. edulis* produced two bands of 450 and 180 bp in length plus some smaller fragments, whereas that from *M. trossulus* produced two bands of 280 and 180 bp as well as many smaller fragments. Hybrids possessed both bands at 450 and 280 bp. Only individuals identified as "pure" *M. edulis* were used for subsequent analyses.

Standard male and recently-masculinized mitochondrial genomes were differentiated with the amplification of a ~750 bp region of the mitochondrial *cox1* gene using the primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and a modified version of HCO2198 that was five bases shorter in the 5' end (5'-TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994; Jha *et al.*, 2007). These sequences (Jha *et al.*, 2007; GenBank Accession Numbers EUO18148 to EUO18204) were compared with previously published recently-

masculinized and standard M-type and F-type *cox1* sequences (GenBank Accession Numbers AY713960, AY130053 and U68773) to identify standard M-type and recently-masculinized bearing-sperm. ClustalW (Thompson, Higgins & Gibson, 1994) was used to align sequences and MEGA 3.0 (Kumar, Tamura & Nei, 2004) was used to generate neighbour-joining and parsimony trees. All of the males whose sequences clustered with the reference recently-masculinized sequence were classified as recently-masculinized (RM) males and those whose sequences clustered with the standard M-type sequence were identified as standard males (SM) (Jha *et al.*, 2007).

The number of amino acid differences between standard and recently-masculnized mtDNAs was determined using the program MEGA 3.0 (Kumar, Tamura & Nei, 2004). *COX1* residues that form the nuclear-encoded protein cytochrome *c* (*CYC*) binding site were extracted from amino acid alignments (ClustalW) including bovine and human *COX1* (GenBank Accession Numbers J01394 and AP_000641). Binding-site residues were identified based on previous studies (Wu *et al.*, 2000; Schmidt *et al.*, 2005).

2.3.3 Enzymatic assays

Ten and nine gonad samples of standard males (SM) and recently-masculinized males (RM), respectively, were used for enzymatic assays. Each gonad sample was weighed and homogenized in 5 volumes of ice cold buffer (50 mM imidazole, 2 mM $MgCl_2$ and 5 mM EDTA, pH 7.5) with three passes of 10 s in a Tekmar homogenizer. Homogenates were centrifuged at 500 *g* during 5 minutes at 4 °C and the supernatant was used for enzymatic analyses. All the manipulations were performed on ice. Citrate synthase (CS), succinate dehydrogenase (complex II or SDH), electron transport system (ETS), which comprise the activity of two enzymes of the respiratory chain, NADH dehydrogenase and cytochrome *c* reductase (complexes I and III), and cytochrome *c* oxidase (complex IV or COX) activities were measured at 15 °C using a

UV/VIS spectrophotometer (Perkin Elmer, Lambda 11) equipped with a thermostated cell holder and a circulating refrigerated water bath. All assays were run in duplicate. Specific activities were expressed in units per mg of protein where one unit (U) of enzyme activity corresponds to 1 μmole of substrate transformed into product per minute. Conditions for the enzymatic assays were as follows:

CS: 100 mmol l^{-1} imidazole-HCl (pH 8.0), 0.1 mmol l^{-1} 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 mmol l^{-1} acetyl CoA and 0.15 mmol l^{-1} oxaloacetate. Activity was measured by following the increase in absorbance due to the oxidation of DTNB at 412 nm and calculated using an extinction coefficient (ε_{412}) of 13.6 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$ (Thibeault, Blier & Guderley, 1997).

SDH: The homogenate was incubated at room temperature for 10 min in a mixture of 50 mmol l^{-1} potassium phosphate (pH 7.4) and 20 mmol l^{-1} succinate. To initiate the reaction, 2 μg ml^{-1} rotenone, 2 μg ml^{-1} antimycin, 2 mmol l^{-1} KCN, 50 μmol l^{-1} dichlorophenolindophenol (DCPIP) and 50 μmol l^{-1} decylubiquinone (DB) were added. Activity was measured by monitoring the increase in absorbance due to the reduction of DCPIP which is coupled to the complex II-catalyzed reduction of DB at 600 nm (ε_{550} = 19.1 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$) (Trounce *et al.*, 1996 with minor modifications).

ETS: 100 mmol l^{-1} sodium phosphate (pH 8.5), 0.85 mmol l^{-1} NADH, 2 mmol l^{-1} iodonitrotetrazolium chloride (INT) and 0.03% Triton X-100. Activity was recorded by following the reduction of INT, which yields an increase in absorbance at 490 nm (ε_{490} = 15.9 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$) (Lannig *et al.*, 2003).

COX: 100 mmol l^{-1} potassium phosphate and 0.05 mmol l^{-1} cytochrome *c* (pH 7.4). Cytochrome *c* was reduced by the addition of dithionite (4 mmol l^{-1}) and the solution was bubbled with air to eliminate the excess of reducing agent (Hodges & Leonard, 1974). Control samples consisted of 50 mmol l^{-1} cytochrome *c* oxidized with potassium ferricyanide (0.05%). Activity was measured by following the oxidation of cytochrome *c* at 550 nm (ε_{550} = 29.1 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$).

Note: All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

2.3.4 Protein assays

Total protein content of each sample was determined by the bicinchoninic acid method (Smith *et al.*, 1985).

2.3.5 Statistical analyses

All statistical tests were performed using Statistica software. Pairwise comparisons (Mann-Whitney) were used to evaluate differences between groups. Statistical significance was assessed at < 0.05 level for all comparisons.

2.4 Results and Discussion

To gain insights on the mechanisms underlying the previously observed association between mtDNA haplotypes [i.e., standard M (SM) and recently-masculinized (RM) mtDNAs] and sperm motility (Jha *et al.*, 2007), we tested the mitochondrial respiratory chain complex activities in SM and RM male gonad homogenates from the same individuals. Shell sizes (width and length) of SM and RM male mussels and the mass, protein content and mitochondrial enzyme-specific activities of SM and RM gonad samples are shown in Table 2.1. A significant increase in mean activity was observed in RM gonad samples for *COX* (complex IV; $P < 0.02$), whereas there was no significant difference in shell sizes between SM and RM male mussels nor in mass, protein content and mean activity of other complexes or citrate synthase (CS) between SM and RM gonad samples. The ratio of *COX* activity to CS was also significantly higher in RM gonad samples ($P < 0.02$). Cs is a reliable nuclear-encoded marker of the number and/or the volume of mitochondria and therefore the ratio of *COX* over CS is a

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convenient way to normalise *COX* activity according to mitochondrial content. Although higher ratios were observed for RM gonad samples for other comparisons of CS corrected activities, the differences were not statistically significant (Table 2.1).

As noted in previous studies (e.g., Venetis *et al.*, 2006; 2007), masculinization event are rare and several male individuals need to be examined to be able to find these "exceptional males" with a RM mtDNA. Of ~ 70 males examined, Jha *et al.* (2007) found 9 recently-masculinized males, a proportion similar to what has been observed along the southern shore of Nova Scotia over the past decade or so (e.g., Hoeh *et al.*, 1997). Nevertheless, one could argue that the use of male gonad tissues, which are always dominated by the paternally-transmitted mtDNA but invariably contaminated with the somatic F mtDNA (Venetis *et al.*, 2006), raises the possibility that they may have sequenced the maternal genome of the male they (and thus we) examined or that enzymatic activities may have been influenced by such a contamination. However, both cases are likely to bias the results against finding any significant differences in mitochondrial enzyme-specific activities and we consider that comparative analyses of standard and recently-masculinized male gonad tissues remain a conservative approach to address the issue of adaptive evolution of M mtDNA in males. Since all the standard F-type reference sequences included in the previous analysis fell out as sister sequences to all of the recently-masculinized M-types (Jha *et al.*, 2007), we also consider that use of the *cox1* gene is adequate to assign males as either standard males or recently-masculinized males.

Table 2.1
Shell sizes (width and length) for standard (SM) and recently-masculinized (RM) male mussels and mass, protein content and enzyme activities in U.mg protein (Mean ± Standard deviation) for SM and RM gonad tissues (N=10 for SM and N=9 for RM). Enzymatic activities were measured at 15 °C. Significant differences ($P < 0.05$) between species are indicated by different letters

Variables	Standard males (SM)	Recently-masculinized males (RM)
Shell size		
Width (mm)	27.4 ± 3	26.1 ± 1.4
Length (mm)	52.5 ± 4.8	52.9 ± 2
Gonad sample		
Mass (mg)	237.1 ± 6.2	234.7 ± 12.5
Protein content (mg)	40.7 ± 6.3	41.9 ± 4.6
CS	0.074 ± 0.021	0.064 ± 0.01
SDH (complex II)	0.029 ± 0.007	0.035 ± 0.006
ETS (complexes I + III)	0.087 ± 0.024	0.082 ± 0.008
COX (complex IV)	0.034 ± 0.012	0.048 ± 0.013[a]
SDH/CS	0.403 ± 0.127	0.536 ± 0.099
ETS/CS	1.197 ± 0.264	1.323 ± 0.278
COX/CS	0.447 ± 0.136	0.768 ± 0.197[b]

Our data strongly suggest that the mitochondrial subunits encoded by the RM mtDNA are associated with higher enzymatic activities and faster sperm motility (Jha *et al.*, 2007) than the gene products of the standard M-type mtDNA in this population of *M. edulis*. Previous studies have shown that sperm motility is dependant on the functionality of the oxidative phosphorylation system and that the sperm-motility phenotype is affected by the mtDNA haplotype (Ruiz-Pesini *et al.*, 1998; Ruiz-Pesini *et al.*, 2000; Montiel-Sosa *et al.*, 2006). For example, it has been demonstrated that asthenozoospermia (i.e., reduced sperm motility), but not oligozoospermia (i.e., reduced number of sperm), is associated with mtDNA haplotypes in humans (Ruiz-Pesini *et al.*, 2000). Therefore, mtDNA differences in

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protein-coding genes that directly affect enzymatic activity or enzymatic assembly could explain the higher mitochondrial enzyme-specific activities in recently-masculinized *M. edulis* gonad samples. In animals, the cytochrome *c* oxidase (complex IV or *COX*) is composed of 10 nuclear- subunits and 3 mtDNA-encoded subunits, these latter forming the "catalytic core" that interacts with the nuclear-encoded cytochrome *c* (*CYC*) for the transfer of electrons from *CYC* to oxygen (Rawson & Burton, 2006). Sequences from Jha *et al.* (2007) (i.e., GenBank Accession Numbers EUO18148 to EUO18204) showed that *COX1* is highly divergent between SM and RM mitochondrial genomes, with pairwise amino acid differences of 7.4% (i.e., 15 amino acid differences were found in the partially sequenced *COX1* protein). Of the 15 amino acid differences, 4 were non-conservative (i.e., charged [SM] vs. neutral [RM]) (fig. 2.1). One of these 4 differences (i.e., residue 139: Ser [RM] vs. Arg [SM]) implied an amino acid residue that is likely to interact with the nuclear-encoded protein *CYC* (Table 2.2). Because cytochrome *c* oxidase complex assembly has been shown to be sensitive to small differences in amino acid sequences not necessarily involved in the catalytic function (i.e., substitutions that could modify the interaction between nuclear and mitochondrial subunits at positions of structural importance to enzyme function), all these changes could potentially have functional significance (e.g., Barrientos *et al.*, 2000).

```
                        10         20         30         40         50         60         70         80
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
M. edulis RM    LIIRIQLGHP GAVFLKRDWF YNVVVTTHAL IIIFFAVIPI LIGAFGNWLI PLLVGGKDII YPRINNLSYW LSPNALYLLI
M. edulis SM    .......... .......... F......... MM........ .......... .......... ...M...R.. ..........
M. edulis F     .......... .......... .......... .......... .......... .......... .......... ..........

                        90        100        110        120        130        140        150        160
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
M. edulis RM    LSFRTDKGVG AGWTIYPPLS VYPYHSGPRI DVLIVSLHLA GLSSLVGAIN FASTNKNIPV LEIKGERAEL YVLRIRVTAV
M. edulis SM    .......... ....V..... R....R...M .....A.... .VR....... .........A .......... ......I..A
M. edulis F     .......... .......... .......... .......... .......... .......... .......... ..........

                       170        180        190        200
                ....|....| ....|....| ....|....| ....|....| ..
M. edulis RM    LLIISIPVLG GGITIILFDR NFNTTFFDPA GGGDPVLFQH LF
M. edulis SM    .......... .......... .......... .......... ..
M. edulis F     .......... .......... .......... .......... ..
```

Figure 2.1 *Mytilus edulis COX1* amino acid sequence alignment as deduced from nucleotide sequence alignement. (RM : recently-masculinised mtDNA; SM : standard male mtDNA; F : female mtDNA).

Table 2.2
Residues in *COX1* interacting with *CYC* in *Mytilus edulis*

Species	Amino acid residues							
	COX1							
	50	**119**	**120**	**131**	**136**	**139**	**140**	**221**
***M. edulis* RM**[a]	R	D	K	P	P	**S**	G	D
***M. edulis* SM**[b]	R	D	K	P	P	**R**	G	D
***M. edulis* F**[c]	R	D	K	P	P	S	G	D
Cow[d]	D	E	A	P	L	A	G	D
Human[e]	N	E	A	P	Y	P	G	D

NOTE.—[a] *Mytilus edulis* recently-masculinized mtDNA; [b] *M. edulis* standard male mtDNA; [c] *M. edulis* female mtDNA; [d, e] *COX1* residues at the binding site of *CYC* in cow and human (data from Wu *et al.*, 2000 and Schmidt *et al.*, 2005).

Differences in enzymatic activities could also be a consequence of differences in other mtDNA-encoded subunits or in tRNAs and rRNAs. If we use the local standard *M. edulis* F mtDNA (Boore, Medina & Rosenberg, 2004; GenBank accession Number AY484747) as a proxy for the recently-masculinized mtDNA (since this particular *M. edulis* RM genome has not been fully sequenced), then the average amount of amino acid sequence divergence between the standard M-type (Breton *et al.*, 2006; GenBank accession Numbers AY823623-24) and the RM mtDNA in *M. edulis* is ~14.7%. Amino acid substitutions have been observed for each of the four respiratory chain complexes composed in part of polypeptide subunits encoded by mitochondrial genes (Breton *et al.*, 2006). These changes could be invoked to explain the apparently higher performance of enzymatic complexes in RM mtDNA-carrying individuals.

On the other hand, our observation that higher ratios of activities of enzyme complexes to CS are associated with higher sperm motility in recently-masculinized male *M. edulis* suggest that motility could also depend on specific

enrichment of the complexes themselves within each of the five mitochondria (Longo & Anderson, 1969; Ruiz-Pesini *et al.*, 1998). We hypothesize that this enrichment could be the result of changes in complex protein levels due to differences in mtDNA replication, transcription and/or translation between SM and RM mitochondrial genomes. To our knowledge, all the RM *Mytilus* mtDNAs examined in detail (see Burzyński *et al.*, 2003; Breton *et al.*, 2006; Burzyński *et al.*, 2006; Venetis *et al.*, 2007) are recombinants possessing both F- and M-type control regions (CR) elements and coding regions consisting of F-type mtDNA. Compared to the SM genome, the multiple CR elements and/or the "F-type" tRNAs and rRNAs present in the RM genome could be responsible for the differences in replication (and expression) efficiency of the RM genome in the male gonad. An increased performance for RM genome in protein synthesis could also be caused by haplotype-associated polymorphism in rRNA or tRNA genes. For example, Breton *et al.* (2006) observed 150 nucleotides differences between F and M *M. edulis* mtDNAs for the 23 tRNA genes sequenced. Coordinated expression of both mitochondrial and nuclear genomes would explain the simultaneous increase of the corrected CS activity of the complex that has no subunit encoded by the mitochondrial genome (i.e., complex II) in RM gonad tissues. Additional studies of these RM and SM polymorphisms (as well as RM and SM polymorphisms in Mytilid species *M. trossulus* and *M. galloprovincialis*) will help to determine if RM variants influence mitochondrial protein synthesis in a way that could explain the higher enzymatic activities of RM mtDNA-carrying male gonad samples.

Our results, combined with those of a previous study by Jha *et al.* (2007), suggest that DUI did not originated because it permits the evolution of mitochondrial adaptations that enhance sperm motility, otherwise, the evolutionarily older, standard M mtDNA (i.e., the haplotype that should have adaptively evolved specifically for male function) should perform better. Alternative hypotheses would be that the paternally-transmitted mtDNA in bivalves might have been retained because of M genome-specific adaptations for

male functions other than sperm motility (Levitan, 2000; Johnson & Yund, 2004) or because of the M genome's obligate participation in sex determination (Saavedra, Reyero & Zouros, 1997; Zouros, 2000; Breton *et al.*, 2007; Jha *et al.*, 2007). It should be noted that these two hypotheses are not necessarily mutually exclusive and both may have played a role in the origin and maintenance of DUI.

Mytilus mussels have a life cycle in which a sedentary adult phase releases gametes into the water column (free-spawning), where fertilization and subsequent planktotrophic larval development take place (Rawson, Slaughter & Yund, 2003). Because sperm must drift via water currents to find eggs, selection could have favoured the retention of the standard M mtDNA for longevity over velocity (Levitan, 2000). This hypothesis is supported by previous findings that slow sperm have increased endurance (Levitan, 2000). The possibility that in nature different combinations of velocities and longevities may confer adaptive advantages under different conditions may explain why recently-masculinized mtDNAs periodically replace existing standard M mtDNAs evolutionarily (e.g., Hoeh *et al.*, 1997; Levitan, 2000; Jha *et al.*, 2007). For example, when sperm are competing for eggs that are quickly fertilized, selection could favour RM-bearing sperm with faster motility. Conversely, when eggs are dispersed and sperm must search longer distances, selection could favour SM-bearing sperm with greater longevity and capable of moving longer distances in a longer period of time. These patterns of sperm traits have been previously observed among three species of *Strongylocentrotus* sea urchins: *S. purpuratus*, which has the fastest but shortest-lived sperm, lives at a high population density where female fertilization rates are near 100% and sperm competition is important. In contrast, *S. droebachiensis*, which lives at the lowest population density where female fertilization success is less than 50%, has the slowest but longest lived sperm. Finally, *S. franciscanus* has intermediate sperm traits, intermediate population density and intermediate levels of female fertilization (Levitan, 2000). Further studies would be needed to verify if sperm traits (and haplotypes) are also adapted

for varying conditions in *Mytilus* populations where both SM and RM haplotypes are found.

A last hypothesis that could be considered to explain the faster sperm motility and higher mitochondrial enzymatic activities in RM gonad samples is that evolutionarily younger M mtDNAs are specialized for selectively advantageous male reproductive functionality but that mutational load eventually reduces the fitness of sperm bearing evolutionarily older M mtDNAs (Jha *et al.*, 2007). Comparisons of F and M nucleotide sequence polymorphism and divergences within and between Mytilid species have shown that the M mtDNA evolves more quickly than the F mtDNA (e.g., Stewart *et al.*, 1996; Quesada, Warren & Skibinski, 1998; Ort & Pogson, 2007). From these studies, it appears that both genomes experience purifying selection, but that this selection is relatively relaxed for the M mtDNA (Stewart *et al.*, 1996; Quesada, Warren & Skibinski, 1998; Ort & Pogson, 2007). These observations led Jha *et al.* (2007) to propose that the fate of M mtDNAs would be determined by their overall fitness, which would be determined by any originally favorable mutations for sperm function as well as by the cumulative effect of slightly deleterious mutations that have accumulated over time (i.e., their genetic load). According to these authors, the genetic load of older M mtDNAs may make it more likely that they will be replaced by a RM recombinant mtDNA. Further studies on functional capacities of the two sperm types in *Mytilus* populations containing mtDNA polymorphism should shed light on the potential adaptive evolution of the paternally-transmitted genomes on sperms traits.

2.5 Acknowledgements

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NSERC scholarships. The experiments comply with the current laws of the country in which the experiments were performed.

CHAPITRE III

MOLECULAR CO-EVOLUTION OF NUCLEAR- AND MITOCHONDRIAL-ENCODED PROTEIN SUBUNITS IN A SPECIES WITH DOUBLY UNIPARENTAL INHERITANCE OF MTDNA

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Résumé

Dans le chapitre précédent, nous avons démontré que, chez les spermatozoïdes de l'espèce *Mytilus edulis*, la combinaison « ADNmt récemment-masculinisé – ADNnu » (qui est essentiellement une combinaison ADNmtF – ADNnu) performe mieux au niveau enzymatique que la combinaison « ADNmt M – ADNnu ». Dans le présent chapitre, nous vérifions si nous obtenons les mêmes résultats au niveau moléculaire. C'est-à-dire que nous avons étudié l'évolution des protéines interactives du système énergétique (c'est-à-dire, produits nucléaires et mitochondriaux en contact physique) afin de vérifier si les portions génétiques qui interagissent co-évoluent ou si la co-évolution du génome nucléaire avec deux types d'ADNmt différents a été favorisée dans une ou l'autre des lignées. Pour ce faire, le gène nucléaire codant pour le transporteur mobile d'électron cytochrome *c* (C*YC*) et des gènes mitochondriaux et nucléaires codant pour des sous-unités protéiques des complexes III (ADNmt = *CYTB*; ADNnu = *C3S6*) et IV (ADNmt = *COX1* et *COX2*; ADNnu = *COX4*) du système de la phosphorylation oxydative ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique. Les données moléculaires semblent démontrer que la co-évolution entre le génome F et le génome nucléaire est mieux couplée, ce qui vient appuyer les résultats enzymatiques.

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3.1 Abstract

Mitochondrial homeostasis depends on the coordinated expression of two physically separated genetic systems, the mitochondrial and nuclear genomes. To maintain efficient mitochondrial function, natural selection should thus continually favor intergenomic coadaptation. In animal species, this coadaptation has been facilitated by the maternal inheritance of mtDNA, which prevents mitochondrial heteroplasmy within an organism and avoids inter-mitochondrial competition between selfish mitochondrial elements that are potentially deleterious to the interests of the nuclear genome. The unusual mtDNA system in marine mussels (Mytiloida), freshwater mussels (Unionoida), and marine clams (Veneroida) challenges this general rule. Species possessing this system, which is referred to as doubly uniparental inheritance (DUI) are characterized by the presence of two highly divergent gender-associated mtDNAs that are inherited either maternally (F mtDNA) or paternally (M mtDNA). This situation makes DUI a unique system for the study of nucleo-mitochondrial coevolution and coadaptation. Here, we explore intergenomic coadaptation at the molecular level in the DUI-containing mussel species *Mytilus edulis*. Three F and M mtDNA-encoded subunits and three nuclear-encoded interacting proteins of the electron transport system have been examined [the mtDNA-encoded cytochrome *b* (*CYTB*), the mtDNA-encoded cytochrome *c* oxidase subunits I and II (*COX1* and *COX2*), the nuDNA-encoded subunit 6 of the complex III (*C3S6*), the nuDNA-encoded protein carrier cytochrome *c* (*CYC*) and the nuDNA-encoded cytochrome *c* oxidase subunit 4 (*COX4*)]. We compare these sequences with mitochondrial sequences and nuclear sequences that have been obtained previously for *M. galloprovincialis*. It appears that the intergenomic coevolution is tighter for the F than for the M lineage, supporting the idea that in terms of relative level of selective constraint, the F mtDNA (and thus the interacting nuclear subunits) is more constrained because it has to perform fully in gonad and soma of both sexes, while the M mtDNA functions primarily in the male gonad (i.e., in sperm).

3.2 Introduction

Animal mitochondrial DNA (mtDNA) typically encodes 13 protein subunits of the respiratory chain complexes and ATP synthase whereas the other subunits of the electron transport system (i.e., ~65 in mammals) are all nuclear-encoded (Garesse & Vallejo, 2001). The maintenance of optimal molecular interactions (i.e., coadaptation) between nuclear and mitochondrial genomes has been inferred from DNA sequence analyses and directly demonstrated through functional assays (see Blier, Dufresne & Burton, 2001 and Rand, Haney & Fry, 2004 for reviews). For example, sequence analyses have shown that the protein subunits of electron transfer complexes III and IV (cytochrome *c* reductase and cytochrome *c* oxidase, respectively) and cytochrome *c*, the protein carrier that connects them, have apparently undergone a period of rapid and synchronic evolution in primates (Wu *et al.*, 2000; Grossman *et al.*, 2001; Doan *et al.*, 2004). Direct demonstration of coadaptation between mtDNA-encoded subunits of cytochrome *c* oxidase and the nuclear-encoded cytochrome *c* came from enzyme activity experiments using genetically isolated copepod *Tigriopus californicus* populations (Burton, Rawson & Edmands, 1999; Edmands & Burton, 1999; Rawson & Burton, 2002; Ellison & Burton, 2006). It was shown that the enzymatic activity of complex IV differed when using *CYC* from different populations as substrate, the activity being consistently higher when complex IV and *CYC* were derived from the same natural population (Rawson & Burton, 2002). These results provided clear evidence that coadaptive evolution occurred for interacting mitochondrial and nuclear proteins, especially for the nuclear-encoded cytochrome *c* (*CYC*) and the mtDNA-encoded subunit 2 (*COX2*) and nuclear-encoded subunit 4 (*COX4*) of the cytochrome *c* oxidase (Wu *et al.*, 2000; Grossman *et al.*, 2001; Rawson & Burton, 2002).

In animal species, coadaptation between the nuclear and the mitochondrial genomes has been facilitated by the strict maternal inheritance (SMI) of the latter (Birky, 2001; but see Xu, 2005 and Elson & Lightowlers, 2006 for cases of paternal leakage in animals). Indeed, uniparental inheritance of mtDNA is one

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mechanism that prevents mitochondrial heteroplasmy within an organism thereby avoiding inter-mitochondrial competition between selfish mitochondrial elements that are potentially deleterious to the interests of the nuclear genome (Hurst & Hoekstra, 1994; Burt & Trivers, 2006). However, the radically different system of doubly uniparental inheritance (DUI) of mtDNA discovered in some species of three evolutionary lineages of bivalved mollusk (Mytiloida, Unionoidea, Veneroida) challenges the paradigm of strict maternal inheritance (see Zouros, 2000 and Breton *et al.*, 2007 for reviews). In species with DUI, fathers also transmit mitochondria containing their paternally inherited mtDNA to their sons and daughters. Male and female zygotes thus inherit distinct maternal and paternal mtDNAs from their parents, but shortly after fertilization, female cells either lose the paternally inherited mtDNA completely or the ratio of M to F mtDNA genomes diminishes drastically (Sutherland *et al.*, 1998) while male zygotes sort the mixture of mtDNAs present such that the paternally inherited mtDNA becomes established in the germ line (Cao, Kenchington & Zouros, 2004; Cogswell, Kenchington & Zouros, 2006). As a result, typical females are essentially homoplasmic for the female-transmitted mitochondrial genome (F mtDNA) whereas males are heteroplasmic for both female- and male-transmitted (M mtDNA) mitochondrial genomes.

The existence in bivalve molluscs of two mitochondrial variants that coexist and simultaneously co-adapt to one nuclear genome makes DUI a unique system for the study of nucleo-mitochondrial co-evolution and coadaptation. The levels of mtDNA sequence divergences between F and M genomes in male bivalves are the highest intra-individual values yet reported (with sequence divergences in *cox1* often >20% and >30%, respectively), even higher than intra- or interspecific values reported in model systems used for the study of intergenomic co-evolution (Breton *et al.*, 2007). Comparisons of F and M nucleotide sequence polymorphism and divergences within and between species with DUI have shown that the M mtDNA evolves more rapidly than the F mtDNA (Rawson & Hilbish, 1995; Hoeh *et al.*, 1996; Liu, Mitton & Wu, 1996; Stewart *et al.*, 1995; Stewart *et al.*, 1996;

Passamonti & Scali, 2001; Passamonti, Boore & Scali, 2003; Ort & Pogson, 2007). A leading explanation for this pattern is that the M mtDNA might experience lower selective constraints than its F mtDNA counterpart because spermatozoa are the only cells for which selection can act directly and exclusively on the mtDNA genome (Stewart *et al.*, 1996; Garrido-Ramos *et al.*, 1998; Dalziel & Stewart, 2002; Venetis *et al.*, 2006).

In theory, the DUI system allows the M mtDNA to evolve adaptively for male-associated functions since, unlike other animals, male mussels do not represent an evolutionary dead-end for mitochondrial genomes (Zeh, 2004; Zeh & Zeh, 2005; Breton *et al.*, 2007). This hypothesis, which has been addressed in two recent studies (Jha *et al.*, 2007; Breton *et al.*, unpublished results), implies a specialization of the M mtDNA for selectively advantageous male reproductive functionality, such as sperm performance (e.g., increased ATP production could lead to increased swimming speed; Zeh & Zeh, 2005). Specifically, both studies explored whether there are any functional (and ultimately evolutionary) consequences associated with the type of mtDNA contained in mussel sperm. Indeed, male mytilid mussels contain two classes of M mtDNAs; "standard" and "recently-masculinized" M mtDNAs (e.g., Hoeh *et al.*, 1997). The latter are recombinant mtDNAs that still possess standard M mtDNA control regions but F mtDNA sequences for the protein coding and RNA genes. These recombinant molecules have invaded the male route of inheritance and are transmitted paternally from generation to generation as new M mtDNAs (Burzyński *et al.*, 2003; Breton *et al.*, 2006; Burzyński *et al.*, 2006; Venetis *et al.*, 2007). The rationale behind studying sperm performance between sperm with standard M-type and sperm with the recently-masculinized M-type was that the high level of amino acid substitution observed between both mitotypes (i.e., 8-9% Stewart *et al.*, 1996) could affect ATP production by the sperm mitochondria which could, in turn, affect sperm motility (Jha *et al.*, 2007; Breton *et al.*, unpublished results). Whereas Jha *et al.* (2007) compared differences in sperm motility parameters between recently-masculinized and standard M mtDNA bearing-sperm in the

species *Mytilus edulis*, Breton *et al.* (unpublished) measured differences in mitochondrial respiratory chain enzyme activities for gonad samples of the same individuals. Somewhat surprisingly, sperm with the recently-masculinized M-type were associated with faster sperm motility and higher enzymatic activities (with significant values for the complex IV) than sperm with the standard M-type mtDNA (Jha *et al.*, 2007; Breton *et al.*, unpublished results). According to the authors, these observations do not support the hypothesis that DUI permits the evolution of mitochondrial adaptations specific to sperm performance, otherwise, the evolutionarily older, standard M genome should perform better. Alternative hypotheses would be that the paternal lineage in species with DUI might have been retained because of M genome-specific adaptations for male functions other than sperm motility (e.g., sperm longevity) or because of the M genome's participation in sex determination (Saavedra *et al.*, 1997; Zouros, 2000; Breton *et al.*, 2007; Jha *et al.*, 2007; Breton *et al.*, unpublished results). Nevertheless, the key observation from these studies is that the recently-masculinized mtDNA/nuDNA combination (which is essentially an F mtDNA/nuDNA combination) results in faster spermatozoa that are capable of more efficient oxidative phosphorylation than the evolutionarily older "standard" M mtDNA/nuDNA combination. These results suggest that intergenomic coadaptation has been more tightly coupled for the F mtDNA/nuDNA combination and somewhat compromised for the M mtDNA/nuDNA combination.

In this study, we explore intergenomic coadaptation at the molecular level in the DUI-containing mussel species *Mytilus edulis*. Three F and M mtDNA-encoded subunits and three nuclear-encoded interacting proteins of the electron transport system have been examined: the only mtDNA-encoded subunit of the cytochrome *c* reductase or complex III (*CYTB*), two of the three mtDNA-encoded subunits of the cytochrome *c* oxidase or complex IV (*COX1* and *COX2*), the nuDNA-encoded subunit 6 of the complex III (*C3S6*), the nuDNA-encoded protein carrier cytochrome *c* (*CYC*) and the nuDNA-encoded subunit 4 of the complex IV (*COX4*) . We compare these sequences with mitochondrial sequences

(complete F and M mtDNAs; Mizi *et al.*, 2005) and nuclear sequences (expressed sequence tags or ETSs; Venier *et al.*, unpublished data; see below) that have been obtained previously for *M. galloprovincialis*. Consistent with sperm motility and enzymatic data (Jha *et al.*, 2007; Breton *et al.*, unpublished data), our results suggest that the intergenomic co-evolution appears tighter for the F than for the M lineage, supporting the idea that in terms of relative level of selective constraint, the F mtDNA (and thus the interacting nuclear subunits) is more constrained because it has to perform fully in the female gonad and somatic cells of both sexes, while the M mtDNA functions primarily in the male gonad (i.e., in sperm).

3.3 Materials and Methods

3.3.1 Collection and genotyping of specimens

Mytilus edulis mussels were collected in an intertidal sandflat in Ste-Luce, Québec, Canada, in June 2006. These animals were then transported in seawater to the Université du Québec à Rimouski. Spawning was induced by transferring individuals into plastic containers containing ~200 ml filtered sea water at 20°C after having rinsed them in room temperature sea water three times and agitated them in a tub for approximately one minute. Sex was determined after spawning: male gametes are white and cloudy in appearance whereas the female spawn consists of yellow- to orange-colored clumps of eggs. Gamete samples were centrifuged at 3000 *g* for 5 min. at 4 °C and the pellet frozen at -80 °C until used. Samples of gill tissue were also taken from all spawned mussels and frozen at -80 °C for further analyses.

For the determination of "pure" *M. edulis* mussels, whole genomic DNA was extracted from frozen gill tissues using a QIAGEN DNA Extraction Kit (QIAGEN Inc., Mississauga, Canada) following the manufacturer's protocol. Extraction efficiency was examined using agarose gel electrophoresis and DNA was stored at -20 °C. "Pure" *M. edulis* were differentiated from *M. trossulus* or hybrids using a PCR-based assay which targets a ribosomal DNA internal

transcribed spacer (ITS) region (Heath, Rawson & Hilbish, 1995; Dalziel & Stewart, 2002). After digestion with the restriction enzyme *HhaI*, the amplified products from *M. edulis* produced two bands of 450 and 180 bp in length plus some smaller fragments, whereas that from *M. trossulus* produced two bands of 280 and 180 bp as well as many smaller fragments. Hybrids possessed bands of both 450 and 280 bp. Only individuals identified as "pure" *M. edulis* were used for subsequent analyses.

3.3.2 Sequencing of mitochondrial genes

Total RNA was extracted from *M. edulis* male and female gametes using a QIAGEN RNeasy Mini kit (QIAGEN Inc., Mississauga, Canada) following the manufaturer's protocol. Extraction efficiency was examined using agarose gel electrophoresis and RNA was stored at -80 °C. Partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*), cytochrome *c* oxidase subunit II (*cox2*) and cytochrome *b* (*cytb*) were amplified and directly sequenced withouth cloning. The primers for *cox2* and *cytb* were designed from the sequences of the complete F and M *M. edulis* mtDNA molecules sequenced by Boore, Medina & Rosenberg (2004) and Breton *et al.* (2006), respectively. F-specific primers were FcytbF (5'-CCC TGT GGT AGG TGA AAG GA-3') and FcytbR (5'-CCT GCT TTA TGG GGA ATT GA-3') for *cytb* and Fcox2F (5'-CTG TGG TTC CCT TCG ATG AT-3') and Fcox2R (5'-CCT GGG GTT TAC TCC ACA AA-3') for *cox2*. M-specific primers were McytbF (5'-TGA GGA GGG TGA ACT GTG TG-3') and McytbR (5'-AGA GCA GTT GCC CAA GAA AA-3') for *cytb* and Mcox2F (5'-TGG CAG TAG GTG TGT TGG TT-3') and Mcox2R (5'-TAT TGG CAA CCG GTT AAT GC-3') for *cox2*. The *cox1* gene was amplified using the primers LCO1490 and a modified version of HCO2198 that was five bases shorter from the 5' end (i.e.. 5'-TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994; Hoeh, Stewart & Guttman, 2002). All amplifications were performed in 50-μl volumes of a solution containing 10-100 ng RNA, 0.6 μM each primer, 10 μl QIAGEN 5X OneStep RT-PCR buffer, 400

μM each dNTP, and 2 μl QIAGEN OneStep RT-PCR Enzyme Mix (QIAGEN Inc., Mississauga, Canada). We used a UNO II thermocycler (Biometra, Goettingen, Germany) with a cycling profile of an initial reverse transcription at 50 °C for 30 min followed by an initial PCR activation step at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min. The amplified products were then sequenced at the Sequencing Platform, McGill University, Québec.

3.3.3 Sequencing of nuclear genes

The primers for the cytochrome *c* (*cyc*), the nuclear-encoded subunit VI of the mitochondrial complex III (*c3s6*) and the subunit IV of the complex IV (*cox4*) were designed from expressed sequence tags (ESTs) of *M. galloprovincialis* (Venier *et al.*, unpublished data; GenBank accession numbers AJ516895, AJ623449 and AJ624375 for *cyc*, *c3s6* and *cox4* respectively). The primers were: cycF (5'-GTC CCA GTC GAA GGC AAC AGC AA-3') and cycR (5'-ACA ATG CTA CTG ATC AAC AAA ATA C-3') for *cyc*, c3s6F (5'-CGA AGC CAG AAG AAG CCT TAG CA-3') and c3s6R (5'- CTA ACT TAA TAT TGT TTC ATG TC-3') for *c3s6* and cox4F (5'-CAT ATA CTG AAG TCC TCT GCT AG-3') and cox4R (5'-CTA TGA CCT TCT TCT TAT ATT CT-3') for *cox4*. Amplifications were performed as described above on a Biometra (UNOII) or a DNA Engine PCT-200 (MJ Research) thermocycler. The amplified products were sequenced directly at the Sequencing Platform of McGill University, Québec.

3.3.4 Data analyses

All the sequences were edited with the program BioEdit version 7.0.4.1 (Hall, 1999) and alignments were made with Clustal W (Thompson, Higgins & Gibson, 1994) using default parameters. Sequences were characterized after alignment and comparison with data from the National Center for Biotechnology Information

databank. Unique mitochondrial (from each gender) and nuclear sequences have been submitted to GenBank (accession numbers EU332444 to EU332545).

Measures of nucleotide polymorphism and divergence at synonymous and nonsynonymous sites, π values, sliding window analysis, tests of neutrality and amino acid distances were obtained using the DnaSP (version 4.10.0; Rozas *et al.*, 2003) and MEGA (version 3.1; Kumar, Tamura & Nei, 2004) programs. Extra- and transmembrane segments of DNA sequences were identified based on previous studies (Andrews *et al.*, 1998; Wu *et al.*, 2000; Rawson & Burton, 2006) or by using hydrophobicity profiles of protein sequences (Kyte & Doolitlle, 1982). Previously sequenced *M. galloprovincialis* mtDNA genes [F and M *cox1*, *cox2* and *cytb* (GenBank accession numbers AY497292 and AY363687 for the complete F and M mtDNAs respectively; Mizi *et al.*, 2005)] and expressed sequence tags [ESTs *cyc* (GenBank accession numbers AJ624095-AJ624375-AJ624894; Venier *et al.*, unpublished data), *c3s6* (GenBank accession numbers AJ623449-AJ625414-AJ625209-AJ625555-AJ624479; Venier *et al.*, unpublished data) and *cox4* (GenBank accession number AJ516895; Venier *et al.*, unpublished data)] were also used for interspecies comparisons.

Statistical tests of neutrality on the basis of the site frequency spectrum (e.g., Tajima's *D* and Fu and Li's *D*) are known to be sensitive to factors other than selection such as small sample sizes and demographic processes (population bottlenecks and/or migration) (Tajima, 1989; Fu & Li, 1993). Because of our small sample sizes and our lack of knowledge of the history of this population of *M. edulis*, we used the one-tailed Fisher's exact test (MEGA version 3.1; Kumar, Tamura & Nei, 2004) to test the hypothesis of positive selection. This is considered an appropriate test for purifying or positive selection or for neutrality when both the number of sequences and the number of the nucleotide substitutions per sequence are small (Zhang, Kumar & Nei, 1997; Nei & Kumar, 2001). This test is based on a comparison of the numbers of synonymous and nonsynonymous substitutions within F-, M-type or nuclear sequences. If the observed number of synonymous differences per synonymous site exceeds the

number of nonsynonymous differences per nonsynonymous site, then the test sets $P = 1$ to indicate purifying selection, rather than positive selection. If the resulting P-value is less than 0.05, then the null hypothesis of neutral evolution (i.e., strictly neutral and purifying selection) is rejected.

Comparisons between F- and M-type mitochondrial sequences were performed by the McDonald/Kreitman (MK) test (McDonald & Kreitman, 1991) as implemented in DnaSP (version 4.10.0; Rozas *et al.*, 2003). This test is based on the assumption that under neutrality, the ratio of nonsynonymous to synonymous fixed differences between F and M types should be the same as the ratio of nonsynonymous to synonymous polymorphisms within types. The neutrality index value was used to measure the deviation from expected polymorphism and divergence ratios, with NI value greater than 1 consistent with negative selection and NI value less than 1 consistent with positive selection (Rand & Kann, 1996).

Tests for gene conversion (i.e., any process that causes a segment of DNA to be copied onto another segment of DNA) and recombination within and between the F and M genes were also performed using the GENECONV program (version 1.81a; Sawyer, 1989). Given an alignment of DNA or protein sequences, GENECONV finds the most likely candidates for aligned gene conversion events between pairs of sequences in the alignment.

3.4 Results

3.4.1 Mitochondrial genes

Three categories of mtDNA variation were examined for *Mytilus edulis* including (1) intraspecies variation among F genomes (i.e., within-gender variation), (2) intraspecies variation among M genomes (also within-gender variation), and (3) intraspecies variation between M and F genomes (i.e., between-gender variation). A total of 1223 base pairs (bp) of mtDNA were sequenced from

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M. edulis gametes, namely 606 bp of *cox1* (12 females and 11 males, GenBank accession numbers EU332488 – EU332510), 290 bp of *cox2* (12 females and 12 males, GenBank accession numbers EU332511 – EU332534) and 327 bp of *cytb* (12 females and 12 males, GenBank accession numbers EU332464 – EU332487). No obvious recombination events were observed within or between F and M genomes for the genes analyzed in this population of *M. edulis*. The numbers of haplotypes obtained and the levels of polymorphism detected in the F and M mtDNAs are summarized in Table 3.1. As observed in other *Mytilus* species (e.g., Stewart *et al.*, 1996; Quesada, Warren & Skinbinski, 1998; Ort & Pogson 2007), levels of synonymous polymorphism within genders exceeded the levels of nonsynonymous polymorphism. Comparisons between F and M genomes (synonymous, nonsynonymous and total π values) gave contrasting results: while nonsynonymous polymorphisms within the M genome were higher than within the F genome for all analyzed gene portions, synonymous polymorphisms were higher within the F genome than within the M genome for *cox1* and *cytb*. Overall, total π values for mtDNA-encoded genes were higher in the M genome compared to the F genome except for *cox1*. The highest level of polymorphism was observed in the *cox2* gene for both F and M genomes.

Table 3.1
Within-gender nucleotide polymorphism in *Mytilus edulis* F and M mtDNA-encoded *cox1*, *cox2* and *cytb*

Genome, Gene Portion (Number of Haplotypes)	Segregating Sites		π Values			Fisher's Exact Test[b]
	Synonymous	Nonsynonymous	Synonymous	Nonsynonymous	Total	
Cox1 F (10)						
All sites	21	0	0.0474	0	0.0113	1.0000
Extramembrane	7	0	0.0451	0	0.0109	1.0000
Transmembrane	14	0	0.0495	0	0.0116	1.0000
Cox1 M (11)						
All sites	19	2	0.0304	0.0008	0.0079	1.0000
Extramembrane	9	1	0.0376	0.0010	0.0102	1.0000
Transmembrane	10	1	0.0246	0.0007	0.0063	0.9827
Cox2 F[a] (8)	12	0	0.0592	0	0.0133	1.0000
Cox2 M[a] (11)	16	8	0.0890	0.0153	0.0312	0.9386
Cytb F (6)						
All sites	8	1	0.0381	0.0007	0.0088	1.0000
Extramembrane	2	0	0.0334	0	0.0069	1.0000
Transmembrane	6	1	0.0403	0.0010	0.0097	1.0000
Cytb M (9)						
All sites	10	5	0.0258	0.0046	0.0092	0.9668
Extramembrane	3	1	0.0238	0.0022	0.0066	0.9719
Transmembrane	7	4	0.0264	0.0057	0.0103	0.9239
Combined F (1.2 kb)	41	1	0.0472	0.0002	0.0111	1.0000
Combined M (1.2 kb)	41	13	0.0477	0.0044	0.0161	1.0000

NOTE.—Number of haplotypes, numbers of segregating sites and average pairwise divergences between haplotypes [π values for synonymous and nonsynonymous sites, and the combination of both] were calculated using program DnaSP.
[a]The portion sequenced for the *cox2* gene did not contain transmembrane segment.
[b]All comparisons by Fisher's exact test gave non-significant values, indicating purifying selection. Average P-values are reported.

According to hydrophobicity profiles of protein sequences and using bovine *COX1*, *COX2* and *CYTB* for comparisons (GenBank accession number NC_006853 for the complete mitochondrial genome; Chang & Ha, unpublished results), the sequenced portion of *M. edulis cox1* corresponded to the first five interspersed membrane bound and extramembrane regions of the 12 membrane bound segments that compose the entire protein, whereas the sequenced portion of *cytb* corresponded to the fourth, fifth and a part of the sixth transmembrane regions of the 8 transmembrane segments that compose the entire protein (McClellan *et al.*, 2005). The partial sequence of *M. edulis cox2* represented the C-terminal extramembrane domain containing the "heme-patch" and a binding site for copper (Cu_a) (Rawson & Burton 2006). For both F*cox1* and F*cytb* genes, transmembrane sites exhibited higher levels of synonymous and nonsynonymous polymorphisms than extramembrane sites (Table 3.1). This pattern was also observed for the M*cytb* gene but not for M*cox1*, which showed higher levels of synonymous and nonsynonymous polymorphisms in extramembrane sites. Consistent with these results, sliding window profiles (window size = 100 bp, step = 10 bp) of nucleotide polymorphism differed substantially between F and M *cox1-cox2-cytb* combined sequences (fig. 3.1). Except for the *cox2* gene portion (nucleotide position 606-896), the locations of maximum diversity were variable between F and M *cox1* and *cytb*, with regions of maximum diversity situated between nucleotide position ~70-160 and ~270-410 for F*cox1*, ~170-230 for M*cox1*, ~1140-1177 for F*cytb* and ~970-1120 for M*cytb*.

Within-gender comparisons assessed by Fisher's exact test (Table 3.1) revealed that purifying selection against nonsynonymous mutations is acting on these F and M *M. edulis* mitochondrial protein-coding genes. However, that the number of nonsynonymous mutations was consistently higher in the M sequences suggests either relatively relaxed selection on the M mitochondrial genes or stronger purifying selection on the F mitochondrial genes.

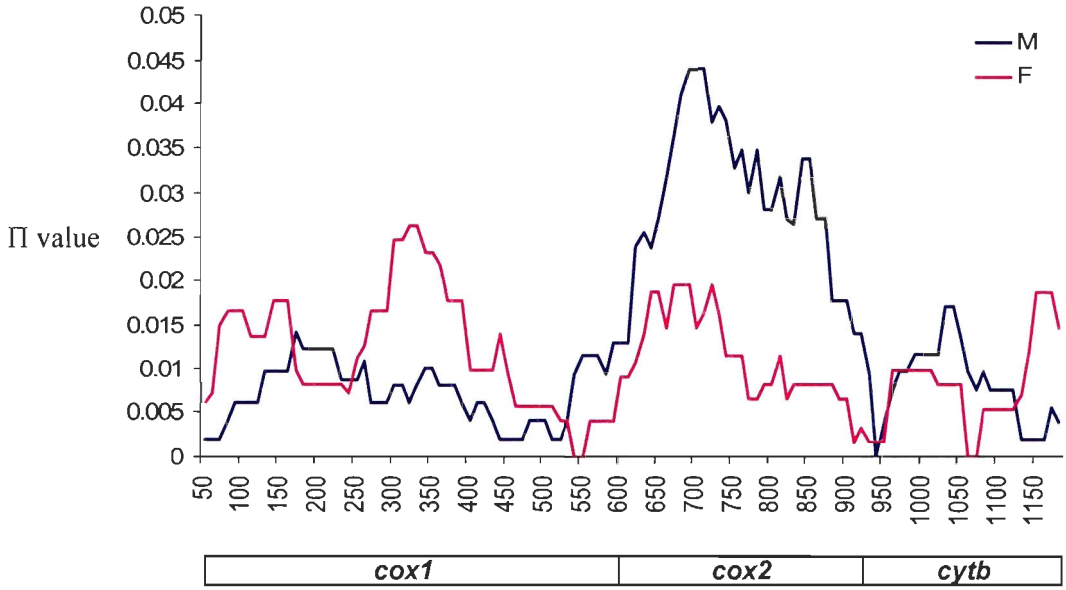

Figure 3.1 Sliding window profiles (100 bp window; 10 bp steps) of *COX1-COX2-CYTB* nucleotide diversity for the M and the F mtDNAs.

Between-gender comparisons by the McDonald and Kreitman's test of neutrality indicated no significant deviation from neutrality for all analyzed gene portions for this population of *M. edulis* (Table 3.2). The nucleotide and amino acid divergences observed between F and M *M. edulis* mitochondrial genes are given in Table 3.3. While the nucleotide divergence for synonymous sites was similar for all genes, the divergence for nonsynonymous sites in *cox2* was considerably higher than for *cox1* or *cytb*; indeed, the D_n/D_s ratio and amino acid distance were twice as high for the *cox2* gene portion analyzed as for *cox1* and *cytb*.

Table 3.2
McDonald and Kreitman test of neutrality between F- and M-type mtDNA sequences

Gene		Substitutions			Neutrality Index (NI)[b]
		Fixed	Polymorphic	Probability[a]	
Cox1					
All sites	Nonsynonymous	8	2	0.727	0.641
	Synonymous	100	39		
Extramembrane	Nonsynonymous	3	1	1.000	0.979
	Synonymous	48	16		
Transmembrane	Nonsynonymous	5	1	0.664	0.452
	Synonymous	52	23		
Cox2	Nonsynonymous	11	8	0.798	1.143
	Synonymous	44	28		
Cytb					
All sites	Nonsynonymous	5	5	0.268	2.556
	Synonymous	48	18		
Extramembrane	Nonsynonymous	0	1	0.446	3.400
	Synonymous	19	5		
Transmembrane	Nonsynonymous	5	4	0.699	1.723
	Synonymous	29	13		
Combined (1.2 kb)	Nonsynonymous	24	15	0.567	1.293
	Synonymous	192	85		

NOTE.— [a]Probability calculated by Fisher's exact test.
[b]Neutrality index is calculated as the ratio of polymorphic to divergent nonsynonymous differences divided by the ratio of polymorphic to divergent synonymous differences.

Table 3.3
Between-gender nucleotide and amino acid divergences in *M. edulis* F and M mtDNA-encoded *cox1*, *cox2* and *cytb*

Comparison	Estimate Type	JC-Corrected Distance (standard error)	D_n/D_s	Amino Acid Distance (standard error)
Cox1 F/M	Synonymous	2.885 (1.174)		
	Nonsynonymous	0.028 (0.009)	0.010	0.077 (0.019)
Cox2 F/M	Synonymous	2.580 (1.000)		
	Nonsynonymous	0.077 (0.021)	0.030	0.179 (0.040)
Cytb F/M	Synonymous	2.202 (0.772)		
	Nonsynonymous	0.033 (0.013)	0.015	0.060 (0.023)

NOTE.—Estimate type refers to synonymous and nonsynonymous category using Nei-Gojobori method (Jukes-Cantor corrected values) as implemented by the program MEGA 3.1. D_n/D_s value is the ratio of nonsynonymous divergence divided by synonymous divergence using JC-corrected values. Amino acid distances were calculated using poisson correction distance (MEGA 3.1).

Alignments of the inferred *COX1*, *COX2* and *CYTB* amino acid sequences of the F and M *M. edulis* mitotypes using bovine *COX1*, *COX2* and *CYTB* as a reference are shown in Figure 3.2. We observed fixed amino acid differences at 14 of the 202 (6.9%) residues between F and M *M. edulis COX1*; 11 (5.4%) of these fixed amino acid replacements occurred in the transmembrane portions of the protein, whereas only 4 amino acid substitutions (2.0%) were found in the extramembrane domains. One substitution in the extramembrane domains involved a residue that may interact with the nuclear-encoded *CYC* (fig. 3.2).

The *COX2* gene showed extensive intergender sequence divergence at the amino acid level; fixed replacement substitutions were found at 11 of the 96 (11.5%) residues. Four substitutions involved residues that may interact with the nuclear-encoded *CYC* protein (one fixed substitution between F and M *COX2* and three polymorphic substitutions within the M lineage). In contrast to the *COX2* protein, fixed amino acid differences were observed at only 4 of the 108 (3.7%) residues between F and M *M. edulis CYTB*; two of these substitutions were found in the transmembrane domains whereas the two other substitutions appear to be found in the extramembrane domains. Amino acid polymorphism within both F

and M lineages were principally found in the extramembrane domain regions (fig. 3.2).

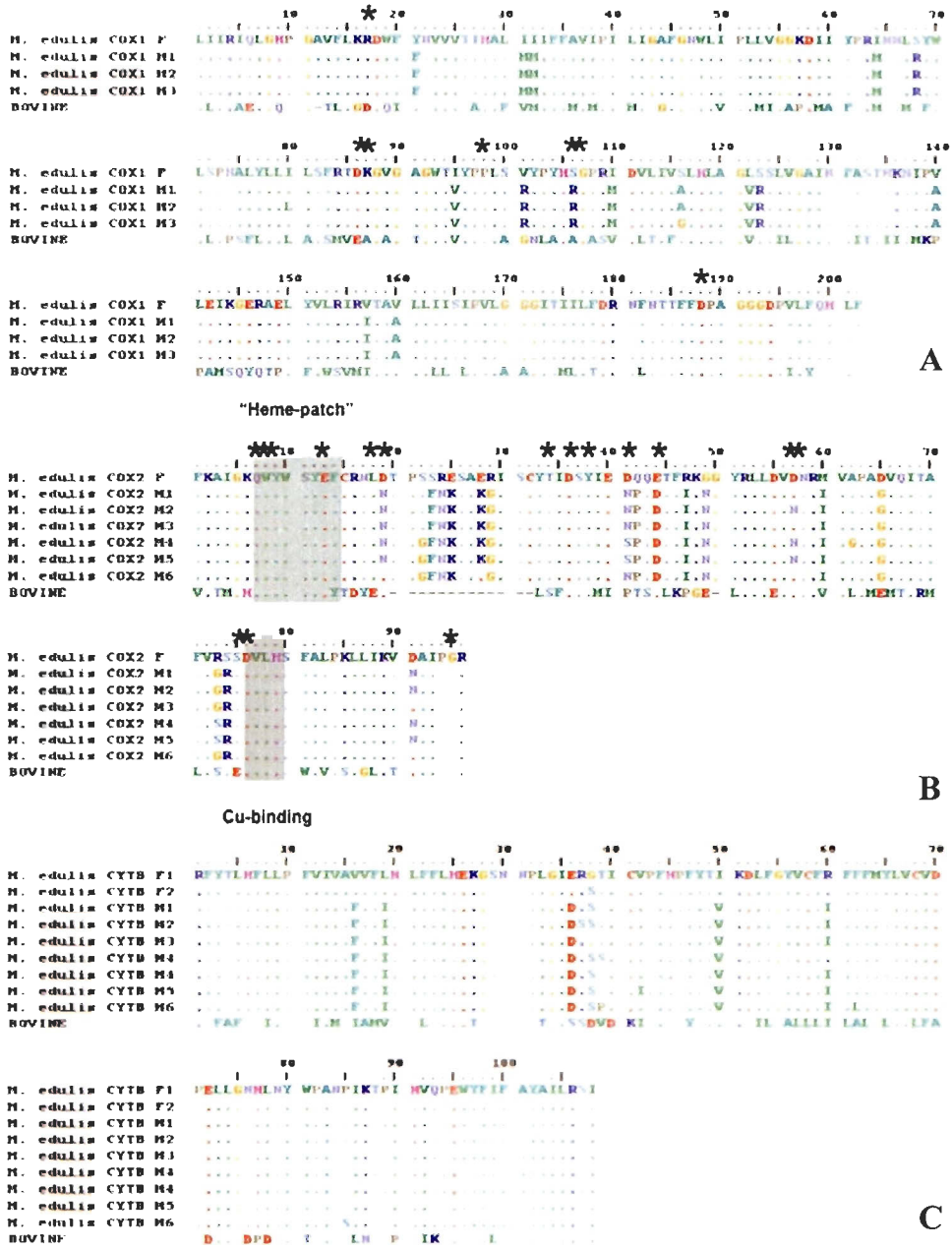

Figure 3.2 *COX1* (**A**), *COX2* (**B**) and *CYTB* (**C**) amino acid sequences for F and M mtDNAs. For comparison the sequences for bovine *COX1*, *COX2* and *CYTB* are also included in the alignments. The relative positions of the transmembrane domains for *COX1* and *CYTB* are shown highlighted in pale grey. The relative positions of the "heme-patch" and Cu-binding residues for *COX2* are shown highlighted in grey. Residues at the binding site of *CYC* are indicated with a star.

3.4.2 Nuclear genes

We obtained sequences for three *M. edulis* nuclear genes encoding proteins of the mitochondrial electron transport system: (1) 361 bp of the *cyc* gene (n = 25, GenBank accession nos. EU332542 - EU332545), which code for the cytochrome *c* protein carrier that connects the mitochondrial complexes III and IV for the transfer of electrons, (2) 420 bp of the *c3s6* gene (n = 30, accession nos. EU332444 -EU332463), which code for the subunit 6 of the mitochondrial complex III (cytochrome *c* reductase) and (3) 418 bp of the *cox4* gene (n = 8, accession nos. EU332535 - EU332541), which code for the subunit IV of the mitochondrial complex IV (cytochrome *c* oxidase). In BLAST searches the inferred amino acid sequences for the three genes showed high similarity to *CYC*, *C3S6* and *COX4* of other species. The *M. edulis CYC* protein had the highest similarity (81-85%) to *CYC* sequences from insect species, including the wasp (*Nasonia vitripennis*; accession no. XP_001606272) and the fruit fly *Drosophila melanogaster* (NP_477176), and a lower degree of similarity (73%) to human *CYC* sequence (NP_061820). For comparison, the *M. edulis C3S6* gene had 56% and 44% similarity to *C3S6* sequences from *D. melanogaster* (NP_573110) and human (NP_006285) respectively, whereas the *M. edulis COX4* gene had 44% and 35% similarity to *COX4* sequences from the same species (NP_995732 and NP_001852 for *D. melanogaster* and human, respectively). From these observations, the *CYC* protein appeared to be the most conserved and the *COX4* protein the least conserved. No replacement substitutions were found within *M. edulis CYC* whereas amino acid differences were found at 6 of the 139 (4.3%) residues within *COX4* and at one of the 139 (0.72%) residues within *C3S6* (data not shown).

Nucleotide polymorphism was examined for the nuclear genes to compare levels of polymorphism with mtDNA genes for this population of *M. edulis*. As for mitochondrial genes, nonsynonymous polymorphisms were relatively rare in nuclear proteins, being considerably higher in *cox4* than in *c3s6* and *cyc* (Table 3.4). Synonymous sites showed high levels of polymorphism in *c3s6* and *cox4*

compared to *cyc*. Fisher's exact test (Table 3.4) was not significant for all analyzed nuclear genes, suggesting that selection against nonsynonymous mutations (i.e., purifying selection) is working within these sequences.

For all nuclear and mitochondrial genes surveyed, the lowest levels of nonsynonymous polymorphism were found in *cyc*, F*cox1* and F*cox2* (i.e., no nonsynonymous polymorphism), and the highest levels of nonsynonymous polymorphism were found in *cox4* and M*cox2* (nonsynonymous π values of 0.863 and 0.890 respectively) (Tables 3.1 and 3.4). Levels of synonymous polymorphism for *c3s6* and *cox4* were similar to those observed for M and F mtDNA-encoded *cox2* genes, respectively (i.e., the highest levels of synonymous polymorphism among the mitochondrial genes analyzed). *Cyc* showed the lowest levels of synonymous polymorphism among all surveyed genes. Overall, the average rate of synonymous substitution of the three nuclear genes was comparable with the average for the three F or M mtDNA genes. In contrast, the average rate of nonsynonymous substitution of the three nuclear genes was 8-fold higher than the average for the three F mtDNA genes and 3.6-fold lower than the average for the three M mtDNA genes (Tables 3.1 and 3.4).

Table 3.4
Nucleotide polymorphism in *Mytilus edulis* nuclear-encoded *cyc*, *c3s6* and *cox4*

Gene	Number of Haplotypes	Segregating Sites		π Values			Fisher's Exact Test[a]
		Synonymous	Nonsynonymous	Synonymous	Nonsynonymous	Total	
Cyc	4	3	0	0.0056	0.0000	0.0012	1.0000
C3s6	20	22	1	0.0863	0.0002	0.0174	1.0000
Cox4	7	13	6	0.0535	0.0046	0.0148	1.0000

NOTE.—Number of haplotypes, numbers of segregating sites and average pairwise divergences between haplotypes [π values for synonymous and nonsynonymous sites, and the combination of both] were calculated using program DnaSP.
[a]All comparisons by Fisher's exact test gave non-significant values, indicating purifying selection. Average P-values are reported.

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3.4.3 Divergence between *M. edulis* and *M. galloprovincialis*

Table 3.5 gives the levels of nucleotide divergence for synonymous and nonsynonymous sites and the amino acid distances observed between *M. edulis* and *M. galloprovincialis* for all genes analyzed. Both nucleotide divergences and amino acid distances were higher for the M genome among the mitochondrial genes surveyed, reinforcing previous observations that the M genome is under relatively relaxed selection compared to the F genome. For the three nuclear-encoded genes, the divergence values for synonymous substitutions were 1.7-fold lower than those observed for the M mtDNA genes and 1.5-fold higher than those observed for the F mtDNA genes. The divergence values for nonsynonymous substitutions were 2.8-fold lower for nuclear genes than for M mtDNA genes and 9.1-fold higher for nuclear genes than for F mtDNA genes. On average, amino acid distances for these three nuclear genes were also lower than those observed for the M mtDNA genes and higher than those observed for the F mtDNA genes.

Table 3.5
Genetic divergence between *Mytilus edulis* and *M. galloprovincialis* mitochondrial and nuclear genes

Comparison	Estimate Type	JC-Corrected Distance (standard error)	D_n/D_s	Amino Acid Distance (standard error)
Cox1 F	Synonymous	0.052 (0.011)		
	Nonsynonymous	0.000 (0.000)	0.000	0.000 (0.000)
Cox1 M	Synonymous	0.111 (0.015)		
	Nonsynonymous	0.002 (0.001)	0.018	0.007 (0.002)
Cox2 F	Synonymous	0.058 (0.020)		
	Nonsynonymous	0.000 (0.000)	0.000	0.000 (0.000)
Cox2 M	Synonymous	0.150 (0.034)		
	Nonsynonymous	0.017 (0.005)	0.113	0.044 (0.013)
Cytb F	Synonymous	0.040 (0.015)		
	Nonsynonymous	0.001 (0.001)	0.025	0.001 (0.001)
Cytb M	Synonymous	0.109 (0.022)		
	Nonsynonymous	0.006 (0.002)	0.055	0.017 (0.006)
Cyc	Synonymous	0.054 (0.024)		
	Nonsynonymous	0.000 (0.000)	0.000	0.000 (0.000)
C3s6	Synonymous	0.123 (0.030)		
	Nonsynonymous	0.003 (0.003)	0.024	0.001 (0.001)
Cox4	Synonymous	0.045 (0.014)		
	Nonsynonymous	0.006 (0.002)	0.133	0.015 (0.005)

NOTE.—Estimate Type refers to synonymous and nonsynonymous category using Nei-Gojobori method (Jukes-Cantor corrected values) as implemented by the program MEGA 3.1. D_n/D_s value is the ratio of nonsynonymous divergence divided by synonymous divergence using JC-corrected values. Amino acid distances were calculated using poisson correction distance (MEGA 3.1).

3.5 Discussion

Several studies have examined the molecular evolution of gender-associated mtDNAs in bivalve species with DUI (see Breton *et al.*, 2007 for a review). The current study extends this research by investigating patterns of co-evolution of the nuclear and mitochondrial genes (both F and M mtDNA) in *M. edulis* that interact to produce the mitochondrial electron transport system (ETS). Specifically, three

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homologous gene regions of the F and M mtDNAs (*cox1*, *cox2* and *cytb*) and three protein-coding regions of the nuclear DNA (*cyc*, *c3s6* and *cox4*) were investigated for a total of 1,223 bp and 1,199 bp respectively. Overall, our results indicated that the average levels of synonymous polymorphism within the population of *M. edulis* were roughly equal for the three sets of genes (i.e., the F and M mtDNA genes and nuclear genes), whereas average levels of nonsynonymous polymorphism were ranked as follows: F mtDNA genes < nuclear genes < M mtDNA genes (i.e., the average level of nonsynonymous polymorphism for nuclear genes fell between those seen for F mtDNA genes and M mtDNA genes). There was, however, considerable variation in levels of polymorphism among individual genes. For example, the highest synonymous, nonsynonymous and total π values were observed for the M mtDNA-encoded *cox2* gene and the lowest values for these parameters were observed for the nuclear-encoded *cyc* gene, respectively.

Previous genetic analyses indicated that on average, mitochondrial DNA genomes have a higher mutation rate than nuclear DNA genomes (see Ballard & Whitlock, 2004 and Korpelainen, 2004 for reviews). However, more detailed examination of patterns of nucleotide polymorphism and divergences within and between some populations and/or species has demonstrated contrasting results (Ballard, 2000b; Betancourt, Presgraves & Swanson, 2002; Willett & Burton, 2004). For example, both synonymous and nonsynonymous substitution rates appeared to be higher for mtDNA-encoded *cox1*, *cox2* and *cytb* than for three nuclear-encoded ETS proteins in the copepod *T. californicus* (Willett & Burton, 2004). In contrast, comparisons of *cox1*, *cox2* and *cytb* with several different nuclear genes for different populations of *D. melanogaster* and *D. simulans* showed higher synonymous rates of substitution but lower nonsynonymous rates for the mtDNA genes (Ballard 2000b; Betancourt, Presgraves & Swanson, 2002). For comparative purpose, we examined nucleotide divergences (synonymous and nonsynonymous) and amino acid distances between *M. edulis* and *M. galloprovincialis* for F and M mtDNA genes and for nuclear genes for

comparison with observed levels of polymorphism within this *M. edulis* population for the same genes. The average levels of synonymous divergence between *M. edulis* and *M. galloprovincialis* suggested higher substitution rates for nuclear genes than for F mtDNA genes and lower substitution rates for nuclear genes than for M mtDNA genes. This pattern was also observed for nonsynonymous divergences and amino acid distances. However, these values varied considerably among genes with the highest divergences observed for the M mtDNA-encoded *cox2* gene and the lowest divergences observed for the nuclearly-encoded *cyc* gene and the F mtDNA-encoded *cox1* gene.

It has been proposed that because of the important role of the mtDNA-encoded subunits in the ETS, a higher mutation rate for mtDNA compared to nuDNA can only explain a rapid rate of amino acid replacement if the fixed substitutions behave neutrally (Willett & Burton, 2004). Alternatively, environmental selection on the ETS could be responsible for increased amino acid divergence in mtDNA-encoded proteins (Willett & Burton, 2004). Our results showed that the M mtDNA-encoded genes evolved more rapidly than the nuclear genes and the F mtDNA-encoded genes, but Fisher's exact test did not provide evidence for positive selection on M mtDNA. Rather, the test supported purifying selection on both F and M *M. edulis* sequences, although purifying selection was stronger for the F mtDNA. Furthermore, the results of the McDonald and Kreitman test suggest that the F and M mtDNA-encoded genes have diverged in a neutral way, supporting earlier reports on species with DUI (Quesada, Warren & Skibinski, 1998; Riginos *et al.*, 2004; Passmonti, 2007), but contrasting other studies, which rejected neutral evolution of gender-associated mtDNAs (Stewart *et al.*, 1996; Quesada, Warren & Skibinski, 1998; Passamonti, Boore & Scali, 2003). For example, positive selection was inferred to have occurred in the marine clam *Tapes philippinarum* based on the observation of fixed replacement mutations in 6 mitochondrial genes between the F and M mtDNAs in this species (Passamonti, Boore & Scali, 2003). These results were interpreted as

evidence for different selective pressures acting on the F and M mtDNAs that reflect their specialized gender-associated roles (Passamonti, Boore & Scali, 2003). However, it has to be noted that population bottlenecks (i.e., accelerated fixation of midly deleterious replacement mutations in small populations that could explain a greater number of fixed amino acid replacements than expected) or recent population expansions (i.e., increased efficacy of purifying selection in growing populations that could explain a lower number of replacement mutations than expected) may affect the reliability of this test and thus account for constrasting results when applying the test to different populations or species (Eyre-Walker, 2002; Passamonti, 2007).

A recent study of complete F and M *Mytilus* mtDNA genomes demonstrated that there was a high degree of correlation in the pattern of amino acid variability between these two gender associated genomes (i.e., regions with high or low amino acid variability were similar in both M and F genomes; Breton *et al.*, 2006). This was interpreted as evidence of co-evolution between nuclear and mitochondrial genomes. The requirement of functioning in the same nuclear background has apparently forced the various genes of the respective F and M mitochondrial genomes to experience similar selective pressures. That said, by studying only amino acid changes one may overlook unequal substitution patterns since nucleotide differences within populations or between closely-related populations or species consist predominantly of synonymous third codon position changes. Mutation hot spots as well as adaptive substitutions are documented for mtDNA and these regions can cause heterogeneous evolutionary rates across genes (Roe & Sperling, 2007). Consequently, to visualize patterns of nucleotide polymorphism throughout *cox1-cox2-cytb*, we used a "parallel" sliding window analysis to detect heterogeneneity in patterns of molecular evolution between the F and M mtDNA genomes. Interestingly, discrepancies in the locations of nucleotide polymorphism peaks occurred throughout the *cox1* and the *cytb* gene portions analyzed. Moreover, differences between F and M *cox1* were also found when polymorphism patterns were examined for transmembrane versus

extramembrane sites. While transmembrane sites exhibited higher levels of synonymous polymorphism than extramembrane sites in F*cox1* (in fact no nonsynonymous polymorphisms were observed), M*cox1* showed higher levels of synonymous and nonsynonymous polymorphisms in extramembrane sites. Although additional analyses such as comparative genomics of F and M mtDNA genes in several populations will be needed to clarify regional nucleotide variation in *M. edulis* F and M mtDNAs, these results suggest the possibility of gender-specific selective constraints associated with the different physiological demands of the different tissues occupied by the F or M mtDNA.

The primary motivation of this study was to explore the nature of intergenomic coadaptation in a species with DUI at the molecular level. Consistent with previous genetic analyses (Stewart *et al.*, 1996; Breton *et al.*, 2006), several amino acid substitutions were observed between F and M mitochondrial genomes. These substitutions were not evenly distributed across genes. While most of the fixed (and polymorphic) substitutions between F*COX1* and M*COX1* were observed in the transmembrane regions of the protein, the transmembrane and extramembrane domains of the F*CYTB* and M*CYTB* proteins presented the same number of fixed replacement substitutions (i.e., two in each domains). Interestingly, one fixed amino acid replacement found in an extramembrane domain of the *COX1* protein (i.e., a non-conservative change F*COX1*-Ser to M*COX1*-Arg) occurred at a site that interacts directly with the nuclear-encoded cytochrome *c*. Similarly, one fixed amino acid replacement between F*COX2* and M*COX2* (i.e., a conservative F*COX2*-Glu to M*COX2*-Asp) also involved an amino acid residue that appears to interact with the cytochrome *c* protein. Since mitochondrial complex assembly has been shown to be very sensitive to small changes in amino acid sequences not necessarily involved in the catalytic function (i.e., substitutions that could modify the interaction between nuclear and mitochondrial subunits at positions of structural importance to enzyme function), all these changes could potentially have functional significance (e.g.,

Barrientos *et al.*, 2000). Because of the important amino acid divergence between F and M mtDNAs, one might expect that nuclear genes that interact closely with mtDNA-encoded protein subunits would also show important amino acid divergences. However, very low (or no) levels of amino acid variation were found in the *CYC* and *C3S6* proteins, a result similar to those observed for the F mtDNA-encoded proteins. The *COX4* protein appeared to be the least conserved of the nuclear genes analyzed, with amino acid variation comparable (or somewhat lower) to the results seen for the M mtDNA-encoded proteins. No evidence for positive selection was found for the *M. edulis* nuclear proteins analyzed.

Previous studies indicated that cytochrome *c* is among the most evolutionarily conserved proteins studied in animal species to date (Rawson, Brazeau & Burton, 2000). In contrast, *COX4* is the least conserved of the nuclear-encoded subunits of the mitochondrial cytochrome *c* oxidase in mammals (Das, Miller & Stern, 2004). To our knowledge, little is known about the molecular evolution (and function) of the subunit 6 of the mitochondrial cytochrome *c* reductase complex (Grossman *et al.*, 2001). Althought our results for *CYC* and *COX4* appear to be consistent with previous observations, rates of evolution observed for the *M. edulis* nuclear-encoded proteins are nevertheless difficult to compare with closely-related invertebrate species with DUI (or even without DUI) because homologous sequences are not available for these taxa. For example, comparisons of nucleotide polymorphism within population for *M. edulis* and the copepod *Tigriopus californicus cyc* genes (Willett & Burton, 2004) suggest that synonymous sites in *M. edulis cyc* have a 2.4-fold lower neutral mutation rate than *T. californicus* (note: no nonsynonymous polymorphisms were observed within populations of either species). Moreover, the nucleotide divergence observed for *cyc* between *M. edulis* and *M. galloprovincialis* was considerably lower than the divergences observed between *T. californicus* populations. Contrary to our results, evidence for positive selection was found for the copepod *CYC* protein (Rawson, Brazeau & Burton, 2000; Willett & Burton, 2004). This was interpreted as

evidence for intergenomic co-evolution with the rapidly evolving interacting mtDNA-encoded genes in this species, although this rapid evolution in mitochondrial subunits was not always associated with rapid divergence in interacting nuclear subunits (e.g., subunits *CYC1* and *RISP* of the mitochondrial complex III) (Rawson, Brazeau & Burton, 2000; Willett & Burton, 2004; Rawson & Burton, 2006).

Our results, combined with previous sperm motility and enzymatic data (Jha *et al.*, 2007; Breton *et al.*, unpublished data), suggest tighter coadaptation between the F mtDNA and the nuclear genome than between the M mtDNA and the nuclear genome. Levels of nucleotide polymorphism and divergence at nonsynonymous sites for nuclear genes, except *COX4*, are roughly equal to those observed for the F mtDNA genes. This suggests coordinated purifying selection on these nuclear genes and F mtDNA imposed by strong functional constraints. In contrast, levels of nonsynonymous polymorphism and amino acid divergence observed for *COX4* fell between those observed for F*COX2* and M*COX2*. These results are interesting because there is evidence that this nuclear subunit is a major regulator of the cytochrome *c* oxidase enzyme, suggesting that functional adaptations of the *COX* enzyme in *M. edulis* could be focused on regulation of oxidative phosphorylation by *COX4* (Wu *et al.*, 2000; Grossman *et al.*, 2001).

At present, a reduction in selective constraint acting on the M mtDNA remains the finest explanation for its great levels of polymorphism and faster rate of evolution in species with DUI (Stewart *et al.*, 1996; Quesada, Warren & Skibinski, 1998; Ort & Pogson, 2007; present study). We suggest that the leading hypothesis of unequal "division of labour" in the DUI system for explaining the faster evolution of the M mtDNA compared to the F mtDNA (Stewart *et al.*, 1996) can also be applied to the DUI intergenomic co-evolution. Indeed, because the F genome as to function in gonads and soma of both sexes, whereas the male genome serves primilary in the male gonad, the natural selection has favoured the evolutionary coadaptation between

the nuclear and the F mitochondrial genomes. Enlarging comparative DUI-containing species sequence data to include other mitochondrial and nuclear subunits of complex III and IV, and also subunits of other complexes will allow us to understand the nature of the forces that have brought about the levels of variation in interacting proteins in species with DUI.

3.6 Acknowledgements

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CONCLUSION GÉNÉRALE

Dans le règne animal, l'ADN mitochondrial est transmis de façon maternelle. La double transmission uniparentale observée chez certaines espèces de bivalves est le seul système mitochondrial animal connu qui échappe à cette règle. L'objectif principal du présent projet de recherche était d'étudier l'évolution moléculaire des génomes mitochondriaux F et M chez les espèces du genre *Mytilus* et de vérifier si les gènes nucléaires et les gènes F et M mitochondriaux qui interagissent pour former le système de la phosphorylation oxydative évoluent de façon concertée (co-évolution). Globalement, nous avons vu que de par son unicité, le système des moules constitue non seulement un excellent modèle pour étudier la co-évolution des génomes nucléaire et mitochondriaux, mais il pourrait également permettre de mieux comprendre les mécanismes impliqués dans la transmission et le développement de certaines maladies associées à la divergence de l'ADNmt des mitochondries. Suite à notre analyse des ADNmt F et M complets chez trois espèces de moules marines (*Mytilus edulis*, *M. trossulus*, et *M. galloprovincialis*) (Chapitre 1), nous avons démontré que les divergences nucléotidiques (et protéiniques) observées entre les ADNmt F et M chez les mâles sont les valeurs «intra-individuelles» les plus élevées jamais rapportées chez les animaux. Ainsi, pour l'ensemble des sous-unités mitochondriales, les divergences mesurées entre les ADNmt F et M sont plus importantes ou quasi équivalentes aux divergences mesurées chez les modèles classiques utilisés pour l'étude de la co-évolution des génomes nucléaire et mitochondrial et pour lesquels une perturbation de l'activité des mitochondries a été observée (fig. 4.1) (Breton *et al.*, 2006; Breton *et al.*, 2007). L'analyse des séquences mitochondriales complètes nous a également permis de vérifier si l'évolution des ADNmt F et M dans un environnement nucléaire commun (ou différent) se traduisait par la conservation (ou non) de certaines de leurs portions (c'est-à-dire une évolution

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congruente des ADNmt F et M). L'étude du génome mitochondrial de type récemment-masculinisé a aidé à l'identification des régions nucléotidiques mitochondriales anciennement de type F susceptibles d'être les premières à présenter des adaptations spécifiques de type M suite à une masculinisation (renversement des rôles). En outre, la séquence mitochondriale complète de l'ADNmt récemment-masculinisé a permis de cibler les régions nucléotidiques qui pourraient être responsables des différentes transmissions de l'ADNmt chez les Mytilidae (c'est-à-dire transmission maternelle ou paternelle).

Il est important de rappeler qu'afin de répondre aux objectifs fixés dans le premier chapitre, deux ADNmt M complets de l'espèce *M. edulis* et un ADNmt de type récemment-masculinisé de l'espèce *M. trossulus* ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique dans le laboratoire du Dr. Gertraud Burger (Centre Robert-Cedergren en Bioinformatique & Génomique, Université de Montréal). Leurs séquences nucléotidiques et protéiniques ont été comparées à celles de l'ADNmt F complet de l'espèce *M. edulis* (Hoffmann, Boore et Brown, 1992) et des ADNmt F et M complets de l'espèce *M. galloprovincialis* (Mizi *et al.*, 2005) déjà disponibles dans la base de données moléculaires «GenBank».

4.1 Principaux résultats obtenus pour le chapitre 1

► L'analyse comparative des ADN mitochondriaux F et M complets chez les espèces du genre *Mytilus* a révélé qu'ils sont identiques au niveau de l'ordre (l'arrangement) des gènes, mais qu'ils sont hautement divergents au niveau des séquences nucléotidiques et protéiniques (voir fig. 4.1).

► Plusieurs des substitutions nucléotidiques observées entre les ADNmt F et M dans les régions codant pour les protéines des complexes mitochondriaux impliquent des changements d'acides aminés. Ces changements devraient entraîner des

modifications des propriétés structurales et fonctionnelles de la chaîne respiratoire mitochondriale.

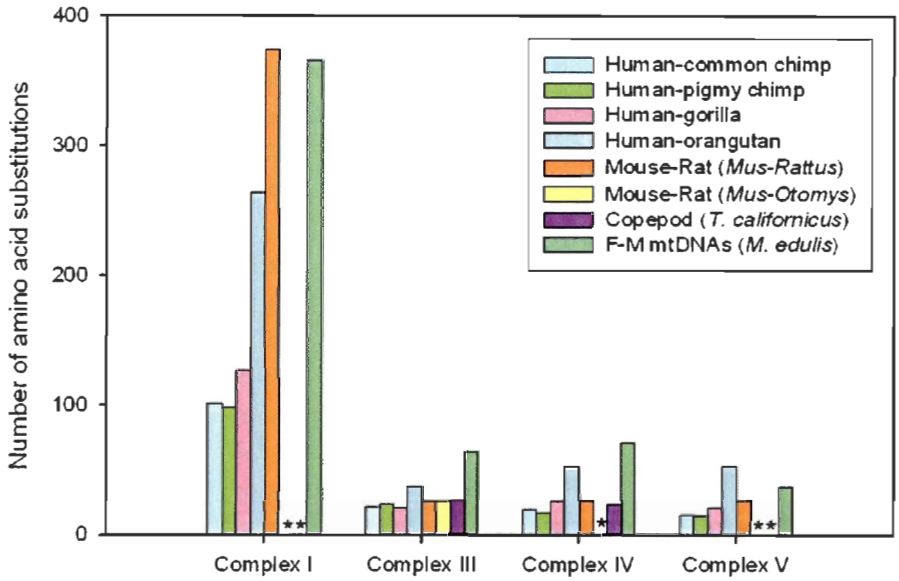

Figure 4.1 Divergences mitochondriales mesurées chez les modèles d'étude de la co-évolution. Les différences entre les séquences protéiniques sont présentées pour les modèles pour lesquels une disruption de l'activité mitochondriale a été observée. Les cybrides humain-chimpanzé et humain-gorille avaient une consommation en O_2 diminuée de 20%, 34% et 27% respectivement, en comparaison au groupe contrôle humain-humain. Ces différences étaient attribuées à une déficience dans l'activité des complexes de la chaîne respiratoire (Kenyon et Moraes, 1997; Barrientos, Kenyon et Moraes, 1998). L'activité enzymatique des complexes I, III et IV chez le cybride souris-rat (*Mus-Rattus*) était à 44%, 37% et 78% respectivement, en comparaison au gr. contrôle souris-souris. L'activité du complexe III chez le cybride souris-rat (*Mus-Otomys*) était à 2% en comparaison au gr. contrôle (McKenzie et Trounce, 2000; McKenzie *et al.*, 2003). L'activité du complexe IV était fortement diminuée chez les hybrides copépodes issus de «backcrosses» (Burton, Rawson et Edmands, 1999; Ellison et Burton, 2006). *Données non disponibles. (Tirée de Breton *et al.*, 2007.)

► La comparaison des taux de substitution nucléotidiques des différents gènes F et M indique que le génome M évolue plus rapidement que le génome F, supportant

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l'hypothèse qu'il expérimente de plus faibles contraintes sélectives que celles agissant sur le génome F. Il est proposé que cette différence dans l'intensité des contraintes sélectives est due à l'inégalité des tâches accomplies par les génomes F et M [c'est-à-dire la fonction obligée de l'ADNmt F dans la gonade femelle et toutes les cellules somatiques comparativement à celle de l'ADNmt M dans les spermatozoïdes seulement] (Stewart *et al.*, 1996).

► Les taux de substitution protéiniques des différents gènes F et M sont hautement corrélés, et ce, que ce soit pour les gènes F et M d'un même environnement nucléaire ou d'un environnement nucléaire différent. Ces résultats suggèrent que les contraintes structurales ou fonctionnelles sont spécifiques aux gènes plutôt qu'aux espèces.

► L'évolution congruente des génomes F et M (c'est-à-dire la corrélation positive des patrons de taux de substitution protéiniques) d'une même espèce indique qu'ils expérimentent des pressions sélectives similaires (mais de différentes intensités, voir ci-haut) qui sont en partie attribuables à une co-existence dans un environnement nucléaire commun. Ces pressions sélectives similaires pourraient entraîner la rétention d'acides aminés particuliers (identiques ou fonctionnellement équivalents) dans les ADNmt F et M à des positions structurales importantes pour les fonctions enzymatiques.

► Les premières régions d'un ADNmt de type récemment-masculinisé les plus susceptibles de présenter des adaptations spécifiques de type M suite à une masculinisation (renversement des rôles) sont celles codant pour les protéines du complexe I puisqu'il s'agit généralement des gènes qui évoluent le plus rapidement chez les Mytilidae.

► La région non-codante ou «région contrôle» qui contient les éléments nécessaires à la réplication et la transcription de l'ADNmt de type récemment-

masculinisé est une portion recombinante qui présente une «région contrôle» de type F et une «région contrôle» de type M. Étant le seul élément différent (ou élément de surplus) entre un ADNmt F standard et un ADNmt de type récemment-masculinisé, cette incorporation d'une «région contrôle» de type M dans une molécule de type F pourrait être responsable de la transmission paternelle d'un molécule autrefois transmise maternellement.

Une des hypothèses proposée pour expliquer l'origine et le maintien du système de la double transmission uniparentale de l'ADNmt chez certains bivalves au cours de l'histoire évolutive est que la lignée mitochondriale paternelle est maintenue car elle permet une adaptation de l'ADNmt M pour des fonctions spécifiques aux spermatozoïdes (par exemple, la motilité) (Zouros, 2000; Zeh & Zeh, 2005; Breton *et al.*, 2007). Afin de vérifier cette hypothèse, nous avons étudié le phénomène de masculinisation ou renversement des rôles qui survient chez la moule bleue *Mytilus edulis*. Tel que présenté précédemment, certains individus mâles de cette espèce possèdent un ADNmt F recombinant (c'est-à-dire qui possède une origine de réplication mâle mais une séquence codante de type F) dans leur gonade qui se transmet de père en fils par les spermatozoïdes comme un ADNmt M standard. En comparant la vitesse de nage et l'activité des enzymes mitochondriales des spermatozoïdes contenant un ADNmt M versus ceux contenant un ADNmt F recombinant (ou de type récemment-masculinisé), Jha *et al.*, (2007) ont démontré que les spermatozoïdes associés à l'ADNmt hypothétiquement adapté pour les fonctions spermatiques (l'ADNmt M standard) possédaient une motilité réduite comparativement aux spermatozoïdes associés à l'ADNmt de type récemment-masculinisé. L'objectif du chapitre 2 était de déterminer, chez les spermatozoïdes (échantillons de gonades) de la moule *M. edulis*, si le remplacement d'un ADNmt M par un ADNmt F (le phénomène de renversement des rôles) avait également un effet sur les capacités métaboliques des mitochondries. Pour ce faire, des dosages

enzymatiques (activité des complexes mitochondriaux I+III, II et IV et de la citrate synthase) ont été réalisés par spectrophotométrie dans le laboratoire du Dr. Pierre Blier (UQAR).

4.2 Principaux résultats obtenus pour le chapitre 2

► Une augmentation significative de l'activité enzymatique de la cytochrome *c* oxydase (complexe IV) a été observée pour les gonades contenant les spermatozoïdes associés à un ADNmt de type récemment-masculinisé tandis que tous les autres paramètres (c'est-à-dire la taille des coquilles, la masse des gonades, le contenu en protéines et l'activité enzymatique des complexes I+III et II et de la citrate synthase) ne présentaient pas de différences significatives entre les gonades contenant des spermatozoïdes de type récemment-masculinisés et M standard.

► Les ratios des activités des complexes I+III, II et IV sur l'activité de la citrate synthase (CS) étaient plus élevés pour les gonades contenant des spermatozoïdes de type récemment-masculinisées, avec une différence significative pour le ratio de l'activité du complexe IV/CS (la citrate synthase est utilisée comme un marqueur reflétant le nombre et/ou le volume des mitochondries, ce qui permet de normaliser les activités enzymatiques par rapport au contenu en mitochondries).

► Les différences importantes au niveau des séquences protéiniques (~8%) observées entre les ADNmt M standard et de type récemment-masculinisé pourraient affecter directement les activités enzymatiques et/ou l'assemblage des complexes de la chaîne respiratoire et donc expliquer les activités enzymatiques supérieures obtenues pour les échantillons de gonades contenant les spermatozoïdes associés à un ADNmt de type récemment-masculinisé.

► Une hypothèse alternative mais non exclusive est que les différences au niveau des séquences nucléotidiques des «régions contrôle» de la réplication et/ou des ARN

ribosomaux et ARN de transfert observées entre les ADNmt M standard et de type récemment-masculinisé pourraient entraîner des différences au niveau de la réplication, de la transcription et de l'expression de l'ADNmt. Une telle situation pourrait entraîner un enrichissement des complexes protéiques de la chaîne respiratoire (avec une expression coordonnée des ADNmt et ADNnu) et conséquemment des activités enzymatiques supérieures pour les échantillons de gonades contenant les spermatozoïdes associés à un ADNmt de type récemment-masculinisé.

► Bien que ces résultats ne réfutent pas l'hypothèse d'une adaptation de l'ADNmt M pour des fonctions mâles autres que la motilité du sperme (par exemple, la longévité), ils nous amènent à considérer l'hypothèse d'un rôle de l'ADNmt M pour la détermination du sexe chez les moules mâles. En effet, il est possible qu'une fois les mitochondries paternelles ségrégées dans les cellules primordiales chez les embryons destinés à devenir des mâles, l'ADNmt M serait impliqué dans la différentiation des cellules germinales et dans le développement de la gonade mâle.

Les résultats du chapitre 2 montrent que la combinaison «ADNmt récemment-masculinisé – ADNnu» (qui est essentiellement une combinaison ADNmtF – ADNnu) performe mieux au niveau enzymatique que la combinaison «ADNmt M – ADNnu». Autrement dit, chez les Mytilidae, la co-évolution intergénomique semble avoir été favorisée pour la lignée mitochondriale maternelle. Ces mêmes résultats se reflètent-ils au niveau moléculaire ? Le chapitre 3 était consacré à l'étude de l'évolution moléculaire des protéines interactives du système de la phosphorylation oxydative (c'est-à-dire les produits nucléaires et mitochondriaux en contact physique) chez l'espèce *Mytilus edulis*. Le gène nucléaire codant pour le transporteur mobile d'électron cytochrome *c* (C*YC*) et des gènes mitochondriaux et nucléaires codant pour des sous-unités protéiques des complexes III (ADNmt = *CYTB*; ADNnu = *C3S6*) et

IV (ADNmt = *COX1* et *COX2*; ADNnu = *COX4*) du système de la phosphorylation oxydative ont été amplifiés par PCR et séquencés à l'aide d'un séquenceur d'ADN automatique dans les laboratoires des Dr. France Dufresne à l'UQAR et Dr. Donald Stewart à l'Université Acadia. Les séquences nucléotidiques et protéiniques sont comparées avec celles de l'espèce *M. galloprovincialis* (Mizi *et al.*, 2005 ; Venier *et al.*, données non publiées) déjà disponibles dans la base de données moléculaires «GenBank».

4.3 Principaux résultats obtenus pour le chapitre 3

4.3.1 Gènes mitochondriaux

► Le polymorphisme total (synonyme et nonsynonyme) est plus important pour les gènes du génome M, excepté pour le gène *cox1* (c'est-à-dire que le gène F*cox1* montre des taux de polymorphisme total plus élevés que le gène M*cox1*).

► Contrairement au gène M*cox1* qui montre des taux de polymorphisme (synonyme et nonsynonyme) plus élevés pour les portions protéiniques extramembranaires, les gènes M*cytb*, F*cox1* et F*cytb* montrent des taux de polymorphisme plus élevés pour les portions protéiniques transmembranaires.

► En accord avec les résultats mentionnés ci-dessus, les maximums de diversité nucléotidique diffèrent entre les gènes F et M *cox1* et *cytb*. Cela suggère que les génomes F et M pourraient expérimenter différentes contraintes sélectives associées aux demandes physiologiques spécifiques aux différents tissus qu'ils occupent.

► Les tests de neutralité indiquent qu'une sélection purificatrice agit sur les génomes F et M. Cependant, vu le nombre de mutations nonsynonymes plus important pour les gènes du génome M, il semble que l'intensité de cette sélection

soit réduite pour ce même génome (ou encore qu'elle soit plus importante pour le génome F).

► La comparaison des séquences en acides aminés montre que 5 substitutions fixes (c'est-à-dire une dans la protéine *COX1* et 4 dans la protéine *COX2*) entre les génomes F et M impliquent des résidus qui sont en contact physique avec la protéine d'origine nucléaire *CYC*. Il est proposé que ces substitutions pourraient avoir un impact sur les propriétés fonctionnelles des mitochondries, ce qui pourrait expliquer les différences obtenues au niveau enzymatique entre les gonades contenant des spermatozoïdes associés à un ADNmt de type récemment-masculinisé (essentiellement de type F) et celles contenant des spermatozoïdes associés à un ADNmt M standard.

4.3.2 Gènes nucléaires

► Le polymorphisme total (synonyme et nonsynonyme) est moins important pour le gène codant pour le cytochrome *c* (*cyc*) comparativement aux gènes *c3s6* et *cox4*.

► Les tests de neutralité indiquent qu'une sélection purificatrice agit sur les gènes nucléaires étudiés.

► L'ensemble des résultats montre que les plus faibles taux de polymorphisme nonsynonyme (c'est-à-dire aucune mutation nonsynonyme) sont observés pour les gènes *cyc* (nucléaire), F*cox1* et F*cox2* (mitochondriaux). Les taux de polymorphisme les plus importants sont observés pour les gènes *cox4* (nucléaire) et M*cox2* (mitochondrial).

► Au total, le taux moyen de polymorphisme synonyme est comparable pour les trois gènes nucléaires et les gènes (F et M) mitochondriaux alors que le taux moyen

de polymorphisme nonsynonyme est 8 fois plus élevé pour les gènes nucléaires que pour les gènes F mitochondriaux, et 3.6 fois moins élevé pour les gènes nucléaires que pour les gènes M mitochondriaux.

4.3.3 Divergences entre *M. edulis* et *M. galloprovincialis*

► Au total, le taux moyen de polymorphisme synonyme est 1.5 fois plus élevé pour les trois gènes nucléaires que pour les trois gènes F mitochondriaux et 1.7 fois moins élevé pour les gènes nucléaires que pour les gènes M mitochondriaux. Le taux moyen de polymorphisme nonsynonyme est 9.1 fois plus élevé pour les gènes nucléaires que pour les gènes F mitochondriaux, et 2.8 fois moins élevé pour les gènes nucléaires que pour les gènes M mitochondriaux.

Les données moléculaires semblent démontrer que la co-évolution entre le génome F et le génome nucléaire est favorisée. La division inégale des tâches accomplies par les génomes mitochondriaux F et M apparaît comme l'hypothèse la plus parcimonieuse pour expliquer cette inégalité de co-évolution. En effet, puisque l'ADNmt F doit fonctionner dans la gonade femelle et toutes les cellules somatiques tandis que l'ADNmt M ne doit fonctionner que dans les spermatozoïdes, la sélection naturelle a favorisé la co-évolution de la combinaison ADNmt F – ADNnu.

L'étude des interactions et de la co-adaptation entre les génomes nucléaire et mitochondrial est un défi majeur de la recherche pour les prochaines années.

L'acquisition de nouvelles connaissances sur les systèmes de transmission mitochondriale inhabituels tels que la DUI permettra de faire d'importantes percées dans ce domaine. Les travaux de cette thèse sont une première étape de défrichage dans la compréhension du réseau de co-évolution intergénomique que représente le système de la DUI. Le premier chapitre s'est intéressé à l'analyse génétique comparative des ADN mitochondriaux complets chez des espèces du genre *Mytilus*. Cette étude est la première à comparer des ADNmt complets pour plusieurs espèces de Mytilidae. Les résultats ont démontré qu'une sélection purificatrice agit sur les ADNmt F et M, mais que cette sélection est moins rigoureuse pour l'ADNmt M. Compte tenu du peu de génomes complets analysés, nous n'avons pas été en mesure de mettre en évidence des différences nucléotidiques régionales résultant des différentes forces sélectives qui agissent sur les ADNmt F et M. Les données de l'étude n'ont également pas permis d'identifier des différences nucléotidiques régionales associées à des adaptations spécifiques des ADNmt F et M pour leur rôle respectif. La comparaison de plusieurs génomes mitochondriaux complètement séquencés pour un plus grand nombre «d'espèces-DUI» pourra permettre d'obtenir un maximum d'informations sur la distribution de la variabilité génétique dans les ADNmt F et M afin de mieux comprendre les différents facteurs agissant sur leur évolution. L'incorporation d'espèces de bivalves non étudiées dans ces analyses permettra également de déterminer l'étendue du système de la DUI au sein de ce groupe.

La séquence complète du génome récemment-masculinisé (M') a permis de démontrer que la région non-codante ou «région contrôle» qui contient les éléments nécessaires à la réplication et la transcription de l'ADNmt M' est une portion recombinante qui présente une «région contrôle» de type F et une «région contrôle» de type M. Nous avons émis l'hypothèse qu'étant le seul élément différent entre un ADNmt F standard et un ADNmt M', cette incorporation d'une «région contrôle» de type M dans une molécule de type F pourrait être responsable de la transmission

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paternelle d'un molécule autrefois transmise maternellement. Jusqu'à maintenant, aucune étude n'a permis de prouver (ou d'infirmer) cette hypothèse. Des analyses génétiques et biochimiques supplémentaires permettront d'élucider le rôle de la région contrôle de type M dans l'induction de la transmission mitochondriale paternelle.

Le chapitre 2 a démontré que le phénomène de renversement des rôles a un impact sur les propriétés fonctionnelles des mitochondries dans les spermatozoïdes de l'espèce *M. edulis*. Nous avons proposé que cet impact est dû aux divergences observées entre les ADNmt M standard et ADNmt M'. Il incombe maintenant de déterminer si ces divergences sont celles associées aux gènes codant pour les protéines du système de la phosphorylation oxydative ou si l'ADNmt M a un effet sur la synthèse des protéines mitochondriales, ce qui pourrait aussi expliquer les différences enzymatiques. En outre, il sera intéressant de vérifier si ces substitutions on un effet sur la respiration mitochondriale et la production d'ATP.

Finalement, les données moléculaires du chapitre 3 semblent démontrer que la co-évolution entre le génome F et le génome nucléaire est mieux couplée chez les Mytilidae. Ces données appuient favorablement les résultats de l'étude enzymatique. Des analyses supplémentaires combinant plusieurs espèces et plusieurs gènes permettront de détecter des zones de sélection positives dans les gènes nucléaires et mitochondriaux et d'identifier les substitutions qui y sont associées. Ces analyses permettront également d'identifier les changements d'acides aminés entre les ADNmt F et M qui peuvent potentiellement avoir un impact sur les propriétés fonctionnelles des mitochondries. Il sera aussi intéressant de vérifier la présence d'isoformes nucléaires chez les espèces possédant la DUI. Ces études constitueront un avancement majeur dans notre compréhension de l'évolution et du fonctionnement du système de la double transmission uniparentale de l'ADN mitochondrial.

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