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L'épigénétique, moteur de l'évolution d'un vertébré asexué

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Thèse intitulée :

L'épigénétique, moteur de l'évolution d'un vertébré asexué

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RÉSUMÉ

L'objectif de cette thèse est de déterminer l'étendue de la variabilité épigénétique, plus particulièrement du polymorphisme de méthylation de l'ADN, non liée à la variabilité génétique dans les populations asexuées en milieu naturel. Cette évaluation nous a permis de mieux cerner l'importance que peuvent avoir les processus épigénétiques en écologie et en évolution. Le modèle biologique utilisé est l'hybride clonal du complexe gynogénétique *Chrosomus eos-neogaeus*. Malgré une homogénéité génétique, une importante variabilité phénotypique est observée entre les hybrides d'une même lignée clonale mais retrouvés dans des environnements différents. L'influence des processus épigénétiques apporte une explication sur ce paradoxe. L'épigénétique se définit comme une modification de l'expression des gènes sans changement de la séquence d'ADN. La diversité des phénotypes peut entre autre s'expliquer par des patrons de méthylation différentiels des gènes et/ou des allèles des gènes entre les hybrides génétiquement identiques. La diversité des lignées épiclonales peut quant à elle s'expliquer par la colonisation de plusieurs lignées épiclonales, s'établir en réponse à l'environnement ou de façon aléatoire. Plusieurs méthodes seront utilisées afin de survoler le génome des hybrides clonaux pour mettre en évidence le polymorphisme de méthylation de l'ADN à l'échelle de l'individu et entre les individus de différentes populations.

Mots-clés: épigénétique des populations, méthylation de l'ADN, héritabilité, sélection, flexibilité génomique, modèle GPG, hybridation, vertébré clonal, complexe *Chrosomus eos-neogaeus*, éléments transposables, MSAP, séquençage au bisulfite.

ABSTRACT

The aim of the thesis is to determine the extent of epigenetic variation, more specifically DNA methylation polymorphism, not linked to genetic variation in natural populations of an asexual vertebrate. This evaluation enables to better understand the importance that plays epigenetics processes in ecology and evolution. The biological model used is the clonal hybrid of the gynogenetic *Chrosomus eos-neogaeus* complex. Even in absence of genetic difference, an important phenotypic variability is observed among hybrids of the same clonal lineage living in different environments. Epigenetics, a modification of genes expression without a change at the DNA sequence, provides an explanation to this paradox. The diversity of phenotypes may be explained by differential methylation patterns of genes and/or alleles among genetically identical hybrids. The diversity of epiclonal lineages may be explained by the colonisation of many epiclonal lineages, established in response to the environment or stochastically. Many methods were used for screening the genome of clonal hybrids in order to highlight DNA methylation polymophism at the scale of an individual and among individuals of different populations.

Key words: population epigenetics, DNA methylation, heritability, selection, genomic flexibility, GPG model, hybridization, clonal vertebrate, *Chrosomus eos-neogaeus* complex, transposable elements, MSAP, bisulfite sequencing.

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LISTE DES SIGLES ET ABRÉVIATIONS

ADN (DNA): Acide Désoxyribonucléique

AFLP: Amplified Fragment Lenght Polymorphism

ARN (RNA): Acide Ribonucléique

CH_3: Groupement méthyle

ET (TE): Éléments Transposables

GPG: General Purpose Genotype

HAT: Histone Acétyle-Transférase

HDAC: Histone Déacétylase

HMT: Histone Méthyle-Transférase

DNMT: ADN Méthyle-Transférase

M : molaire

mM: millimolaire

min: minute

MSAP: Methylation Sensitive Amplified Polymorphism

MS-SSCA: Methylation-Sensitive Single Strand Conformation Analysis

ng: nanogramme

Pb (bp): Paires de bases

PCR: Réaction de Polymérase en Chaîne

pmol: picomol

RdRP: ARN Polymérase ARN Dépendante

s: seconde

siRNA: petits ARN interférents

SSCP: Single Strand Conformation Polymorphism

≠: n'est pas égale à

%: pourcentage

µl: microlitre

°C: degré celsius

À Marco et Luca, mes deux amours.

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1. INTRODUCTION

Ce projet de thèse porte sur l'évaluation de l'importance des processus épigénétiques en écologie et en évolution. Ce sujet sera traité par une étude de l'étendue de la variabilité épigénétique, plus particulièrement du polymorphisme de méthylation de l'ADN, sur des populations naturelles d'un modèle vertébré. Afin de cerner spécifiquement les variations épigénétiques non liées à la variabilité génétique, un poisson à reproduction clonale soit les hybrides du complexe *Chrosomus eos-neogaeus* a été utilisé. Les thèmes permettant de cerner les différents aspects de cette étude seront abordés dans l'introduction qui suit. Premièrement, une définition de l'épigénétique, la terminologie associée à ce domaine d'étude ainsi que les différents types de modifications épigénétiques seront discutés. Suivra une description détaillée des mécanismes moléculaires spécifiquement associés à la méthylation de l'ADN et des différents facteurs qui influencent les profils de méthylation de l'ADN à l'échelle de l'individu et entre les individus. Les exemples classiques de la littérature démontrant le lien entre la variation phénotypique et épigénétique viendront étayer l'importance de cerner l'étendue de la variation épigénétique non liée à la variation génétique. Le concept d'héritabilité de la variabilité épigénétique sera de plus présenté en faisant le parallèle entre les plantes et les mammifères de façon à comprendre la rareté de ce phénomène chez ces derniers. Le complexe *C. eos-neogaeus* sera présenté et les caractéristiques qui font de ces hybrides un modèle d'intérêt dans cette étude seront décrites. L'objectif et les hypothèses de ce projet de thèse seront finalement énoncés. En terminant, une brève description des quatre chapitres suivants permettra d'apprécier dans son ensemble le travail effectué durant cette thèse de doctorat.

1.1 L'épigénétique

L'épigénétique a dans ses premiers instants été décrit comme l'étude de l'épigenèse, c'est-à-dire de tous les évènements de régulation des gènes liés au développement qui mènent du zygote à l'état adulte et qui, en partant du matériel génétique, façonnent le phénotype (Waddington 1953). Les processus épigénétiques ont une importance cruciale lors du développement puisque c'est par le biais de l'expression différentielle des gènes que les cellules au bagage génétique identique des organismes pluricellulaires peuvent être fonctionnellement et structurellement hétérogènes (Jaenisch and Bird 2003). Une définition plus récente de l'épigénétique est l'étude des changements mitotiquement (méta) stables et potentiellement méiotiquement héritables de l'expression des gènes qui ne peuvent être expliqués par des changements de la séquence d'ADN (Richards 2006; Bird 2007). L'ensemble des marques épigénétiques qui régulent l'expression des gènes d'un individu forment l'épigénome. Les processus épigénétiques incluent les modifications «programmées» qui permettent la différentiation cellulaire et les épimutations.

1.2 La structure de la chromatine et les modifications épigénétiques

La chromatine est un complexe d'ADN et de protéines et celle-ci peut prendre différentes conformations. Les deux conformations principales sont l'hétérochromatine, l'état condensé et transcriptionnellement inactif, et l'euchromatine, l'état relâché qui peut être transcriptionnellement actif ou inactif. La condensation de l'ADN en hétérochromatine peut être permanente, il s'agit de l'hétérochromatine constitutive qui se retrouve dans des régions du génome composées de peu de gènes (télomères et centromères). La condensation de l'ADN

est aussi facultative, c'est-à-dire que la chromatine sera sous cette conformation dans certains types de cellules à un moment spécifique du développement.

Plusieurs modifications épigénétiques sont connues. Celles-ci se regroupent en deux classes soient les processus qui engendrent une modification de la structure de la chromatine et les processus qui engendrent une modification de l'ADN (sans changement de la séquence des nucleotides). Ces différents processus épigénétiques sont intimement reliés et engendrent un remodelage de la chromatine (hétérochromatine, état inactif compacte ↔ euchromatine, état actif relâché). Dans la première classe, les modifications de la structure chromatinienne sont engendrées par des complexes de remodelage de la chromatine et des complexes de modifications des histones. La liaison de complexes de remodelage de la chromatine et l'activité d'enzymes histone acétyle-transférase (HAT) est essentielle à l'initiation de la transcription de l'ADN en ARN. La répression de la transcription est possible suivant l'activité d'enzyme histone déacétylase (HDAC) et histone méthyle-transférase (HMT).

Dans la seconde classe, la répression de la transcription de l'ADN est causée par une modification de l'ADN suivant l'activité d'enzymes ADN méthyle-transférases (DNMT) et la liaison de protéines « methyl binding domain » qui se lient aux groupements méthyles des cytosines et empêchent la liaison des facteurs de transcription (Boyes and Bird 1991). Ces deux composantes de la machinerie de méthylation de l'ADN sont essentielles dans l'établissement, le maintien et la lecture des patrons de méthylation. La méthylation de l'ADN agit de concert avec la

déacétylation et la méthylation des histones. Ces mécanismes de répression de la transcription interagissent afin d'enclencher la propagation de l'état compact et inactif de la chromatine.

1.3 La méthylation de l'ADN

La méthylation de l'ADN est un mécanisme épigénétique corrélé à l'expression des gènes (Razin and Riggs 1980). Bien que tous les nucléotides puissent être méthylés, l'ajout de groupements méthyles (CH_3) sur la cytosine du dinucléotide CpG (ainsi que sur la cytosine du brin complémentaire) est le plus répandu (Martienssen and Colot 2001). La méthylation de l'ADN est présente chez tous les eucaryotes à l'exception des levures. Chez les vertébrés, elle est particulièrement dense sur l'ensemble du génome à l'exception des îlots CpG des régions promotrices des gènes transcriptionnellement actifs (Suzuki and Bird 2008).

L'activité de différentes enzymes ADN méthyle-transférases est essentielle à l'établissement et au maintien des patrons de méthylation. Cinq gènes sont impliqués dans la réalisation de ces deux fonctions primordiales. La méthylation « *de novo* » cible les sites non méthylés des dinucléotides CpG (DNMT3a, DNMT3b, DNMT3L). Ces enzymes n'ont pas de préférence pour les sites hémiméthylés (Okano et al. 1998) et sans ces protéines la méthylation «*de novo*» n'est pas possible (Okano, Xie, and Li 1998). Afin d'assurer le maintien de la méthylation lors de la réplication cellulaire, l'enzyme DNMT1 cible quant à elle les fragments hémiméthylés (Riggs 1975). L'inactivation de l'enzyme DNMT1 cause une perte importante de la méthylation du génome chez la souris (Li et al. 1993). De plus, l'inactivation de ces gènes est létale

chez la souris. La fonction de l'enzyme DNMT2 reste quant à elle mal connue à ce jour.

Comme le degré de méthylation de l'ADN est négativement corrélé au degré d'expression des gènes, une continuité de phénotypes peut être ainsi produite (Kalisz and Purugganan 2004). Lorsqu'une variation dans le degré de méthylation entre les allèles d'un gène est présente, ces allèles sont appelés des épiallèles. Cette variation entre les allèles de différents individus peut être le résultat du nombre ou de la distribution des sites méthylés sur la séquence spécifique du gène. Un allèle ayant un niveau ou un patron de méthylation variable sera exprimé de façon différente entre les individus et il peut en résulter la production d'un continuum de nouveaux phénotypes. La production de nouveaux phénotypes épialléliques a une implication importante dans l'évolution des plantes (Kalisz and Purugganan 2004). Un exemple de régulation épiallélique de l'expression d'un gène résultant en la production de nouveaux phénotypes est retrouvé chez la linaire commune (*Linaria vulgaris*). Une variation du degré de méthylation du gène associé à la symétrie de la fleur, gène *Lcyc*, est responsable de l'apparition du phénotype à symétrie radiale à partir de la forme sauvage à symétrie bilatérale (Cubas et al. 1999). Un autre exemple d'apparition de nouveaux phénotypes suivant une régulation épiallélique est retrouvé chez les gènes de pigmentation du maïs (*Zea mays*) (Chandler et al. 2000).

1.4 L'intégration des signaux intrinsèques

Chez les organismes pluricellulaires on reconnait deux types de cellules: les cellules germinales et les cellules somatiques. Les cellules germinales sont responsables de la

transmission de l'information génétique aux générations suivantes tandis que les cellules somatiques constituent l'organisme lui-même et sont fonctionnellement et structurellement hétérogènes. Comme la différentiation cellulaire n'a pas pour résultat la délétion de parties du génome, la question à répondre est comment arrive-t-on à produire différents types de cellules?

Les cellules germinales sont pour la grande majorité à l'état de repos et ne répondent pas aux stimuli externes afin de préserver l'intégrité de leur épigénome. Par contre, elles répondent à toute une panoplie de signaux internes ce qui permet une reprogrammation adéquate pour l'initiation et la réalisation du programme de développement cellulaire. Dans un premier temps, le maintien de la totipotence des cellules germinales est assuré par la supression de la grande majorité des marques épigénétiques. Suivant la fécondation, une deuxième phase de déméthylation survient sur l'ensemble du génome sauf pour les gènes sous empreinte génomique; processus observé entre autre chez les mammifères (Niemitz and Feinberg 2004). Finalement, le programme de développement cellulaire, basé sur des marques épigénétiques programmées (code épigénétique), est initié.

Lors du développement, la majorité des marques de méthylation sont semblables entre les tissus somatiques (pour les gènes domestiques «*housekeeping*») mais certaines différences sont visibles sur des séquences spécifiques (pour les gènes tissus-spécifiques). L'absence ou la perte programmée de méthylation sur des séquences spécifiques résulte en l'activation de la transcription de leur gène cible au cours du développement. Des expériences d'invalidation génique «*knockout*» des

enzymes DNMT chez la souris ont démontré que sans ces enzymes, les cellules embryonnaires sont viables mais vont mourir lors de l'induction de la différentiation (Li, Beard, and Jaenisch 1993; Okano, Xie, and Li 1998). Lors du développement, les îlots CpG des gènes domestiques restent hypométhylés tandis que les îlots CpG des gènes tissus-spécifiques seront méthylés à des moments et dans des cellules spécifiques afin de permettre la différentiation cellulaire (Bird 2002). L'importance de la méthylation de l'ADN comme processus de régulation de l'expression des gènes est soulignée par les modifications spatiales et temporelles de ces patrons épigénétiques afin de permettre la différentiation cellulaire de l'embryon jusqu'à l'état adulte.

1.5 L'intégration des signaux extrinsèques

La méthylation de l'ADN est une marque réversible donc plus facilement modifiable en comparaison à l'apparition d'une mutation de l'ADN. La fréquence des épimutations serait de deux ordres de magnitude plus grands que les mutations somatiques (Bennett-Baker et al. 2003). Une épimutation peut se définir comme la modification de l'état épigénétique initial d'une séquence d'ADN. Les épimutations peuvent survenir pendant le développement et/ou au cours de la vie adulte des organismes. Richards (2006) a défini trois types de variation épigénétique en fonction de leur dépendance par rapport à la variation génétique (effets en *cis* ou en *trans*). Dans la mesure où l'état épigénétique est strictement déterminé par le génotype, la variation épigénétique est décrite comme obligatoire. Lorsque l'état épigénétique a une plus forte probabilité d'apparaître dans un contexte génomique spécifique, il est décrit comme facilité. Enfin, si la variation épigénétique n'est aucunement sous

l'influence du génotype, celle-ci est décrite comme pure. L'état de méthylation des gènes peut être modifié par des processus stochastiques et/ou en réponse à l'environnement. Les épimutations qui en résultent peuvent avoir un effet sur le phénotype des individus et le résultat peut être avantageux (par exemple afin de permettre la plasticité phénotypique; Angers et al. 2010) ou désavantageux (par exemple lors de la perte de l'empreinte génomique; Ubeda and Wilkins 2008). Dans les paragraphes suivants, quelques exemples des trois types de variation épigénétique seront décrits. Ces exemples permettront aussi d'illustrer les effets de l'environnement sur le façonnement de l'épigénotype et les modifications phénotypiques qui en résultent.

Un exemple de variation épigénétique obligatoire est celui de la floraison hâtive en absence de vernalisation chez *Arabidopsis*. La floraison est contrôlée par des processus endogènes et des signaux environnementaux. La vernalisation, une exposition prolongée à des températures froides et à des journées plus longues, est un signal environnemental qui induit une floraison hâtive. Plusieurs gènes sont associés à la floraison chez *Arabidopsis*. La variabilité naturelle de la floraison est entre autre liée à la variation allélique de deux gènes: *FRIGIDA* (*FRI*) et locus de floraison C (*FLC*) (Boss et al. 2004). Le gène *FLC* est un répresseur de la floraison et celui-ci est négativement régulé par la vernalisation tandis que le gène *FRI* est lié à la régulation de *FLC* (Michaels and Amasino 1999; Sheldon et al. 2002). Les plants qui ont une floraison tardive possèdent des allèles dominants pour les deux locus tandis que la floraison hâtive est possible si, pour au moins un locus, l'allèle récessif est exprimé. La souche Landsberg *erecta* (L*er*) possède un allèle nul pour le gène *FRI* mais la

floraison est tout de même hâtive (Johanson et al. 2000). La floraison hâtive en absence de vernalisation et de l'expression d'allèle récessif pour le gène *FRI* est occasionnée par la présence d'un élément transposable dans le premier intron du gène *FLC*. La présence de cette insertion en *cis* cause une plus faible expression du gène *FLC* (Michaels et al. 2003) suivant l'action de petits ARN interférents (siRNA) (Liu et al. 2004). En présence de cet élément transposable, la floraison est toujours hâtive chez cette souche. L'état épigénétique est donc strictement déterminé par le génotype puisque l'expression du gène *FLC* est obligatoirement régulée par les siARN produits en présence de l'élément transposable.

Un exemple de variation épigénétique facilitée est celui du gène *Agouti* chez la souris. L'insertion de l'élément retroviral *IAP* dans le pseudoexon 1A en amont du gène *Agouti* promouvoit l'expression d'épiallèles métastables tel que A^{vy}, la couleur du pelage des souris en est affectée (Millar et al. 1995). Ce changement de phénotype est corrélé au degré de méthylation de l'élément retroviral *IAP* ainsi, une mosaïque de phénotypes peut-être produite de brun (normal, *IAP* est méthylé) à jaune (mutant, *IAP* est non méthylé) (Morgan et al. 1999). L'hypométhylation de l'élément retroviral *IAP* aura un effet sur la couleur du pelage et de plus, cause l'obésité et l'apparition de tumeurs (Duhl et al. 1994). La diète est un des facteurs reconnus comme ayant un effet important sur la modification de l'épigénome dans ce modèle donc ultimement, sur la modification du phénotype (Feil 2006). Il a été démontré qu'une diète riche en groupement méthyle donnée aux mères va produire le phénotype normal pour le gène *Agouti* même en présence de l'élément retroviral *IAP* (Wolff et al. 1998). En absence

de cet élément retroviral, le phénotype sauvage est exprimé tandis que la régulation épigénétique de celui-ci facilite l'apparition de toute une gamme de phénotypes.

Un premier exemple de variation épigénétique pure induite par l'environnement est l'induction d'une floraison hâtive suivant l'effet de la vernalisation (Sheldon et al. 2000). Comme mentionné plus haut, le gène de floraison C (*FLC*), un répresseur de la floraison, joue un rôle primordial dans la réponse à la vernalisation. Suivant une exposition prolongée à des températures froides et à des journées plus longues, le gène *FLC* sera réprimé. Conséquemment, la floraison de deux plantes génétiquement identiques ne sera pas synchronisée si celles-ci ne sont pas soumises toutes les deux à la vernalisation. Il a été démontré que l'activité du gène *FLC* est entre autre contrôlée par la méthylation de l'ADN (Sheldon et al. 1999). L'induction d'une floraison hâtive est fonction de la vernalisation, un signal environnemental qui influence le patron d'expression du gène *FLC* par l'altération de son statut de méthylation.

Un autre exemple de variabilité épigénétique pure est retrouvé chez les abeilles (*Apis mellifera*). Dans ce système deux types de femelles sont produits à partir de larves génétiquement identiques: les ouvrières stériles et les reines fertiles. Afin de produire des reines fertiles, les larves doivent être nourries avec de la gelée royale. Il a été démontré que l'inactivation de l'enzyme DNMT3 lors du développement par des siARN avait pour résultat de produire une proportion plus importante de reines (Kucharski et al. 2008). Une étude détaillée du statut de méthylation des dix dinucléotides CpG des exons cinq, six et sept du gène *Dynactine*

p62 a révélé que le degré de méthylation de cette partie du génome des reines est significativement moins élevé que celui des ouvrières. Afin de produire des ouvrières, certains gènes seraient réprimés par la méthylation de l'ADN. Ces résultats démontrent que la détermination du statut social n'est aucunement liée au génotype car les deux phénotypes sont produits à partir de larves génétiquement identiques. Dans ce cas-ci, le statut social est fonction d'un signal environnemental ayant une influence sur le statut de méthylation de certains gènes d'importance dans la détermination du phénotype.

1.6 Héritabilité entre les générations

1.6.1 Les mammifères

Chez les mammifères, les épimutations qui seront transmises d'une génération à l'autre doivent: 1) être présentes dans les cellules germinales et surpasser l'étape de supression des marques épigénétiques qui se produit lors de la gamétogenèse ainsi que lors de l'initiation du programme de développement cellulaire (Morgan et al. 2005) ou 2) occasionner le transfert de l'information essentielle au rétablissement des marques épigénétiques à la génération suivante (voir section sur les paramutations). Malgré ces obstacles, l'héritabilité de l'information épigénétique entre les générations est claire et démontrent que la molécule d'ADN elle-même n'est pas l'unique déterminant de notre phénotype. Dans les sections suivantes, quelques exemples seront décrits.

Dans le cas du locus agouti chez la souris, il a été observé que le nombre de souriceaux au phénotype mutant (jaune et obèse) était plus élevé chez les femelles au phénotype mutant (Wolff et al. 1998). Les auteurs avaient tout d'abord proposé une influence de l'environnement maternel sur l'établissement des patrons épigénétiques. Une étude subséquente a par contre démontré l'héritabilité par le transfert de l'information épigénétique dans les gamètes femelles (Morgan et al. 1999). Il semblerait par contre que la supression incomplète des marques de méthylation de l'ADN ne soit pas le processus par lequel l'héritabilité de l'information épigénétique est possible (Blewitt et al. 2006).

Une étude effectuée sur des rats exposés à un contaminant environnemental (Endocrine-Disrupting Chemicals) a démontré que ce contaminant a un effet sur l'épigénome des lignées cellulaires germinales (Crews et al. 2007). Cette étude propose que les facteurs environnementaux vont promouvoir l'altération de l'épigénome sur plusieurs générations et ce, même en absence du contaminant aux générations successives. Les auteurs concluent également que les femlles sont capables de détecter les mâles contaminés jusqu'à trois générations suivant l'exposition et de choisir leur partenaire en discréminant les mâles contaminés (sélection sexuelle). Ces modifications épigénétiques ont donc un impact important sur la viabilité des populations et l'évolution de l'espèce.

Un exemple de transfert de l'information épigénétique entre les générations est le cas des soins maternels chez les rats. Il a été observé que les femelles peuvent avoir deux types de comportement en regard des soins maternels qui sont dispensés à la

progéniture. Les «bonnes mères» vont materner significativement plus leur progéniture en comparaison aux «mauvaises mères». À l'âge adulte, la progéniture des «bonnes mères» va démontrer une meilleure réponse au stress (Liu et al. 1997). Lors de la réalisation d'expériences d'échange de la progéniture entre les deux types de mères, il a été observé que la progéniture des «bonnes mères» élevée par des «mauvaises mères» sera «stressée» alors que la progéniture des «mauvaises mères» élevée par de «bonnes mères» sera normale (Francis et al. 1999). Cela indique que ce phénotype n'est pas codé par le génome et est transmis de la mère à sa progéniture (Meaney 2001). Cette réponse au stress est médiée par une boucle de rétroaction négative qui est contrôlée par l'expression du gène récepteur des glucocorticoïdes (*RG*). Une étude du statut de méthylation des dinucléotides CpG du promoteur de ce gène et plus particulièrement, du site de liaison du facteur de transcription *NGFI-A* a permis de visualiser que la méthylation est toujours plus importante chez la progéniture des «mauvaises mères». Les auteurs ont pu conclure à un lien de causalité entre l'acétylation des histones, l'hypométhylation de l'ADN de la région promotrice de ce gènes et la liaison du facteur de transcription ce qui permet l'expression du gène *RG* et une meilleure réponse au stress de la progéniture des «bonnes mères» (Weaver et al. 2004).

L'exemple par excellence d'héritabilité épigénétique entre les générations est le cas des paramutations. Une paramutation se définit comme la modification épigénétique héritable en *trans* d'un allèle (allèle sensible, sera réprimé) par l'allèle homologue (allèle paramutateur, cause de la paramutation) d'un gène (Chandler, Eggleston, and Dorweiler 2000). L'état épigénétique lié à la paramutation conduit à

un réarrangement de la chromatine donc à une modification de l'expression des gènes. À l'origine, les paramutations seraient un mécanisme de défense cellulaire apparu afin de réprimer des éléments génomiques aux effets délétères comme les éléments répétés et les éléments transposables. L'action de ces petits ARN interférents serait particulièrement importante dans les lignées germinales considérant l'hypométhylation des gamètes et de l'embryon. La plupart des cas de paramutation doivent avoir un effet beaucoup plus similaire au locus *Kit* en termes de pénétrance et d'héritabilité (voir détail plus bas). Cela rend difficile l'observation de ces phénomènes. Les paramutations sont sans doute des processus plus fréquents que les observations réalisées à ce jour et pourraient représenter un mécanisme permettant le transfert de l'état d'expression des gènes (allèles des gènes) à la progéniture (Chandler 2007).

Un premier exemple de paramutation suivant une régulation post-transcriptionnelle est celui du locus *Kit* qui code pour un récepteur tyrosine kinase chez la souris (Rassoulzadegan et al. 2006). Des lignées de souris hétérozygotes, allèle mutant Kit^{tm1Alf} / allèle sauvage Kit^{+}, présentent un phénotype à queue et pattes blanches. Le phénotype mutant est causé par une insertion du gène rapporteur *lacZ* dans un des deux allèles chez les hétérozygotes. Lorsque présente, cette insertion est exprimée au détriment de l'allèle sauvage *Kit*. Lors d'un croisement entre un individu hétérozygote et un individu homozygote sauvage ou entre deux hétérozygotes, une plus grande proportion de juvéniles au phénotype queue et pattes blanches est observée. Ces individus nommés paramutants (*Kit**) peuvent présenter un génotype homozygote pour l'allèle sauvage. L'allèle mutant est un allèle paramutateur

produisant des ARN de taille anormale et sans queue poly-A qui dégradent les ARNm normaux produits par l'allèle sauvage *Kit* (allèle sensible). Ces petits ARN interférents sont transférés au zygote par le cytoplasme des gamètes mâles et femelles. Ainsi, lors d'un croisement entre un individu paramutant et un individu sauvage tous deux homozygotes pour l'allèle sauvage, une certaine proportion de juvéniles au phénotype mutant est observée. Cela démontre que le phénotype queue et pattes blanches est héritable même en absence de l'allèle paramutateur simplement par le transfert des ARN interférents d'une génération à l'autre. Par contre, en absence de l'allèle paramutateur Kit^{tm1Alf}, il y aura dilution du phénotype au fil des générations. Une étude détaillée de la méthylation des îlots CpG et de la structure de la chromatine de la région promotrice du locus *Kit* n'a pas révélée de différence entre les individus homozygotes, paramutants et hétérozygotes mutants. Les auteurs suggèrent qu'une autre région promotrice pourrait être impliquée dans la régulation de la transcription du locus *Kit*. Le ou les mécanisme(s) qui mène(nt) à l'expression de l'insertion au détriment de l'allèle *Kit* chez les hétérozygotes n'est pas connu.

1.6.2 Les plantes

Chez les plantes, les lignées germinales sont dérivées des lignées somatiques ou végétatives, elles sont produites plus tardivement au cours du développement et la supression des marques épigénétiques est moins importante (Richards 2006). Les épimutations sont donc plus facilement présentes et maintenues dans les gamètes ce qui rend moins problématique la transmission de celles-ci entre les générations. Un exemple classique d'épimutation méiotiquement héritable est celui de la transmission des marques de méthylation de l'ADN du gène *Lcyc* qui est responsable de la

symétrie de la fleur chez la linaire commune *Linaria vulgaris* (Cubas, Vincent, and Coen 1999).

Un deuxième exemple de paramutation, cette fois suivant une régulation transcriptionnelle, est celui du Locus *b1* qui code pour un facteur de transcription de pigments mauves chez le maïs (Stam et al. 2002). Les plants sauvages *B-I* présentent un phénotype mauve suivant une forte transcription du locus *b1* tandis que les plants mutants *B`* présentent un phénotype clair suivant une faible transcription du locus *b1*. *B-I* et *B`* sont deux épiallèles. Tous deux présentent sept répétitions en tandem de 853pb spécifiques au locus *b1* et se retrouvent en amont du site d'initiation de la transcription de ce locus. Chez le plant sauvage *B-I*, les répétitions en tandem ne sont pas méthylées tandis que chez le plant mutant *B`* elles le sont. Chez un hétérozygote allèle sauvage *B-I* / allèle mutant *B`,* l'allèle mutant *B`* va agir comme allèle paramutateur en induisant la méthylation de l'allèle sauvage *B-I* (allèle sensible). Une ARN polymérase ARN dépendante (RdRP) encodée par le gène *mop1* (la médiatrice de paramutation), et les répétitions en tandem sont les deux éléments essentiels afin de convertir allèle sauvage *B-I* en allèle paramuté *B`** (Alleman et al. 2006). La transcription des éléments répétés méthylés de l'allèle paramutateur *B`* par *mop1* produit des petits ARN interférents qui induisent cette modification épigénétique. Les allèles paramutés *B`** sont ensuite capables d'agir comme allèle paramutateur en induisant la méthylation des allèles sauvages sensibles *B-I*. Ainsi, le croisement entre un plant homozygote sauvage *B-I* et un plant hétérozygote mutant produit exclusivement des plants hétérozygotes mutants, *B`/ B`**. Ce cas de paramutation est extrêmement stable et démontre 100% de pénétrance. La méthylation des répétions de

l'allèle paramutateur est de plus méiotiquement héritable (Stam et al. 2002). Contrairement au locus *Kit* chez la souris, le passage des ARN interférents dans les gamètes n'est dans ce cas-ci pas essentiel afin de permettre l'héritabilité entre les générations.

1.7 Sources de variation épigénétique et variabilité phénotypique

Plusieurs sources de variation épigénétique sont reconnues. Premièrement, la variabilité épigénétique sera intimement liée à la variabilité génétique par des éléments agissant en *cis* et en *trans*. Par contre, la proportion de variation phénotypique associée à des processus épigénétiques n'est pas uniquement fonction de la variabilité épigénétique liée à la variabilité génétique. Pour un génotype donné, il peut y avoir variation autour d'un phénotype optimal en fonction d'un gradient environnemental (norme de réaction). La capacité d'un génome à intégrer les signaux de l'environnement au cours de la vie d'un organisme et à modifier le phénotype en conséquence (plasticité phénotypique) ou la capacité d'un génotype à donner différents phénotypes dans différents environnements (flexibilité développementale ou variabilité phénotypique), sont des processus qui donnent une certaine flexibilité au génome afin de faire face aux changements environnementaux. Les processus épigénétiques représentent donc un système qui permet la variation phénotypique en absence de variabilité génétique. Cependant, il y a très peu d'information sur l'étendue de la variabilité épigénétique non liée à la variabilité génétique dans les populations en milieu naturel. Finalement, la portion héritable de la variabilité épigénétique totale sera façonnée par les forces évolutives qui agissent dans les populations (Richards 2008). À l'échelle de la population, la variabilité épigénétique

observée sera fonction des processus stochastiques, de l'environnement, de la variabilité génétique, de la fréquence des épimutations, de l'intensité de la dérive épigénétique et de la sélection.

1.8 Le modèle biologique

Le modèle biologique utilisé dans cette étude est le poisson à reproduction clonale *Chrosomus eos-neogaeus*. Dans la prochaine section, le complexe sera présenté et les caractéristiques qui font de ces hybrides un modèle d'intérêt pour les fins de cette étude seront détaillées.

1.8.1 Les hybrides clonaux *Chrosomus eos-neogaeus*

Le complexe *Chrosomus eos-neogaeus* (Pisces, Cyprinidae) provient d'hybridations ancestrales entre les femelles *C. neogaeus* (ventre citron) et les mâles *C. eos* (ventre rouge du nord) (Figure 1). Ces évènements d'hybridation ont donné naissance à des lignées d'hybrides exclusivement femelles (Dawley et al. 1987) (Figure 1). Ces hybrides se reproduisent de façon asexuée par gynogenèse (Goddard et al. 1998). La gynogenèse implique la formation d'œufs diploïdes sans méiose par les hybrides femelles. Le développement des œufs doit être induit par un spermatozoïde d'une des deux espèces parentales. Le matériel génétique du mâle n'est toutefois pas intégré dans l'œuf; les juvéniles ainsi produits sont identiques à leur mère (clones) (Goddard and Dawley 1990).

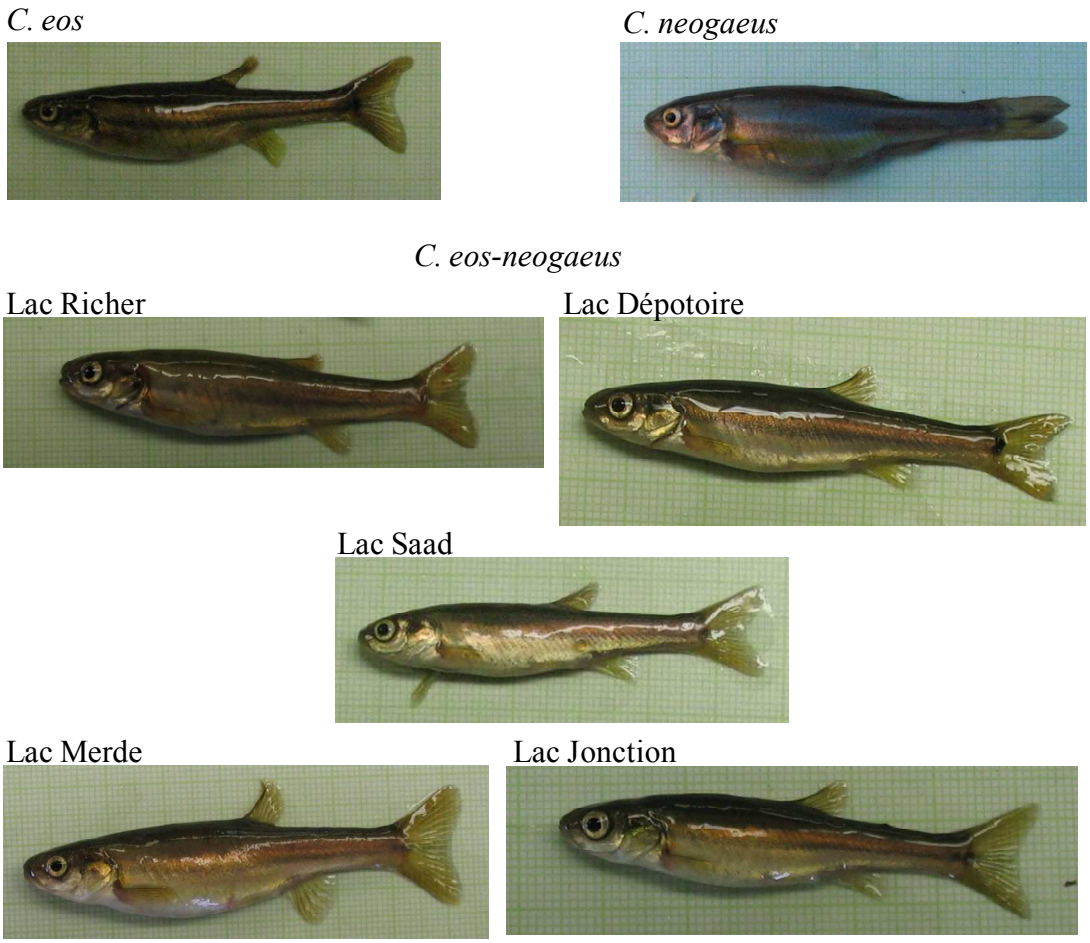

Figure 1. Représentation photo des deux espèces parentales (femelles), *C. eos* et *C. neogaeus*, ainsi que des hybrides *C. eos-neogaeus* de cinq populations.

1.8.2 Répartition géographique

Les membres du complexe *Chrosomus* sont retrouvés sur une grande répartition géographique et sont soit en sympatrie ou en allopatrie dans la plupart des lacs du centre et du nord-est de l'Amérique du Nord (Scott and Crossman 1973). *C. eos* et les hybrides, sont les types de *Chrosomus* principalement retrouvés dans les lacs des Laurentides (Binet and Angers 2005). Les deux espèces parentales sont décrites comme évoluant dans des eaux fraîches ayant un pH allant de neutre à légèrement

acide de petits cours d'eau, d'étangs, de ruisseaux et de lacs marécageux (Scott and Crossman 1973). On les retrouve dans une multitude d'environnements très différents et sont donc potentiellement soumis à des pressions environnementales variables. Ces variations environnementales auront possiblement un effet important sur le façonnement de l'épigénome puisque l'environnement est reconnu comme ayant un effet déterminant sur l'apparition des épimutations.

1.8.3 Niche écologique et variabilité phénotypique

L'étude de Schlosser et al. (1998) a démontré que l'espèce parentale *C. eos* et les hybrides diploïdes occupent des niches écologiques différentes; la fréquence relative des membres du complexe varie selon une succession d'environnements. Tous les membres du complexe préfèrent habiter la zone littorale de milieux bien oxygénés mais les hybrides diploïdes sont moins sensibles au stress relatif aux conditions anoxiques en comparaison à *C. eos* (Schlosser et al. 1998; Doeringsfeld et al. 2004). En comparaison avec les espèces parentales, les hybrides diploïdes devraient être en mesure de tolérer une plus grande gamme de conditions environnementales dû à leur forte hétérozygotie (hétérosie ou vigueur des hybrides) (Lynch 1984; Hotz et al. 1999).

Il est reconnu dans la littérature que la morphologie, un des nombreux aspects de l'expression du phénotype, peut s'associer à la niche écologique (Schluter 1993). En montrant la relation entre les différences morphologiques et la variation dans l'utilisation de l'habitat, il est possible d'interpréter l'interaction entre les conditions environnementales et la variabilité génétique et/ou épigénétique dans le succès des

organismes. Les hybrides diploïdes du complexe *C. eos-neogaeus* démontrent une grande variabilité de phénotypes (Doeringsfeld et al. 2004). La caractérisation morphologique des hybrides a démontré que ceux-ci présentaient une variabilité aussi importante que les espèces parentales sexuées et ce, en absence de variabilité génétique entre les individus (c'est-à-dire pour une même lignée clonale) (Figure 2). Les hybrides diploïdes sont morphologiquement intermédiaires en comparaison aux espèces parentales. Il est par contre intéressant de noter que certains hybrides sont morphologiquement très similaires à *C. eos* tandis que d'autres hybrides sont morphologiquement très similaires à *C. neogaeus*. Bien qu'observée, la variabilité phénotypique en absence de diversité génétique reste inexpliquée à ce jour.

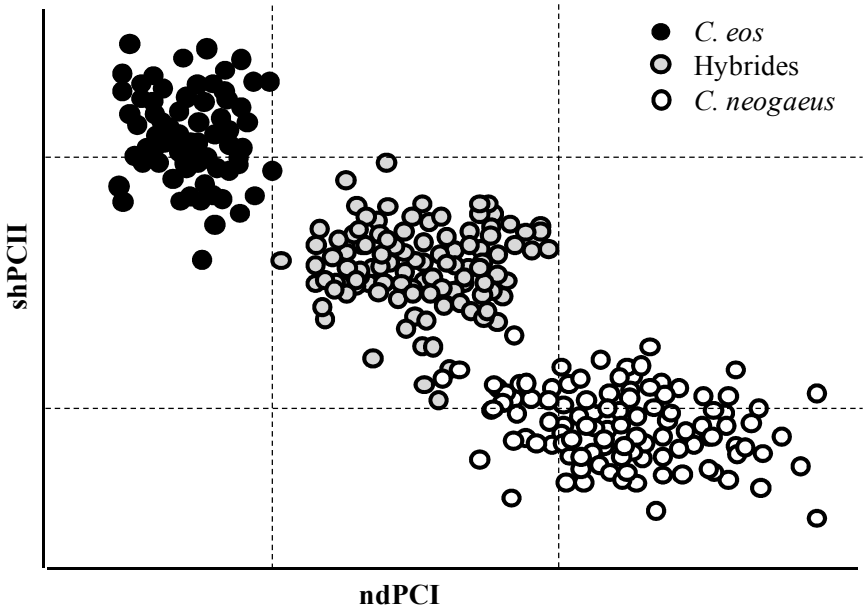

Figure 2. Relation entre les biotypes (*C.eos*, *C.neogaeus* et hybrides *C.eos-neogaeus*) pour des caractères morphologiques quantitatifs (shPCII) *versus* des caractères morphologiques qualitatifs (ndPCI) (Figure modifiée à partir de Doeringsfeld et al. 2004).

1.8.4 La fidélité au site de natalité

Les membres du complexe *Chrosomus* démontrent un comportement de fidélité au site de natalité à une fine échelle géographique (Massicotte et al. 2008). Ce comportement a été mis en évidence par la réalisation conjointe d'expérience de marquage-recapture et d'analyses génétiques. Les résultats démontrent que les individus d'un lac sont organisés en plusieurs unités reproductives occupant chacune une niche reproductive distincte. Ces différentes unités se retrouvent dans des environnements aux conditions environnementales potentiellement variables. Le comportement de fidélité au site de natalité a donc des implications importantes sur l'écologie et l'évolution des membres de ce complexe asexué en structurant les populations à une fine échelle géographique.

1.8.5 Expression différentielle des allozymes

Dans une étude précédente, les auteurs ont quantifié l'expression des allozymes (allèles *C. eos* et *C. neogaeus*) de deux enzymes (Glucose-6-phosphate isomérase, GPI et Phosphoglucomutase, PGM) chez les hybrides diploïdes du complexe *C. eos-neogaeus* (Letting et al. 1999). Les résultats indiquent une expression plus importante de l'allèle *C. eos* chez les hybrides diploïdes. L'expression significativement plus importante de l'allèle *C. eos* est fonction de l'enzyme et du tissu à l'étude. Les résultats suggèrent l'implication de processus de régulation de l'expression des allèles des gènes chez les hybrides diploïdes.

1.8.6 Intérêts du modèle biologique

En résumé, ce modèle biologique animal présente des avantages certains pour les fins de la présente étude. Premièrement, l'uniformité génétique entre les hybrides d'une même lignée clonale permet de faire l'étude de la variabilité épigénétique non liée à la variabilité génétique en milieu naturel. Contrairement aux études effectuées sur les jumeaux monozygotes, il est possible de comparer les profils épigénétiques de plusieurs individus d'une même lignée clonale par population et pour plusieurs populations naturelles. De plus, la variabilité phénotypique observée implique des processus de régulation de l'expression des gènes indépendants de la variabilité génétique ce qui suggère un effet des processus épigénétiques. Enfin, une même lignée clonale se retrouve dans des environnements différents sur une grande répartition géographique (Angers and Schlosser 2007). Cela permet de faire l'étude des effets de l'environnement sur la variation épigénétique entre les hybrides clonaux. Il est aussi important de mentionner la présence d'un allèle de chacune des espèces parentales (*C. eos* et *C. neogaeus*) pour tous les gènes (hémizygotes) chez les hybrides diploïdes clonaux.

1.9 Objectif général et hypothèses

L'objectif de cette thèse est de déterminer l'étendue de la variabilité épigénétique, plus particulièrement du polymorphisme de méthylation de l'ADN, non liée à la variabilité génétique dans les populations asexuées en milieu naturel afin de mieux cerner l'importance des processus épigénétiques en écologie et en évolution. Afin de répondre à cet objectif, des expériences visant à quantifier la variabilité épigénétique à l'échelle de l'individu et entre les individus de différentes populations seront

réalisées. Les trois hypothèses suivantes ont été testées sur le complexe *C. eos-neogaeus* dans les articles 2, 3 et 4.

Notre première hypothèse est qu'en absence de variabilité génétique, la variabilité épigénétique (polymorphisme de méthylation de l'ADN) entre les individus est le résultat des effets conjoints des processus stochastiques et de l'environnement.

Notre deuxième hypothèse est que la variabilité épigénétique observée est façonnée par les conditions environnementales auxquelles les individus sont exposés.

Notre troisième hypothèse est que l'intérêt des processus épigénétiques est de permettre la flexibilité (plasticité) à partir d'un génome fixe.

1.10 Structure de la thèse et contribution des auteurs

Les quatre prochains chapitres sont présentés sous forme d'articles scientifiques. Le premier article intitulé «Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after» a été coécrit avec deux autres auteurs, Bernard Angers et Émilie Castonguay. Cette revue de littérature invitée représente une suite logique à l'introduction de la thèse et la complète en faisant un survol détaillé de la relation entre le polymorphisme de méthylation de l'ADN, le phénotype et l'environnement et discute de l'importance des processus épigénétiques en écologie et en évolution. Cet article a été publié en 2010 par le journal *Molecular Ecology*.

Les trois autres articles découlent quant à eux des résultats obtenus dans le cadre de ce projet de doctorat. Le deuxième article intitulé «DNA methylation: a source of random variation in natural populations» a été coécrit avec deux autres auteurs, Bernard Angers et Emma Whitelaw. Il porte sur l'évaluation de l'étendue de la variabilité épigénétique non liée à la variabilité génétique sur des populations asexuées en milieu naturel. Les facteurs responsables du polymorphisme de méthylation de l'ADN à l'échelle de l'individu et entre les individus de différentes populations sont discutés. J'ai effectué les manipulations en laboratoire, l'analyse des données et rédigé l'article. Bernard Angers a participé à l'analyse des données et les deux co-auteurs ont participés à la rédaction de l'article. Cet article a été publié en 2011 par le journal *Epigenetics*.

L'article trois intitulé «Variable DNA methylation of transposable elements in *Chrosomus eos-neogaeus* hybrid genome» a été coécrit avec Bernard Angers. Dans le cadre de la thèse, il est pertinent d'étudier la méthylation des ET ces loci sont spécifiquement réprimée par ce processus épigénétique. De plus, cet article propose de démêler les effets de la réunion de génomes interspécifiques de ceux associés à d'autres processus pouvant intervenir lors de la formation du zygote. Le niveau et le patron de méthylation de copies paralogues d'un élément transposable présentes chez les hybrides *C. eos-neogaeus* et les espèces parentales, *C. eos* et *C. neogaeus*, ont été comparés. Les résultats permettent de conclure en l'absence d'une différence significative des profils de méthylation entre les hybrides et les espèces parentales. Cela suggère que des processus autres que la réunion de génomes inter-spécifiques, tel que les effets maternels et/ou paternels, occasionneraient la perturbation des

processus épigénétiques responsables du contrôle des éléments transposables. J'ai effectué les manipulations en laboratoire, l'analyse des données et rédigé l'article. Bernard Angers a participé à l'analyse des données et à la rédaction de l'article. Cet article est accepté pour publication (avec corrections) par le journal *Genetica*.

Le quatrième article intitulé «General Purpose Genotype or how epigenetics extend the flexiblity of a genotype» a été coécrit avec Bernard Angers. Il discute du lien entre les processus épigénétiques, la flexibilité d'un génome clonal et la valeur adaptative en absence de différence génétique face à des changements de l'environnement chez les vertébrés asexués. L'observation de polymorphisme de méthylation de l'ADN chez des hybrides *C. eos-neogaeus* d'une même lignée clonale mais provenant de différentes populations indique un effet de l'environnement sur le façonnement de l'épigénome. Deux groupes de populations ont été identifiés sur la base des profils de méthylation. Une différence de pH a été observée entre les lacs permettant de faire le lien entre les profils de méthylation et une condition physico-chimique de l'environnement local. J'ai effectué les manipulations en laboratoire, l'analyse des données et rédigé l'article. Bernard Angers a participé à l'analyse des données et à la rédaction de l'article. Cet article a été publié en 2011 par le journal *Genetics Research International*.

2. ARTICLE 1

Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after

Bernard Angers, Emilie Castonguay et Rachel Massicotte

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Résumé

Les organismes font souvent face aux changements de l'environnement en produisant des phénotypes alternatifs. Les processus épigénétiques, tel que la méthylation de l'ADN, peuvent contribuer à la variation phénotypique induite par l'environnement en modifiant l'expression des gènes. Les changements de la méthylation de l'ADN, au contraire des mutations de l'ADN, peuvent être influencés par l'environnement; sont stables dans le temps à l'échelle d'un individu et présentent différents niveaux d'héritabilité. Ces caractéristiques font de la méthylation de l'ADN un processus moléculaire potentiellement important afin de répondre aux changements environnementaux. L'objectif de cette revue de littérature est de présenter les implications de la méthylation de l'ADN sur la variation phénotypique suivant un changement de l'environnement. Plus spécifiquement, nous explorons les concepts épigénétiques qui concernent un changement de phénotype en réponse à l'environnement et l'héritabilité de la méthylation de l'ADN, en particulier l'effet Baldwin et l'accommodation génétique. Avant d'adresser ce point, nous reportons les différences majeures de la méthylation de l'ADN entre les taxa et le rôle des changements de la méthylation de l'ADN dans la production et le maintien de la variation phénotypique induite par l'environnement. Nous présentons aussi les différentes méthodes qui permettent la détection du polymorphisme de méthylation de l'ADN. Nous croyons que cette revue de littérature intéressera les écologistes moléculaires car elle met en lumière l'importance des processsus épigénétiques en écologie et en évolution.

Abstract

Organisms often respond to environmental changes by producing alternative phenotypes. Epigenetic processes such as DNA methylation may contribute to environmentally induced phenotypic variation by modifying gene expression. Changes in DNA methylation, unlike DNA mutations, can be influenced by the environment; they are stable at the time scale of an individual and present different levels of heritability. These characteristics make DNA methylation a potentially important molecular process to respond to environmental change. The aim of this review is to present the implications of DNA methylation on phenotypic variations driven by environmental changes. More specifically, we explore epigenetic concepts concerning phenotypic change in response to the environment and heritability of DNA methylation, namely the Baldwin effect and genetic accommodation. Before addressing this point, we report major differences in DNA methylation across taxa and the role of this modification in producing and maintaining environmentally induced phenotypic variation. We also present the different methods allowing the detection of methylation polymorphism. We believe this review will be helpful to molecular ecologists, in that it highlights the importance of epigenetic processes in ecological and evolutionary studies.

2.1 Introduction

The interactions between an organism and its biotic and abiotic environment are continuous and diversified. Environmental conditions often change rapidly and in unpredictable ways, challenging the organism's survival and reproduction. When possible, individuals can move to escape unfavourable conditions. Alternatively, the maintenance of internal equilibrium by physiological homeostasis or individual genetic variability (Hedrick 1998) can provide a buffer against environmental changes. However, many organisms respond to environmental changes by modifying their original phenotype. This may encompass changes in an individual's development, morphology, physiology or behaviour (Agrawal 2001; Price et al. 2003).

Alternative phenotypes produced during the lifetime of an individual can be achieved through regulation of the expression of a specific gene or activation of an alternative gene pathway (Schlichting and Pigliucci 1993; Pigliucci 1996). An important property of environmentally induced phenotypes is that the associated variations in gene regulation are not necessarily heritable—the gene is always transmitted but not necessarily its expression state. On the other hand, the capacity to respond to environmental cues is often heritable, indicating a genetic basis for this process. The ability to produce alternative phenotypes could have evolved to maximize the fitness of individuals in variable environments (Dudley and Schmitt 1996; Debat and David 2001).

Although many examples of environmentally induced phenotypes have been described and the associated changes in gene transcription measured (Mori et al. 2005; Derome et al. 2006; Sumner et al. 2006), the underlying mechanisms responsible for regulating which genes' expressions should change in response to a specific environmental variation are still poorly understood.

Changes in gene expression may occur through epigenetic modifications (Jaenisch and Bird 2003). Epigenetic modifications refer to changes in gene expression that are stable throughout mitoses but also reversible and that occur without changes in the underlying DNA sequence. The most direct way of tagging a gene for expression or silencing is to place a chemical mark directly on its DNA. DNA methylation is indeed the most studied and probably the best understood type of epigenetic modification (for an overview of other types of epigenetic modifications, see box 1). Such a mechanism could represent a way to allow phenotypic variability in a changing environment without having to rely on genetic variation.

Phenotypic variation is central in ecology and evolution and often plays a role in adaptation, niche shift, population dynamics, and evolutionary diversification (Agrawal 2001; Debat and David 2001; Price, Qvarnstrom, and Irwin 2003; West-Eberhard 2003; Pigliucci et al. 2006). The objective of this review is to present the role of DNA methylation in creating phenotypic variation driven by environmental changes. DNA methylation exists in all living organisms with important differences among and even within taxa. We describe the extent and the differences in DNA methylation across taxa. We then examine the processes responsible for creating

variation in DNA methylation and how they link the environment with phenotypic changes through modulation of gene expression. We present the tools and framework available to measure DNA methylation polymorphism in natural populations and to assess its evolutionary importance. Finally, we present how concepts related to phenotypic change in response to the environment, namely the Baldwin effect and genetic accommodation, can be explained by heritable (or not) changes in DNA methylation patterns and we discuss the evolutionary relevance of these epigenetic processes.

2.2 DNA methylation

DNA methylation, the incorporation of a methyl group (CH_3) to specific nucleotides, is the most widespread epigenetic modification. Indeed, DNA methylation is detected throughout all domains of life, in Eubacteria, Archea, and Eukaryotes. The establishment and maintenance of DNA methylation is achieved by specific enzymes known as DNA methyltransferases. The sequence similarity of these enzymes in bacteria, plants, and animals suggests a common origin (Ponger and Li 2005). Among them, the DNMT3 family is responsible for the establishment of methylation patterns on DNA (*de novo* methylation) and the DNMT1 family ensures the maintenance of these marks during DNA replication. The role of other methyltransferases is less clear (e.g., DNMT2) or is specific to given taxa (e.g., chromomethylases [CMT] in plants or DNMT 4, 5 in fungi; (Ponger and Li 2005).

Though DNA methylation may appear to be a hallmark of all living organisms, numerous differences have been reported among and even within taxa.

First, there are differences in which nucleotides are methylated and at what molecular position. Depending on the organism, the methyl group may be incorporated on the N6 position of the adenine or at different positions on the molecular structure of the cytosine (N4 or C5) by using distinct DNA methyltransferases. Methylation of adenine is found in Eubacteria and Archea, but in Eukaryotes it is restricted to some unicellular organisms (e.g., *Tetrahymena*; (Hattman 2005) and the chloroplastic genome of land plants. However, methylation at the C5 position of cytosine is common throughout all domains of life and is the only DNA modification convincingly reported in multicellular Eukaryotes (Suzuki and Bird 2008 and references therein). The distribution of 5-methylcytosine is not random in the genome, and organisms with DNA methylation differ in the type of sequences that are methylated. For example, methylation occurs mainly at the CpG dinucleotide (adjacent cytosine and guanine linked by a phosphate) in vertebrates but also at CpNpG sequences in plants and at CpNpN (N could be A, C, G, or T) sequences in plants and fungi (Ito et al. 2003).

Organisms also differ in the pattern of DNA methylation across their genome (Suzuki and Bird 2008 and references therein). For instance, in vertebrates, methylated sites are distributed globally across the genome: all types of DNA sequences (genes, transposable elements, intergenic DNA) are subject to methylation. The exception to this global methylation is short unmethylated regions, the CpG islands, that represent only a small fraction of the genome (1–2%) and that are generally associated with housekeeping genes. In other animals, methylation has a mosaic pattern, with methylated domains interspersed with unmethylated domains.

The highest methylation levels are observed in plants, where up to 50% of cytosines can be methylated in certain species. In maize, for example, these high levels of methylation are associated with the large number of transposons present in the genome. However, other plants such as *Arabidopsis thaliana* display mosaic patterns of DNA methylation similar to what is seen in non-vertebrate animals (Chan et al. 2005).

The additional layer of information provided by DNA methylation alters neither the DNA sequence of the gene nor that of the RNA or the protein, but can actually regulate the expression of the gene. First, the level of DNA methylation in the promoter region—a sequence upstream of a gene required for its transcription—is generally negatively correlated to levels of gene expression. Repression of transcription is expected to occur when specific proteins—the methyl-CpG-binding proteins—bind to the methylated promoter instead of the transcription factors and subsequently recruit chromatin remodelling complexes; this action eventually closes the chromatin to gene transcription (Boyes and Bird 1991; Weaver et al. 2004). However, DNA methylation was also found to be targeted on the transcription units of actively transcribed genes in *A. thaliana*, where it is likely to reduce transcriptional noise by preventing spurious initiation of transcription (Bird 1995; Weber et al. 2005; Zilberman et al. 2007). In addition, it seems that not only the amount but also the pattern of methylation (in terms of which specific CpG dinucleotide is methylated) are important in determining levels of gene expression. For instance, in a study by Weaver et al. (2004), where they compared the methylation status of CpG dinucleotides in the promoter of the glucocorticoid receptor gene when it was

expressed and when it was not expressed, the variation in the methylation status of a single CpG dinucleotide was found to be relevant in determining whether the gene would be expressed. Similarly, comparisons between active and inactive X chromosomes in female humans revealed that the active X chromosome was overall more methylated than the inactive X chromosome. This observation first seemed counterintuitive since higher levels of methylation are generally associated with an increase in silencing. However, a closer look at the methylated sequences revealed that the promoters of the inactive chromosome were hypermethylated whereas the extra methylation on the active X chromosome was actually located on the gene bodies (Hellman and Chess 2007).

Gene regulation by DNA methylation is involved in different functions (Colot and Rossignol 1999; Suzuki and Bird 2008). First, DNA methylation can serve as a protection system against transposable elements. This role has been convincingly reported in plants and fungi and it might also be present in mammals (Suzuki and Bird 2008). Also, the genes required for transposition of mobile elements are generally heavily methylated for repression. In mammalian dosage compensation (Avner and Heard 2001), DNA methylation is involved in the inactivation of one of the two female X chromosomes, which leads to the expression of a single X chromosome, therefore mimicking the situation prevailing in males. DNA methylation is also involved in genomic imprinting (Li, Beard, and Jaenisch 1993; Wilkins 2005). Genomic imprinting has been detected in mammals and in some plants and is established during gametogenesis, where sex-specific methylation alters the expression of hundreds of genes. In the zygote, imprinted genes are either

expressed only from the allele inherited from the mother or from the allele inherited from the father. Most imprinted genes are required for normal development. Finally, the role of DNA methylation is not restricted to endogenous gene regulation. In bacteria, for example, DNA methylation serves to protect the bacterial genome from invasion by extracellular DNA. Indeed, while bacterial restriction endonucleases cleave the foreign DNA, they do not recognize the methylated sequences of the bacterial genome.

2.3 How does DNA methylation affect phenotype?

Epigenetic processes are crucial in coordinating changes in gene expression leading to cell lineage differentiation during an organism's development (Bird 2002). However, DNA methylation is not exclusively influenced by intrinsic signals during development. Indeed, examples of spontaneous or environmentally induced changes in methylation profiles are increasingly reported, and these are the object of the current review. Numerous studies have highlighted the relevance of such processes in creating phenotypic variation (Cubas, Vincent, and Coen 1999; Rakyan et al. 2003; Chong and Whitelaw 2004; Weaver et al. 2004; Blewitt et al. 2006; Manning et al. 2006; Richards 2006; Whitelaw and Whitelaw 2006; Crews et al. 2007; Vaughn et al. 2007; Kucharski et al. 2008; Jablonka and Raz 2009). Through DNA methylation, the genome can integrate environmental signals and as a result, these extrinsic signals can potentially directly modify the phenotype without changing the underlying DNA sequence.

Changes in gene expression through DNA methylation may have profound phenotypic repercussions. For example, studies with plants in which the establishment and the maintenance of methylation marks were disrupted by mutagenesis or chemical treatment yielded phenotypically aberrant individuals, thus giving evidence for a correlation between DNA methylation and the phenotype (Fieldes and Amyot 1999); Kalisz and Purrugganan 2004 and references therein). The list of alternative phenotypes resulting from alternative DNA methylation states of a same gene is continuously growing (e.g., Jablonka and Raz 2009). Alternative methylation states of a same gene, known as epialleles, have been associated with variations in individual behaviour, physiology, and morphology as well as development (Weaver et al. 2004; Anway et al. 2005; Jeon 2008). How DNA methylation can cause phenotypic variation through the modification of gene or gene pathway expression is exemplified by the Colorless non-ripening (*Cnr*) gene, a component of the regulatory network controlling fruit ripening in tomato. The *Cnr* phenotype differs from the wild-type phenotype by a colourless and mealy pericarp. Comparison of the cytosine methylation patterns of the wild-type and *Cnr* phenotypes revealed that cytosines at the promoter of the *Cnr* gene (SQUAMOSA promoter binding protein-like genes) are extensively methylated in all individuals carrying the *Cnr* phenotype whereas they are largely unmethylated in wild-type fruits (Manning et al. 2006).

2.4 How do variations in DNA methylation appear?

There are key events at which such variations in DNA methylation patterns may occur. First, the DNMT1 enzymes responsible for copying the methylation marks

during DNA replication have an error rate of 5% of CpG per cell division (Riggs et al. 1998; Bird 2002) compared to 10^{-9} per nucleotide per cell division for DNA polymerase. Errors in the replication of the initial epigenetic state of a gene lead to epigenetic variations among cells of the same tissue, a process that can lead to phenotypic variegation. Such spontaneous and random epigenetic errors may provide a large spectrum of alternative methylation states for the same genetic sequence. For instance, a dominant mutation at the *Agouti* gene (A^{vy} allele) in mice results in an extensive variation of coat colouration (Morgan et al. 1999). The A^{vy} allele displays a variable degree of expression that is linked to the level of methylation of a transposable element inserted upstream of the *Agouti* gene: if the transposable element is hypomethylated across cells, A^{vy} is ectopically expressed and the coat colour is more yellow; if the transposable element is hypermethylated, *Avy* is expressed normally and the coat colour is normal. Because the methylation level of the transposable element displays extensive variation, the expression of the *Agouti* gene varies from cell to cell, which leads to a large variation in the coat colouration of an individual (e.g., mottling; Morgan et al. 1999). The extent of the variegated yellow and normal coat is linked to the random methylation of the A^{vy} allele among cell lineages.

Secondly, a nonmethylated sequence can be *de novo* methylated (or vice versa). *De novo* methylation is induced by intrinsic developmental signals. However, it may also appear randomly (as a spontaneous variation) or be induced by environmental signals throughout the life of an individual. For example, in an isogenic strain of mice containing the A^{vy} allele, extensive phenotypic variation can be

observed among individuals, indicating inter-individual variations in random *de novo* methylation at the *Agouti* gene (Morgan et al. 1999; Blewitt et al. 2006). Another spectacular example of alternative *de novo* methylation leading to the production of distinct phenotypes can be observed in social insects. In the honeybee (*Apis mellifera*), phenotype is determined environmentally via the feeding of royal jelly to larvae meant to be fertile queens but not to larvae meant to be sterile workers. Kucharski and collaborators (2008) induced the inactivation in bee larvae of the enzyme responsible for *de novo* methylation and thereby demonstrated that the normal developmental pathway was to provide sterile individuals, but that interruption of the spread of methylation at a precise moment during the development led to the production of fertile individuals.

Interestingly, the variations observed in agouti mice can also be influenced by the environment. Maternal nutrient supplementation with a diet rich in methyl donors during gestation will globally increase the levels of DNA methylation at the transposable element associated with the *Agouti* gene and increase the proportion of the progeny with a normal phenotype (Wolff et al. 1998). Inversely, neonatal exposure to bisphenol A decreases methylation, therefore shifting the coat colour toward the mutant phenotype (Dolinoy et al. 2007). However, these effects of environment are not similar to those observed in social insects in that not all individuals exposed to these conditions will exhibit a given alternative phenotype. In addition, this epigenetically induced phenotypic variation has profound consequences on the individual's fitness because the effects of the *Agouti* gene are pleiotropic, and mice with a hypomethylated transposable element will be obese, have a non-insulin-

dependent diabetic-like condition, and have a propensity to develop a variety of tumours.

These previous examples highlight how the environment and DNA methylation may trigger different gene expression patterns to produce different phenotypes from genetically identical individuals. In these examples, the epigenetic state is important in determining the phenotype; the genotype by itself cannot explain the phenotype.

2.5 Heritability of methylation

One of the properties of DNA methylation marks is their transmission through mitosis: these marks are conserved during DNA replication by the action of DNMT1. This situation refers to mitotic epigenetic inheritance and concerns transmission within an individual's lifetime (Crews 2008). However, for epigenetic variation to affect inheritance, meiotic transmission is also required. Some examples of meiotically transmitted methylation marks have indeed been reported. The transgenerational effects of DNA methylation require either a direct or indirect alteration of methylation in the germ line. This is termed meiotic epigenetic inheritance (Crews 2008). Therefore, in addition to perpetuating a change in gene expression throughout an individual's life, DNA methylation could also transmit the effect of the environment on gene expression to further generations, even in the absence of initial stimulus (Crews et al. 2007; Jablonka and Raz 2009). An interesting characteristic of DNA methylation is that inheritance of the variants appears to be highly variable among affected genes as well as among taxa (Rakyan et al. 2002).

Depending on the genotypic context (see Richards 2006 for discussion) and the taxon, some methylation marks will be transmitted across generations whereas others will be limited to the lifetime of an individual.

DNA methylation has most extensively been studied in mammals, where methylation patterns are erased (to some extent) and reset twice during development. There is a first global genome demethylation during gametogenesis (but see (Flanagan et al. 2006) and a second one during the period following fertilization. Erasure of methylation patterns has also been shown to occur during zebrafish development (MacKay et al. 2007), suggesting a common pattern in vertebrates. Therefore it seems that the DNA methylation marks that these organisms acquire during their life will not be transmitted to their progeny. However, the extent of erasure of epigenetic marks was found to vary among multicellular organisms, and even in mammals this erasure is not absolute (Richards 2006; Hitchins et al. 2007). Indeed, in organisms where the gametes appear later in development and where there is a less extensive erasure of epigenetic marks, such as plants, a higher propensity for methylation mark transmission is expected (Richards 2006). In support of this, most epialleles have been detected in plants (Kalisz and Purugganan 2004).

Though it seems that DNA methylation by itself is a process whose effects are in many cases limited to the lifetime of an individual, DNA methylation can interact at several levels with other mechanisms that may indirectly promote its transmission (Blewitt et al. 2006). Indeed, it seems that DNA methylation influences and could be influenced by small regulatory RNAs. For example, previous observations that the

environment can affect microRNA expression could be accounted for by the fact that environmentally induced DNA methylation regulates the expression of microRNA genes. Therefore, the effects of environmentally induced methylation could be indirectly propagated across generations via RNA molecules that are transmitted in the cytoplasm of gametes. Evidence for such indirect effects of DNA methylation has not yet been identified, but inheritance of a phenotypic variant via RNA can be observed in paramutation (Rassoulzadegan et al. 2006; Chandler 2007). Paramutations are defined as the modification of the effective expression of an allele (paramutated allele) by another homologous allele (paramutator allele). An example of this is found in mice where an engineered allele of the kit locus (paramutator allele) leads to the production of small interfering RNAs (siRNA) that degrade messenger RNA produced by the wild-type allele (paramutated allele) (Rassoulzadegan et al. 2006). The wild-type allele's DNA sequence remains unchanged, but there is loss of effective expression of the gene. This silencing of the wild-type allele leads to a white-tipped tail and white feet phenotype. Interestingly, through transmission of these siRNAs in the cytoplasm of the gametes, progeny that inherited the wild-type allele can also display the alternative phenotype. These siRNA will then interfere with the expression of the wild-type alleles in the next generation. However, in absence of the paramutator allele, the phenotype is diluted each generation.

2.6 How to study DNA methylation

While the field of cancer epigenetics demonstrated more than a decade ago that changes in DNA methylation and gene regulation occur in cancer, there is still a

dearth of studies addressing the importance and the frequency as well as the heritability of epigenetic variation in natural populations (Kalisz and Purugganan 2004; Richards 2008). Most current knowledge on DNA methylation comes from the comparison of epigenetic profiles of individuals of a same species with highly divergent phenotypes. The examples presented in the previous paragraphs are isolated and spectacular cases. There is as yet no sense of how widespread these types of phenomena are or of the importance of the challenge they present the current evolutionary theory. The implications of a widespread heritable environmentally induced epigenetic variation are potentially quite important. This section is meant as an overview of the methods available for quantifying DNA methylation polymorphism and for investigating the effects of methylation on phenotypic variation.

The amounts of DNA methylation in a genome have traditionally been analyzed by high-performance liquid chromatography (HPLC). Although this technique has successfully been used to assess global changes of methylation in different experimental contexts (Cai and Chinnappa 1999), it does not allow the detection of the methylation state at the single gene level.

The tools for investigating variations in DNA methylation at the gene level are available and can easily be incorporated into any laboratory studying DNA polymorphism (Liu and Maekawa 2003; Suzuki and Bird 2008). Methylation is a chemical mark added to the DNA, and there is no complementary nucleotide specific to methylated cytosines. It is not possible to detect the presence of methylation by

directly using classic PCR-based analyses or sequencing because methylated and non-methylated cytosines are indistinguishable. However, methylated cytosines can be labelled prior to PCR amplification. Two different approaches can be envisaged: methods using endonucleases with different sensitivities to methylation or methods where non-methylated cytosines are chemically altered.

The presence of a methyl group on their restriction site can affect the capacity of certain bacterial endonucleases to recognize this site. Methylation sites can be identified by comparing the restriction fragment patterns generated by enzymes that have the same restriction site but different sensitivities to the methylation of this site. The isoschizomeric enzymes HpaII and MspI, for example, both recognize the CCGG sequence, but HpaII is unable to cut the DNA when the internal cytosine is methylated. Surveys using Methylation Sensitive Amplified Polymorphism (MSAP) (Xiong et al. 1999), a variant of the AFLP (Vos et al. 1995), can then be performed without further treatment. A limitation of this technique is that the endonuclease only detects differences in methylation that occur at its restriction site.

The gold standard for the detection of methylation polymorphisms remains sodium bisulfite treatment of DNA prior to PCR analyses. This chemical treatment allows the conversion of unmethylated cytosines to uracil while methylated cytosines remain unchanged (Frommer et al. 1992). Sequencing of treated and untreated DNA allows the identification of all methylated cytosines in a given sequence. To screen for variations in DNA methylation at the scale of a population, bisulfite treatment can be used prior to SSCP (Maekawa et al. 1999), methylation-allele–specific PCR using

primers ending on a CpG dinucleotide (methylation-sensitive PCR) or microarray analysis (Yamamoto and Yamamoto 2004).

However, except in model species or well-known gene pathways (Lister et al. 2008; Meissner et al. 2008; Lister et al. 2009), the question is not so much how but where to look for methylation differences in the genome. Surveys for candidate sequences can be achieved with AFLP-based techniques by comparing across individuals restriction fragment patterns generated by enzymes with different sensitivities to methylation or by comparing DNA treated or untreated with sodium bisulfite.

Screening for variations in DNA methylation patterns is different from screening for variations in DNA sequence because methylation patterns are time and tissue specific. Indeed, even though the cells of multicellular organisms are genetically identical, they present structural and functional heterogeneity. A developmental program may lead to the production of more than 200 different cell phenotypes, most of which can be accounted for by variations in DNA methylation (Bird 2002; Meissner et al. 2008). Epigenetic regulation of gene expression is also thought to be a dynamic process, with the methylation status of a gene potentially changing in response to developmental and environmental cues and aging (Fraga et al. 2005). Therefore, not only are different cell types within a given organism likely to have very different DNA methylation patterns (different epigenomes or methylomes), but fluctuations in time can also be expected even within the same cell. For instance, the analysis of the methylation polymorphism of the human major

histocompatibility complex (MHC) revealed that a significant proportion of these genes show variegation (tissue-specific methylation profiles) in addition to inter-individual epigenetic variation (Rakyan et al. 2004).

DNA methylation can be influenced or not by the environment, is more or less independent of the associated genetic background, and displays different levels of heritability. Once inter-individual variations in DNA methylation have been detected, it is of interest to characterize these elements to determine the evolutionary significance of these variations. DNA methylation is affected by the environment and the genotype as well as by their interaction (Richards 2006), and can therefore be considered as a phenotypic trait (Gorelick 2004, 2005). The quantitative genetics framework can thus be used to establish the relative importance of the environment, the genotype, and their interaction on this phenotypic trait. The variation of a given phenotype can be measured by controlling for genetic background and environment; for instance, by using an experimental design involving several replicates of a genotype (clones, full siblings) maintained in controlled environmental conditions. A common garden experiment can assess developmental flexibility while reciprocal translocation of environmentally induced phenotypes can be used to investigate plasticity (Cai and Chinnappa 1999). Following this kind of approach, “pure” epigenetic marks are those whose presence correlates with the environmental conditions whereas marks that are independent from the environment are referred to as genetically obligate marks (Richards 2008).

A similar challenge is faced when trying to study the effect of DNA methylation on the phenotype since the phenotype is influenced by DNA variations, the environment, and DNA methylation as well as by the interactions of these elements (Gorelick 2004, 2005; Bossdorf et al. 2008; Richards 2008). Variations in DNA methylation can be correlated with phenotypic variation by using approaches similar to those discussed in the previous paragraph. In addition, in the case of heritable methylation marks, the environment experienced by previous generations must also be known since it can affect the offspring's phenotype (Gorelick 2004). Alternative approaches can also be considered to investigate the role of DNA methylation on phenotype, such as those involving treatments that affect the establishment or the maintenance of methylation marks (Fieldes and Amyot 1999; Kucharski et al. 2008).

2.7 Methylation, ecology, and evolution

The relevance of phenotypic variation in the domains of ecology and evolution is now widely accepted. Environmentally induced phenotypes are coined either phenotypic plasticity or developmental flexibility. Phenotypic plasticity refers to the capacity of an individual to change its phenotype throughout its life in response to a change in environments (Callahan et al. 1997). On the other hand, developmental flexibility is the production of different phenotypes from individuals harbouring a similar genotype, depending on the environmental conditions experienced during their development (Bradshaw 1965). The effects of developmental flexibility (the reaction norm) are most readily observed when comparing the phenotypes of

genetically identical individuals reared in different environments (Thoday 1953). The ability to produce such phenotypic variation is an evolving property, and evolutionary changes can also be mediated by phenotypic variation (Debat and David 2001; Young and Badyaev 2007).

Because of its role in gene regulation and the creation of phenotypic variation as well as its versatility, DNA methylation is expected to contribute to the persistence and evolution of populations in multiple ways. Indeed, epigenetic variation creates phenotypic differences that have an effect on individual fitness and therefore can be acted upon by natural selection (Crews et al. 2007). In addition, DNA methylation may allow individuals to use different strategies in fluctuating environments depending on its degree of inheritance. DNA methylation, unlike genetic modifications, may occur rapidly in response to environmental changes and could therefore represent a potential way to cope with environmental stress on very short time scales, possibly even during the lifetime of an individual (Rando and Verstrepen 2007). Because it could then allow individuals to produce alternative phenotypes in response to environmental change, DNA methylation would be a relevant process even in the absence of inheritance.

Following changes in the environment, alternative methylation patterns may be established on certain sensitive alleles, possibly giving the individuals that possess them an alternative phenotype (Figure 3a). The frequency of advantageous methylation-sensitive alleles will therefore increase in subsequent generations, thereby increasing the number of individuals apt to react to environmental

fluctuations. In this scenario, there is no inheritance of the methylation marks: DNA methylation modulates gene expression in response to the environment, while the genetic background provides heritability of the genes required for flexibility. It is the flexibility of the phenotype that is selected for rather than the result of the flexibility itself (Figure 3b). This type of scenario, known as the Baldwin effect (Simpson 1953) reviewed in (Crispo 2007), could be advantageous in unstable or highly heterogeneous environments.

Production of phenotypic variants through DNA methylation may help in exploring alternative environments and consequently in providing a wider niche. For example, some authors showed how environmentally induced phenotypes (heritable or not) may initiate the transition from one adaptive peak to another by allowing the exploration of the adaptive landscape without leaving the high fitness peak linked to the underlying genotype (Pal and Miklos 1999). DNA methylation could provide an additional process to induce a peak shift that, unlike genetic drift, does not require demographic reduction or small population size (shifting balance theory) (Wright 1932). Regulation of gene expression through DNA methylation may also provide asexual organisms with developmental flexibility, possibly explaining observations where populations of clonal organisms were found to display as much phenotypic variability as closely related sexual species (Doeringsfeld et al. 2004; Gorelick 2010).

In what circumstances is the inheritance of an environmentally induced phenotype advantageous? Predictability (variability across generations) and reliability (variability within generation) of the environmental conditions are components

expected to influence selection on phenotypic variation (West-Eberhard 2003). While a fluctuating environment should favour a high level of plasticity (Baldwin effect, Figure 3b), new environmental conditions that are stable both within and across generations should favour genetic assimilation (Young and Badyaev 2007). Genetic assimilation refers to a reduction of the variability around a new phenotype following environmental changes (Figure 3c). For example, the heritability of an environmentally induced phenotype becomes favourable if the variability around the new phenotype is low and the phenotype is close to its optimum (Waddington 1961; Pal and Miklos 1999; Crispo 2007). However, this process requires long-term heritability of the marks responsible for the apparition of the new environmentally induced phenotype. The efficiency of transmission of such marks or cellular memory is essential to enhance the strength of the phenotypic selection (Pal 1998). Because of the delay between the induction and the selection of the new phenotype, the environment must be stable for a period at least as long as the organism's generation time in order for it to be adaptive (Lachmann and Jablonka 1996). In completely random or highly fluctuating environments, the persistence of induced phenotypes for several generations even in the absence of the inducing environmental conditions that produced the phenotype seems to represent the optimal strategy when compared to strictly fixed (genetic) or completely inducible (plastic) strategies (Jablonka et al. 1995). An epigenetic inheritance system could ensure the transmission of the marks responsible for the environmentally induced phenotype from parents to their progeny. Such a mechanism may facilitate the transition between individual plasticity and long-term evolutionary innovations and allow adaptation at an intermediate time frame.

When inherited across generations, variation in DNA methylation becomes similar to genetic variation and serves as a template to natural selection and other evolutionary forces such as drift and migration. However, because of its reversibility and more labile nature, the persistence of DNA methylation is not expected to be as stable as DNA mutations over a long period of time. During the process of genetic assimilation, the environmentally induced phenotype becomes genetically assimilated, and the environmental signal as well as the epigenetic marks are no longer required to produce it (Waddington 1953). While the role of DNA methylation may only be transient, it remains crucial in initiating the exploration of the adaptive landscape by inducing phenotypic variation in response to the new environmental conditions (Figure 3c).

2.8 DNA methylation and modern evolutionary synthesis

Cases of environmentally induced heritable variations in DNA methylation causing phenotypic differences with an impact on fitness have been reported (Crews et al. 2007; Crews 2008). These findings challenge the existing theory of evolution (modern synthesis) that supports the view that the information that is transmitted changes only at random without any direction from the environment toward any phenotypic outcome. Indeed, it rather seems the environment could be responsible for both the apparition of variation and its selection (Richards 2006; Jablonka and Lamb 2008).

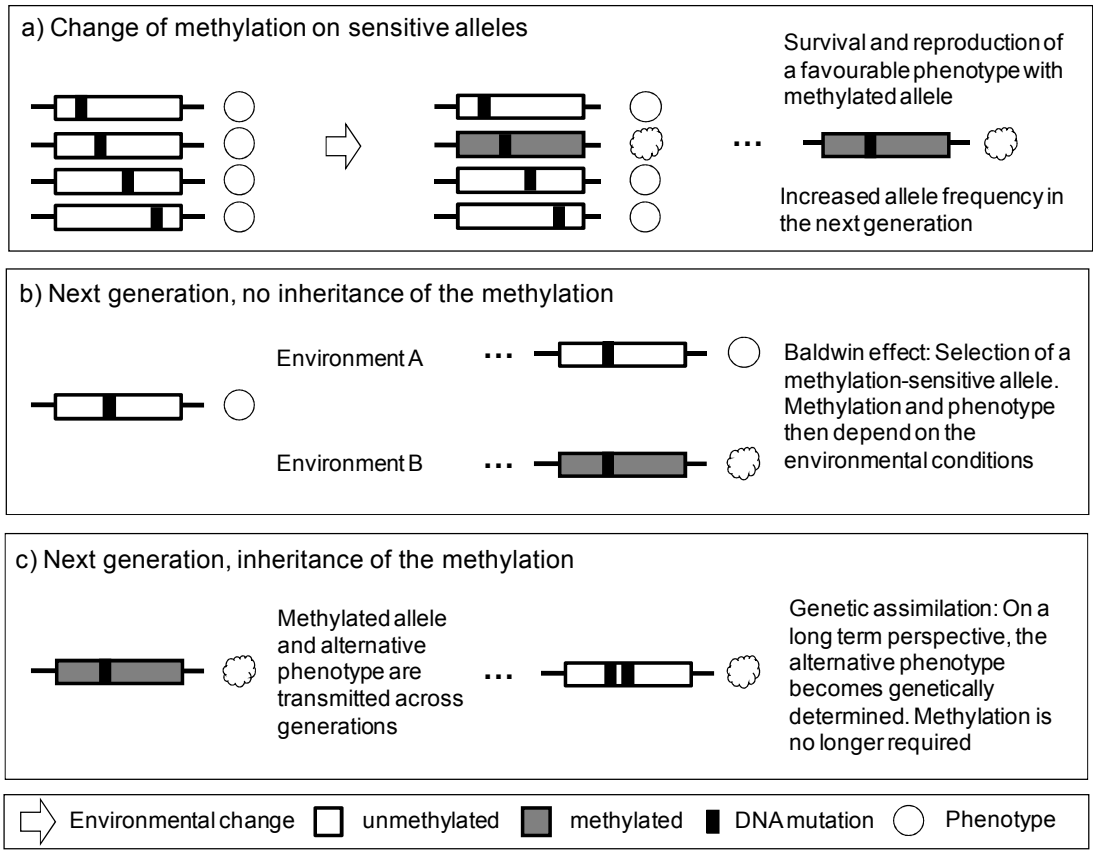

Figure 3. Consequences of the inheritance (or not) of methylation marks following an environmental change. a- As a consequence of a given environmental change, the methylation of some alleles will be modified depending on their sensitivity. If the resulting alternative phenotype is advantageous, the frequency of this allele will increase in the next generation as a result of natural selection. b- In the next generation, in the absence of inheritance of the new methylation marks, the initial phenotype will be restored in environment A whereas the alternative phenotype may reappear in environment B (for the same reason it appeared in A) because the selected allele is more sensitive to environmentally induced epigenetic variation. c- In the next generation, in the presence of inheritance of the new methylation marks, the alternative phenotype is maintained for several generations until a mutation replaces the effect of methylation.

A major source of confusion regarding the effect of environment on phenotype is the belief that all environmentally-induced changes produce a phenotypic adaptation. The effect of larval diet on the methylation patterns and the phenotype of social insects (Kucharski et al. 2008) and how the nursing by mother rats reduces stress in their offspring (Weaver et al. 2004) are examples of how the environment, can act as an intrinsic cue to modify development, resulting in a predetermined phenotypic change (Figure 4a).

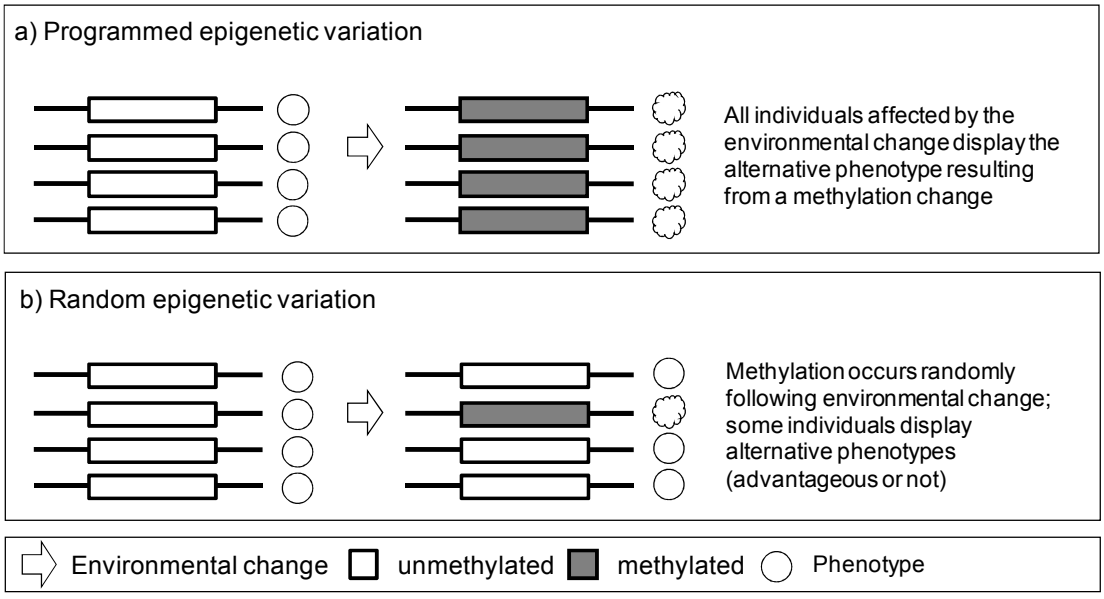

Figure 4. Environmentally induced DNA methylation and phenotypic consequences. a- An environmental cue can act as an intrinsic signal in initiating a particular developmental pathway, resulting in a predetermined phenotype as observed in social insects. b- Environmental changes act randomly among individuals, resulting in distinct phenotypes upon which selection can act.

However, environmental changes do not always result in a predictable phenotypic outcome (Figure 4b). Individuals as well as genes are likely to display different thresholds or sensitivities to a given environmental signal (Sollars et al. 2003), and a given gene could be affected differently by environmentally induced methylation in different individuals, resulting in distinct phenotypes on which selection can act. The resulting phenotypes are thus not always favourable. For example, induction of aberrant DNA methylation by environmental toxicants during critical developmental periods has been known to lead to inappropriate gene expression and disease pathogenesis in later life (Dolinoy, Huang, and Jirtle 2007; Perera et al. 2009).

DNA methylation and other epigenetic processes appear not to be in disagreement with the theory of modern evolutionary synthesis. Such processes allow organisms to use the environment to modify development as a "signal of a programmed change" (or modify their development in response to environment). But environmentally driven methylation changes are expected to occur as randomly as mutations, and according to the outcomes of these changes, the phenotype will be selected for or against. Even though environment appears to be responsible for both the creation of and selective pressures affecting variation, these two processes are independent.

2.9 Conclusion

Many of its characteristics make DNA methylation a versatile mechanism for modifying gene expression and phenotype. Methylation marks are enzymatically

modifiable, can change rapidly, and are reversible, which is not the case with DNA mutations. They are also conservatively replicated through mitosis and, in some cases, through meiosis as well. It is unclear which characteristics of DNA methylation prevail in natural populations and how they impact evolutionary processes. However, because of the variety of genes, genotypes, and organisms involved, and because of the spatial and temporal heterogeneity of the environment, it is expected that a broad range of conditions exists where non-heritable methylation marks permit rapid adjustment to the environment or where, in certain circumstances, transgenerational marks lead to local adaptation and promote divergence until speciation (Jablonka and Lamb 1991; Jablonka and Lamb 1995; Pal and Miklos 1999; Pigliucci and Murren 2003; West-Eberhard 2003; Schlichting 2004; de Jong 2005; Bonduriansky and Day 2009). This also suggests that all organisms do not react in the same way to environmental changes due to evolutionary differences in DNA methylation among taxa or to different genetic backgrounds among individuals and populations.

DNA methylation and other epigenetic processes also suggest that a reconsideration of the nature of the heritable material is needed (Gorelick and Laubichler 2008; Bonduriansky and Day 2009), but they are not otherwise in disagreement with the modern evolutionary synthesis. Though it may seem to add to the complexity of carrying out biological research, taking into account the additional layer of information provided by DNA methylation has already provided insightful explanations to biological phenomena that could not be accounted for by variations in

DNA sequence. The study of DNA methylation and other epigenetic processes may soon be an integral component in the study of any biological process.

2.10 Box 1. Other epigenetic processes affecting phenotype

DNA methylation is an essential gene regulation process that can influence an individual's phenotype. However, several taxa, including model organisms such as the fruit fly *D. melanogaster*, the nematode worm *C. elegans*, and the yeast *S. cerevisiae*, have undetectable or very low levels of DNA methylation. These organisms nonetheless display extensive phenotypic variation, indicating that DNA methylation is not the only process responsible for phenotypic variation. In these organisms, as well as in organisms for which DNA methylation is present, other epigenetic processes are important in determining the phenotypic outcome. These other processes may affect gene expression at the transcriptional level, as does DNA methylation, or at the post-transcriptional level.

DNA is intimately associated with histone proteins; modifications of the histone tails are known to control the packaging of DNA, therefore regulating the access to the genes for transcription. Polycomb and Trithorax systems include numerous genes that encode for proteins modifying histone tails to a repressed (barrier to gene transcription) or active (accessible to transcription) chromatin state, respectively. It is thought that these genes are important in regulating the expression of house-keeping genes and developmentally important genes such as the Hox genes (Meissner et al. 2008). This epigenetic system and its effects on phenotype have been extensively studied in *Drosophila* (Hauenschild et al. 2008). It is also important to

note that DNA methylation and Polycomb/Trithorax are interwoven epigenetic systems (Meissner et al. 2008).

Phenotypic variation may also be due to the release of previously hidden genetic (Rutherford and Lindquist 1998) or epigenetic (Sollars et al. 2003) variation in response to stressful environmental conditions. For instance, under normal environmental conditions, the protein chaperone Hsp90 assures correct folding and function of proteins in spite of mutations the proteins might contain in their sequence. The variability buffered by such a mechanism can be revealed if Hsp90 function is compromised (for instance, following an environmental stress), therefore resulting in an increase in phenotypic variation.

3. ARTICLE 2

DNA methylation: a source of random variation in natural populations

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Résumé

Les processus épigénétiques (par exemple, la méthylation de l'ADN) ont été proposés comme des mécanismes évolutifs potentiellement importants. Cependant, avant de conclure sur leur importance en évolution, l'indépendance de la variation épigénétique et de la variation génétique ainsi que l'étendue du polymorphisme de méthylation de l'ADN en milieu naturel doivent être évalués. Nous avons évalué ces deux points sur des populations naturelles d'un poisson clonal, *Chrosomus eos-neogaeus*, pour lequel des individus génétiquement identiques sont retrouvés dans des environnements distincts. Un survol du génome a confirmé l'uniformité génétique des individus alors qu'un niveau substantiel de variabilité entre les individus a été détecté pour la méthylation de l'ADN. L'étude de l'état de méthylation des dinucléotides CpG d'un fragment de retrotransposon a confirmé une différence marquée dans la composition épialléliques entre les tissus et les individus. Cette étude apporte une évidence supplémentaire de variation épigénétique en absence de variation génétique et démontre que ce processus peut être une source de variation aléatoire dans les populations en milieu naturel.

Abstract

Epigenetic processes (e.g., DNA methylation) have been proposed as potentially important evolutionary mechanisms. However, before drawing conclusions about their evolutionary relevance, we need to evaluate the independence of epigenetic variation from genetic variation, as well as the extent of methylation polymorphism in nature. We evaluated these in natural populations of a clonal fish, *Chrosomus eos-neogaeus*, for which genetically identical individuals may be found in distinct environments. A genomic survey confirms the genetic uniformity of individuals, whereas a substantial level of inter-individual variation results in DNA methylation. Survey of the methylation status of the CpG dinucleotides of a fragment of a retrotransposon confirmed a marked difference in epiallelic composition amongst tissues, as well as amongst individuals. This study provides further evidence of epigenetic variation in the absence of genetic variation and demonstrates that this process can be a source of random variation in natural populations.

3.1 Introduction

Epigenetic processes are crucial in regulating gene expression leading to cell differentiation during development (Bird 2002). However, the epigenome is not exclusively influenced by the standard developmental program (Jaenisch and Bird 2003), and evidence accumulates on the environmental effects on methylation profiles and gene expression (Waterland and Jirtle 2002; Weaver et al. 2004; Crews et al. 2007; Dolinoy, Huang, and Jirtle 2007; Kaminen-Ahola et al. 2010; Verhoeven, Jansen et al. 2010). The genome is able to integrate environmental changes and, as a result, this extrinsic signal can potentially modify the phenotype without changing the underlying DNA sequence. Numerous studies have highlighted the relevance of such process in creating phenotypic variation (Cubas, Vincent, and Coen 1999; Rakyan et al. 2003; Chong and Whitelaw 2004; Weaver et al. 2004; Blewitt et al. 2006; Manning et al. 2006; Richards 2006; Crews et al. 2007; Kucharski et al. 2008; Jablonka and Raz 2009; Kaminen-Ahola et al. 2010) one of the cornerstones of evolution (Debat and David 2001). Thus, epigenetic processes enable the interaction of the genome with the environment and may influence the phenotype of an individual via the regulation of gene expression.

DNA methylation is one of the most studied epigenetic marks (Razin and Riggs 1980; Boyes and Bird 1991; Weaver et al. 2004), although almost nothing is known about the extent of DNA methylation polymorphism in natural populations. Quantification of this variability is particularly important to determine the biological relevance of methylation variation in promoting the phenotypic diversification of individuals under different natural environmental conditions.

Another major concern about the implications of epigenetic processes in evolution is their dependence on genetic variation (Richards 2008). While the relevance of epigenetic variation that is completely linked to genetic variation is questionable, pure (or facilitated) epigenetic variation may provide an additional evolutionary process (Richards 2008; Angers et al. 2010). Before arguing about the specific effect of epigenetic variation in modifying the phenotype, pure (or facilitated) epigenetic variation must be observed in natural populations (Richards 2006). However, working in natural populations of sexual organisms raises some difficulties, especially when trying to disentangle epigenetic from genetic variation.

The environment can influence development and lead to predetermined phenotypic changes, as exemplified by the role of the diet in the determination of the honeybees reproductive status (Kucharski et al. 2008). In order to be consistent with the synthetic theory of evolution (Pigliucci 2007), the effect of the environment must lead to some random methylation changes that could potentially be adaptive (Angers, Castonguay, and Massicotte 2010). The effect of DNA methylation has been extensively studied following the observation of some aberrant phenotypes that were first thought to be the result of DNA mutations (Cubas, Vincent, and Coen 1999; Manning et al. 2006), as well as in few model organisms including human (Morgan et al. 1999; Sheldon et al. 1999; Fraga et al. 2005). However, the randomness of environmentally-driven methylation changes as a source of variation in natural populations remains largely unknown.

The aim of this project is to assess the extent of epigenetic variation independent of the genetic variation in natural populations of a vertebrate species. To rule out the effect of DNA polymorphism on the methylation profile, naturally occurring genetically identical individuals (clones) were studied. The all-female *Chrosomus eos-neogaeus* (Cyprinidea, Pisces; formerly *Phoxinus*)(Strange and Mayden 2009) resulted from a few ancestral hybridization events between female finescale dace (*C. neogaeus*) and male northern redbelly dace (*C. eos*) (Dawley, Schultz, and Goddard 1987). The diploid *C. eos-neogaeus* hybrids are pseudogamous and reproduce clonally via gynogenesis (Goddard and Dawley 1990). The presence of only a few clonal lineages throughout heterogeneous habitats represents an exceptional natural system to investigate naturally occurring epigenetic variation not related to genetic variation.

3.2 Results

3.2.1 Genetic vs. epigenetic variation in asexual hybrids

We first assessed the global level of DNA methylation over the *C. eos-neogaeus* genome using a wild-type mouse genome as a reference. Digestion pattern obtained from isoschisomeric endonucleases that are sensitive to CpG methylation (HpaII) or not sensitive to CpG methylation (MspI) provided contrasting results, indicating abundant methylation in the genome of hybrids. However, we observed similar level of DNA methylation between *Chrosomus* hybrids and wild-type mouse genomes (Figure S1). Similar results have been reported in zebrafish, *Danio rerio* (Suzuki and Bird 2008).

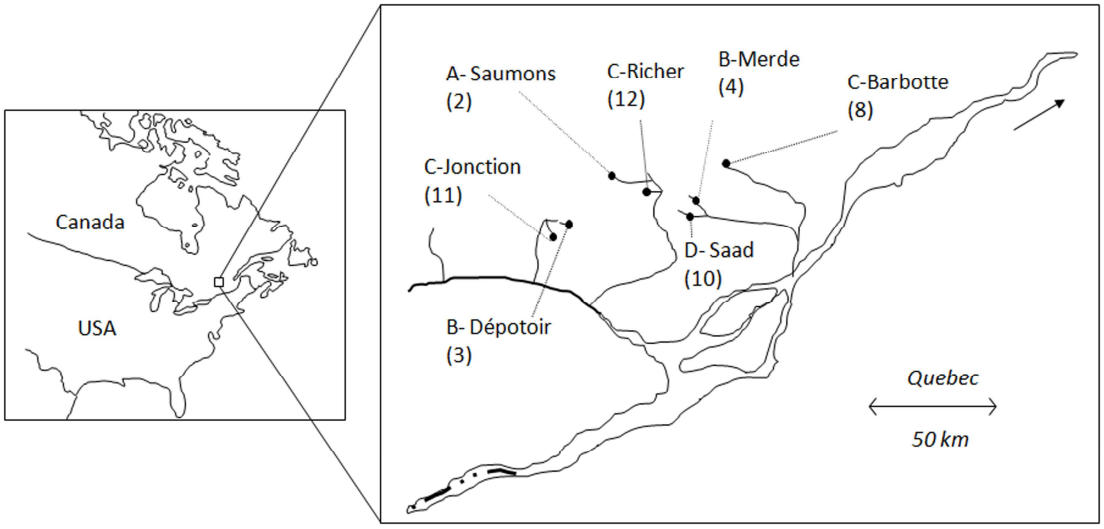

Figure 5. Sampled lakes in the Laurentian Lakes, Quebec, Canada. The number of gynogens hybrids sampled per lake is indicated in parentheses. Capital letters refer to the habitats characterization according to Schlosser et al. (1998) A- pond of moderate depth, B- a shallow beaver pond, C- a moderately deep area of open water upstream from a beaver dam, D- pond of moderate depth with flooded standing and fallen trees.

We then compared the extent of genetic and epigenetic variation across the *C. eos-neogaeus* genome with a MSAP analysis performed on four tissues of four individuals from distinct populations (Figure 5). A total of 630 reproducible fragments detected between 150 and 600 bp were assessed. The presence of genetic polymorphism was detected at only 6 of these singleton fragments representing 1.5 mutations per individual (Tableau S1). This leads to a level of genetic variation of 1.7 x 10^{-4} changes per nucleotide, considering the 14 nucleotides surveyed per fragment (8 nucleotides of restriction sites and 6 nucleotides of the selective primers). This low level of genetic variability is similar to what is observed in other clonal organisms

(Singh et al. 2002), and largely contrasts with sexual species, for which 60% or more of the loci are polymorphic (Gagnaire et al. 2009).

On the other hand, 93 fragments (14.76%) revealed methylation polymorphism among tissues (Tableau S1). Interestingly, for a given tissue, 76 of these fragments are also variable among individuals representing 4.5 epimutations per tissue per individual (Tableau S1). Assuming a similar number of MseI and MspI/HpaII restriction sites over all fragments and considering that, per fragment, a single CpG is surveyed for methylation change, this leads to a level of epigenetic variation of 7.1×10^{-3} methylation changes per CpG per tissue that are possibly distinct from those encoded by the developmental program.

To determine if methylation polymorphism occurred in regions of transcriptional activity, a total of 15 loci were randomly selected for sequencing amongst the 76 fragments variable among individuals (Tableau 1). Eleven fragments displayed a strong homology with zebrafish sequences (taxonomically close to *Chrosomus*) (Tableau 1). Ten sequences could be classified as CpG rich regions as they had a ratio of observed-over-expected number of CpGs higher than 0.6, a density of G+C higher than 0.5 and were longer than 200 bp (Bird 1986). These sequences are generally located within genes, in gene regulatory regions, or within transposable elements.

Tableau 1. Sequence analysis of the 15 candidate loci obtained from MSAP.

Fragments	Size (bp)	Nb. CpG	CpG Ratio (Obs./Exp.)	C + G content (%)	Accession number, sequence homology and coverage
A1	301	18	0.95	54	NF
A2	357	18	0.81	48	NF
B1	224	8	0.57	54	NF
B2	377	19	0.81	48	BX120005; 81% Hist2h2l and n.c.r.; 100%
E1	320	9	0.45	43	CT37172, 93% n.c.r. pbx3a gene; 90%
H1	368	22	0.96	45	787 hits, unidentified repetitive element
I1	287	21	1.17	55	AL732455, 93% n.c.r. putative zinc finger protein; 78%
I2	289	6	0.33	46	XM691587; 89% BTB/POZ domain and n.c.r.; 48%
I3	340	22	1.04	49	BX248505; 80% 5'end n.c.r.putative TBC1protein; 100%
I4	338	11	0.52	56	NM_001128347; 93% putative SHANK2 protein; 100%
I5	358	17	0.76	49	FP236359; 74% no annotation; 69%
I6	377	18	0.76	50	160 hits; 57% DIRS1 group retransposons; 79%
J1	356	10	0.42	48	NF
J2	447	16	0.57	50	FP243361; 83% no annotation; 84%
N1	334	20	0.96	54	BC162697, 82% atonal homolog 1b; 59%

- NF, no homology detected to zebrafish genome sequences in databases
- n.c.r., non-coding region
- Exact identification of two out of 15 candidates was not possible because different clones of a given fragment provided distinct sequences. Such a drawback is, however, frequent when sequencing fragments from AFLP/MSAP because distinct fragments of the same size cannot be distinguished from one another on the denaturing polyacrylamide gel (Meksem et al. 2001).

3.2.2 DNA methylation profiles

We further investigated the extent of DNA methylation polymorphism at a candidate locus (I6; Tableau 1), characterized by its homology with a retrotransposon from the DIRS1 group (Goodwin and Poulter 2001), detected in *Tetraodon nigrivis* and *Danio rerio*. Nearly 80% of the I6 sequence is homologous to DIRS1. However, no homology was detected at the 5' extremity (77 bp), while a large deletion characterized the 3' region (Figure S2). Other DIRS1 sequences detected in *C. eos-neogaeus* did not present these characteristics and are more similar to those of *T. nigrivis* and *D. rerio* (Figure S2). The I6 locus has not been detected in other lineages of hybrids (A4, A5, and A6) (Angers and Schlosser 2007), and nor in any of the *C. eos* or *C. neogaeus* parental species sampled from the same lakes (Figure 5). These characteristics suggest that I6 locus is an incomplete fragment of a recent transposition of DIRS1 in this specific hybrid lineage. Finally, SSCP analysis of genomic DNA confirmed the absence of genetic variability among 14 individuals belonging to seven populations (Figure 5) and strongly suggests that this is a single copy locus (Figure S3A, B). This characteristic added to the fact that transposable elements are targeted by DNA methylation makes this locus a very interesting candidate to study epigenetic variation.

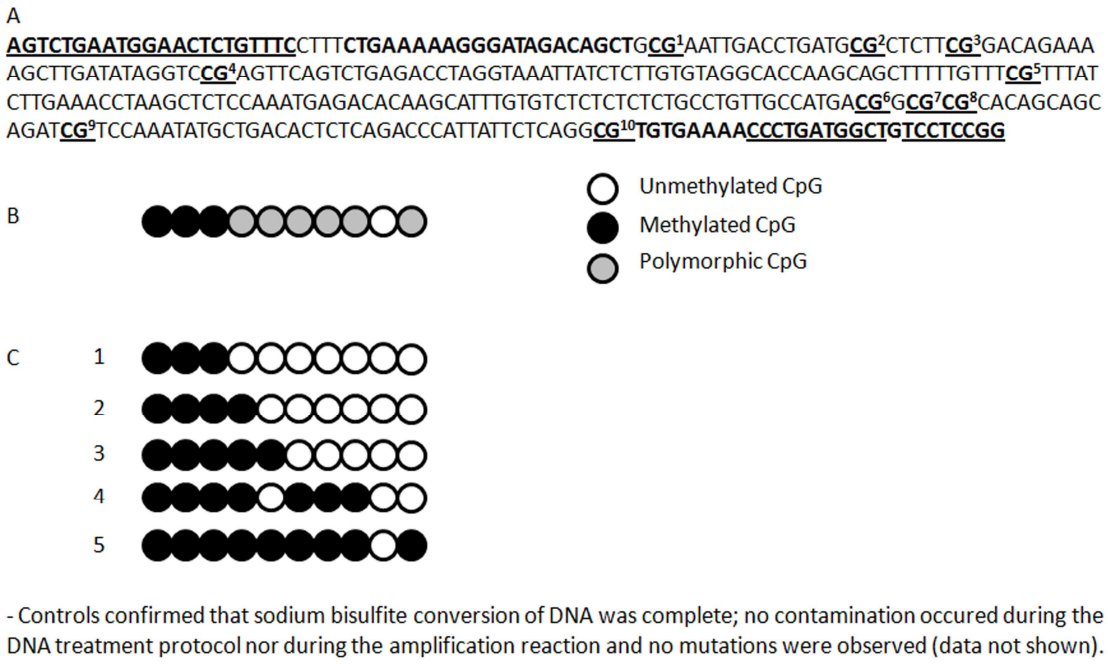

Figure 6. Methylation polymorphism at the candidate locus I6. (A) Sequence of I6 locus with primers detail and location of CpG dinucleotides; (B) Location of invariant (unmethylated or methylated) and polymorphic CpGs along I6 sequence; (C) Variation of the methylation profile among the 5 epialleles detected.

Methylation polymorphism along the sequence of I6 locus (Tableau 1 and Figure 6A) was assessed by bisulfite treatment of DNA, MS-SSCA gel followed by sequencing of the observed epivariants. Contrasting with the lack of variation on genomic DNA, MS-SSCA revealed distinct electromorphs (Figure S3C, D). Bisulfite sequencing of epivariants (51 bands excised from MS-SSCA gels) recovered five epialleles. Two epialleles largely predominate (epiallele 3, 39.2% and epiallele 5, 49%; Figure 6C) while epialleles 1, 2, and 4 were detected in a given tissue for only a few individuals (Figure 7). Sequencing also revealed that epialleles only differ by their methylation profile, no nucleotide mutations were observed and bisulfite

conversion was complete. Epiallele 3 harbours four unmethylated CpGs that are otherwise methylated for epiallele 5 (Figure 6C). The others epialleles differ by one or two methylated sites from the predominant epialleles (Figure 6C) and are always associated with one of the predominant epivariants.

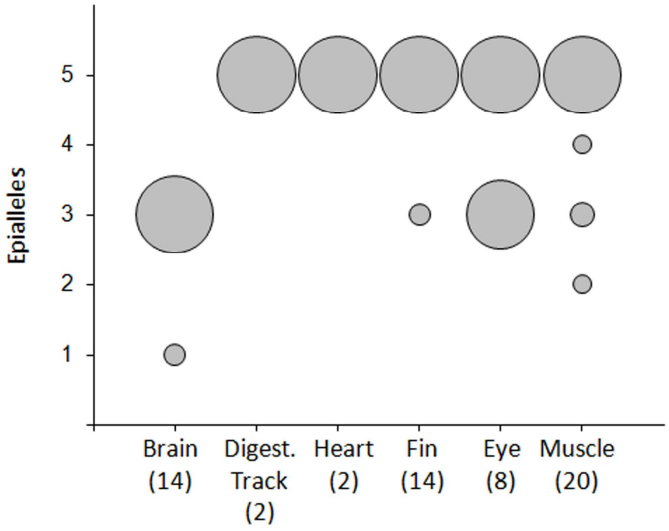

Figure 7. Epiallele frequency distribution for the six tissues analyzed. For each tissue, the diameter of the circles represents the percentage of individuals displaying a given epiallele. The total percentage for a tissue can be different from 100% due to variegation. The number of individuals analyzed per tissue is given in parentheses.

The six tissues analysed varied in their methylation profiles. The heavily methylated epiallele (epiallele 5; Figure 6C) characterises the heart (2 individuals), fin (14 individuals), digestive track (2 individuals) and muscle (20 individuals) (Figure 7) while the less methylated epiallele (epiallele 3; Figure 6C) is present by

itself only in the brain (14 individuals) (Figure 7). Both epialleles 3 and 5 (Figure 6C) occur in the eye (8 individuals) (Figure 7).

While most of the individuals display the same methylation pattern for a given tissue, inter-individual variation was detected on all tissues for which at least eight individuals were assessed. One individual revealed different methylation profiles for the brain (7%) or the fin (7%), two individuals for the eye (25%) and five for the muscle (25%) (Figure 8). For a given tissue, the alternate methylation profile is the same among individuals. We failed to detect any relationship between the epivariants and the lake of origin, which are supposedly representative of different environments. Similarly, there is no relationship between epivariants and the different tissues analyzed for a given individual.

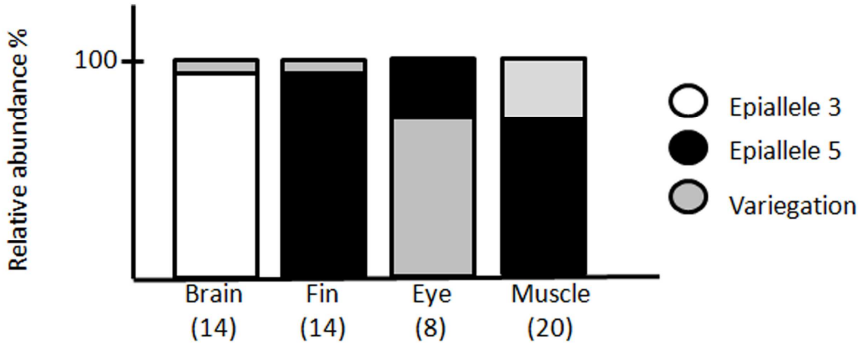

Figure 8. Methylation polymorphism among individuals. The relative abundance (in percentage) of the individuals displaying epialleles 3, 5 or variegation is given per tissues for which at least eight individuals were assessed. The number of individuals analyzed per tissue is given in parentheses.

3.3 Discussion

3.3.1 Genetic vs. epigenetic variation in asexual hybrids

The global level of DNA methylation of *C. eos-neogaeus* appears similar to that of wild-type mice, indicating the model organism used in this study does not exhibit an abnormal methylation level that could have been related to its hybrid status or asexual mode of reproduction. Similarly, studies performed on asexual dandelions lineages did not report abnormal DNA methylation changes from parents to F1 (Verhoeven, Van Dijk et al. 2010). This is consistent with studies in other plants species such as *Arabidopsis thaliana* (Cervera et al. 2002). This is in sharp contrast with the very low levels of CpGs methylation observed in *Daphnia magna* hybrid complexes (ranging from 0.13 to 0.81%) (Vandegehuchte et al. 2009). Such low levels of DNA methylation observed in *Daphnia* is however far from what is observed in vertebrates, plants and other invertebrate species in general (Suzuki and Bird 2008).

Our survey of the epigenome of *C. eos-neogaeus* clonal hybrids reveals a high level of variation at the inter-individual level. These results suggest that epigenetic processes such as DNA methylation may provide an important source of variability. Numerous studies have reported epigenetic variation via MSAP analysis (Salmon et al. 2005; Zhao et al. 2007; Verhoeven, Jansen et al. 2010; Verhoeven, Van Dijk, and Biere 2010). Although methylation variations are not related to sequence variation around CCGG motifs for most of these studies, other *trans* effects may have a significant influence on the epigenetic polymorphism detected. In all instances, it remains unknown to what extent DNA methylation polymorphism reflects phenotypic divergence.

Contrasting with epigenetic variation, the extremely low level of genetic variation observed is comparable to previous results for *C. eos-neogaeus* hybrids (Binet and Angers 2005), and confirmed the genetic uniformity of sampled individuals. Accordingly, both the effects of *cis* and *trans*-acting genetic factors as sources of the observed epigenetic variation could not be invoked (Verhoeven, Van Dijk, and Biere 2010). Such observations support the idea that there is some pure (or facilitated) epigenetic variation in natural populations for vertebrates (Richards 2006). This is consistent with a previous study performed on monozygotic twins (Fraga et al. 2005). The important distinction to be made here is that we investigated DNA methylation polymorphism in natural populations, over a larger number of genetically identical individuals in heterogeneous environmental conditions.

3.3.2 DNA methylation profiles

Epigenetic variation among individuals might be the result of two distinct processes. First, the incapacity to maintain the original epigenetic profile across mitotic divisions leads to a mosaic of epigenetic states among cells of the same cell type, also known as variegation (Rakyan et al. 2002). Such stochastic epimutations are expected to be frequent, based on the error rate of DNA methyl-transferase (Bird 2002). Secondly, the influence of the environment in shaping the epigenome is increasingly reported in controlled environments (e.g., temperature (Sheldon et al. 2002), diet (Feil 2006), behavior (Meaney 2001), and chemicals (Crews et al. 2007)).

In our study, the observed epigenetic variation appears to be the result of both the effect of stochastic and environmental epimutations on clones belonging to different populations. Epialleles 1, 2 and 4 likely arise from stochastic epimutations since they are at very low frequency and differ at a few CpG from the common epivariants. Such a situation has already been reported in a study performed on human germ cells (Flanagan et al. 2006). On the other hand, most of the variation observed among tissues as well as among individuals involves differences at the very same four CpG dinucleotides, making stochastic epimutations unlikely. These results suggest that the establishment of the methylation profile results in either one of the two alternate epigenetic states (epialleles 3 or 5). Such an effect resembling an on/off switch has already been observed from the epigenetic programming of CpG 16 of exon 1_7 at a glucocorticoid receptor gene promoter in the rat (Weaver et al. 2004). Furthermore, on our study, most of the individuals displayed the same profile for a given tissue, while some tissues show distinct profiles. Interestingly, transposable elements are thought to be targeted by DNA methylation in order to suppress their transcription, and, as a result, suppress their transposition elsewhere in the host's genome. Rather than being actively methylated for repression, our results suggest that this portion of transposable elements seem to acquire the methylation status of the insertion site. That the variation at the locus studied here could be the result of its proximity to a tissue-specifically expressed gene remains speculative in absence of its exact genomic position. Nevertheless, it suggests that non random processes such as intrinsic signals may in part be responsible for the establishment of these profiles among tissues.

Epivariants did not correlate with either the lake of origin or the individual suggesting that different cell types may react differently to environmental signals. The epigenome largely differs amongst cell lineages within a given tissue, among tissues within a given individual as well as among individuals within a given population. Excluding genetic variation as the cause, this suggests that whatever the process responsible for these methylation changes, individuals as well as genes are likely to display different thresholds or sensitivities to a given environmental signal (Sollars et al. 2003). Such a situation has already been reported in humans (Fraga et al. 2005), and mice (Morgan et al. 1999). Both the influence of stochastic and environmental epimutations has been proposed as source of epigenetic variation for these two models species. In contrary to the variation observed among tissues, the inter-individual variation detected at this locus (in term of either epialleles 3 or 5) for a given tissue likely occurred randomly. Such randomness is in sharp contrast with the environmentally induced programmed epigenetic variation observed in honeybees (Kucharski et al. 2008), and rats (Weaver et al. 2004), and appears much more like DNA mutations.

3.4 Conclusions and evolutionary implications

An abundant source of pure (or facilitated) epigenetic variation, in term of cytosine methylation, was detected among clonal organisms from natural populations. Epimutations that occur following an environmental change seem to establish in a random fashion as do genetic mutations. However, the epimutations may represent a faster process of variation than mutations as observed for this clonal vertebrate. Even if pure (or facilitated) epigenetic variation remains difficult to estimate for sexual

organisms, such variation is expected to have a similar impact on the evolutionary processes of these organisms.

3.5 Material and methods

3.5.1 Ethics statement.

A scientific permit (#2007-04-25-108-15SP) was delivered by the Ministère des Ressources naturelles et de la faune du Québec to Bernard Angers regarding sampling and proper handling of the specimens.

3.5.2 Genetic uniformity of sampled *C. eos-neogaeus*.

Fish from seven lakes belonging to different watersheds of the St Lawrence River, Quebec, Canada were sampled (Figure 5). These lakes were known to contain either one or both parental species (*C. eos* and *C. neogaeus*) as well as gynogens and triploid hybrids (Binet and Angers 2005; Angers and Schlosser 2007). Previous studies revealed no current event of hybridization even in lakes where both parental species are present (Binet and Angers 2005; Angers and Schlosser 2007). A genetic survey of nine microsatellite loci confirmed that the hybrids sampled belong to the same clonal lineage (Angers and Schlosser 2007). The lakes sampled were each classified as one of the four different types of environment according to a characterization previously used to describe *C. eos-neogaeus* populations (Schlosser et al. 1998).

3.5.3 Genetic identification of sampled *C. eos-neogaeus* and DNA extraction.

Sampled fish were identified using genetic markers (Binet and Angers 2005). Hybrids, characterized by the presence of one set of chromosomes from *C. eos* and one set from *C. neogaeus,* were identified using species specific primers. Gynogen hybrids (diploid) were then discriminated from triploid hybrids according to the ploidy of the nuclear genome by using eleven hypervariable microsatellites (Binet and Angers 2005; Angers and Schlosser 2007). We extracted and purified total DNA from all tissues (brain, muscle, fin, eye, digestive track and heart) of gynogenetic hybrids (Orkin 1990).

3.5.4 DNA methylation level in *C. eos-neogaeus*.

The level of DNA methylation over the whole *C. eos-neogaeus* genome was compared to that of a wild-type mouse and a mutant Dnmt1 mouse. The enzyme responsible for the maintenance methylation is not active in the mutant Dnmt1 mouse, and, as a result, the intensity of DNA methylation is highly reduced (Ashe et al. 2008). DNA was digested with MspI or HpaII restriction enzymes. The quality of the initial DNA used for digestion was assessed by the uncut DNA controls. The effectiveness of the digestion reaction with HpaII was visualized by the comparison with the Dnmt1-/- mouse DNA restriction pattern. Migration was performed on a 1% agarose gel (O'Neill et al. 1998).

3.5.5 Methylation-Sensitive Amplified Polymorphism (MSAP) analysis.

MSAP analysis was performed on four tissues (brain, eye, fin and muscle) from four individuals belonging to four populations (lakes Jonction, Merde, Richer, and

Barbotte) (Figure 5). DNA was digested with HpaII. Using a subsample of the previously digested DNA to cut with MspI, enabled us to visualize the presence of an internal restriction site that is methylated. For a given sample, the absence of fragment for both treatments indicated the presence of a mutation at a restriction site. Full methylation of both cytosines and hemi-methylation of the internal cytosines were not investigated by MSAP. As a consequence, it was impossible to distinguish them from unmethylated sequences. Fragments that displayed methylation polymorphism among samples at restriction sites were identified by the presence/absence banding pattern between the two treatments (MseI/HpaII and MseI/HpaII/MspI) (Figure S4). Aliquots (4 µl) of each sample for each primer combinations were loaded on 6% polyacrylamide gels (19:1 acrylamide to bisacrylamide) containing 8 M urea and 1X TBE gels.

3.5.6 Candidate loci sequencing.

Fifteen of the fragments that displayed methylation polymorphism were excised from the gel and placed in separate collecting tubes containing purified water. The eluted DNA was used as a template for reamplification with the appropriate selective primer combinations. The PCR products were then purified (QIAquick PCR purification kit) and cloned into the plasmid pGEM-T vector (Promega Corp.), followed by transformation in *E. coli* competent cells (strain JM109) according to the manufacturer's protocol. For each fragment, an average of three colonies was sequenced (CEQ^{TM} 8000 Genetic Analysis System, Beckman Coulter). The sequences of the fragments were compared against available sequences of the National Centre of Biotechnology Information (NCBI).

3.5.7 Bisulfite conversion of DNA, candidate locus amplification, MS-SSCA and bisulfite sequencing.

Sodium bisulfite treatment of 200-500 ng of DNA was performed according to MethylCode^{TM} Bisulfite Conversion Kit protocol (Invitrogen). A negative control was included for each bisulfite conversion protocol to make sure that no contamination occurred during treatment of genomic DNA. The candidate locus was amplified using a fully nested PCR approach with two sets of primers designed for the specific amplification of bisulfite converted DNA (Tableau S2). A negative control was also included for each PCR to make sure that no contamination occurs during the amplification reaction. Each of the two runs of nested amplification reactions contained 10 pmol of each primer (round 1 I405Bis1F x I600Bis1rev) then (round 2 I405Bis1FN x I405Bis1RB), 2.5 mM of each dNTPs, 10x *Taq* DNA polymerase buffer (Invitrogen), 0.16 unit of *Taq* DNA polymerase (Invitrogen) and 2 µl of bisulfite converted DNA or run one PCR products in a final volume of 50 µl. The PCR protocol for both runs of nested amplification was 92 °C for 3 min, followed by 35 or 45 cycles at 92 °C for 30 s for run one and run two respectively, 52 °C for 30 s, and 68 °C for 45 s and a final extension at 68 °C for 5 min. Sequence differences are expected according to the position of methylated and non-methylated cytosines following the bisulfite treatment. Differences were visualized on a Single Strand Conformation Polymorphism (SSCP) gel (Kinoshita et al. 2000; Dobrovic et al. 2002). PCR products were electrophoresed at 4 °C for 15 hours on a 5% polyacrylamide (37.5:1 acrylamide to bisacrylamide) with 5% glycerol. Each sample was amplified and electrophoresed at least two times. Polymorphic fragments were then excised from the gel and placed in a collecting tube containing purified water.

The eluted DNA was used as template for reamplification using the internal primers set (run two of the fully nested PCR). The PCR products were then purified (QIAquick PCR purification kit) and sequenced (CEQ^{TM} 8000 Genetic Analysis System, Beckman Coulter). We also collected and duplicated sequencing for each of the polymorphic fragments.

3.6 Supplementary information

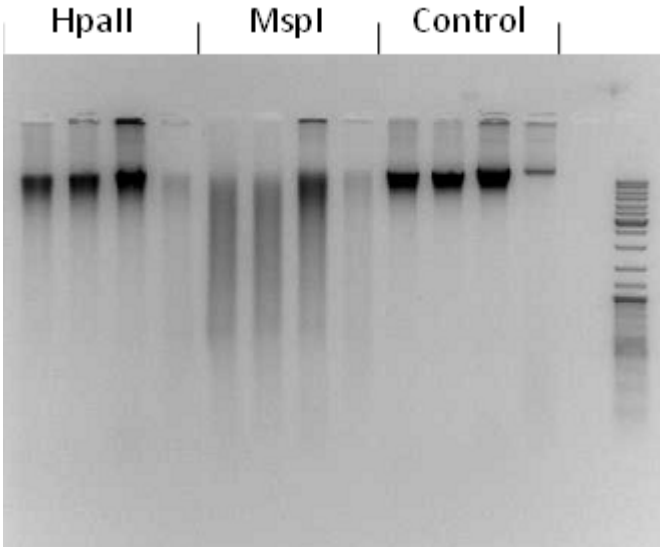

Figure S1. Restriction digests of genomic DNA with HpaII, MspI and control. Migration on a 1% agarose gel. From left to right for each treatment: two *Chrosomus eos-neogaeus* hybrids (muscle), mice (spleen), mice Dnmt1 -/- (whole embryo).

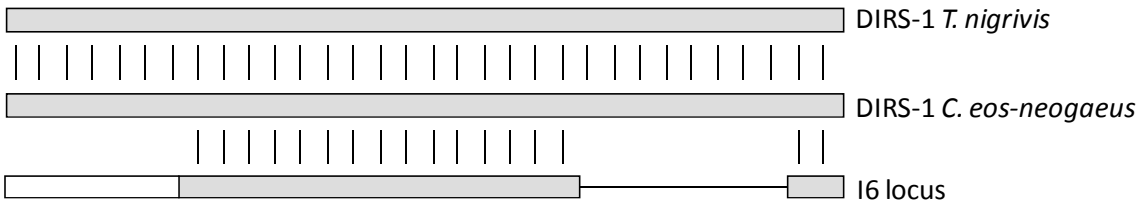

Figure S2. Homology detected among *T. nigrivis* DIRS-1 TE*, C. eos-neogaeus* DIRS-1 TE, and I6 locus. Region of I6 without homology to DIRS-1 TE is indicated by the white surface and deletion by the horizontal line.

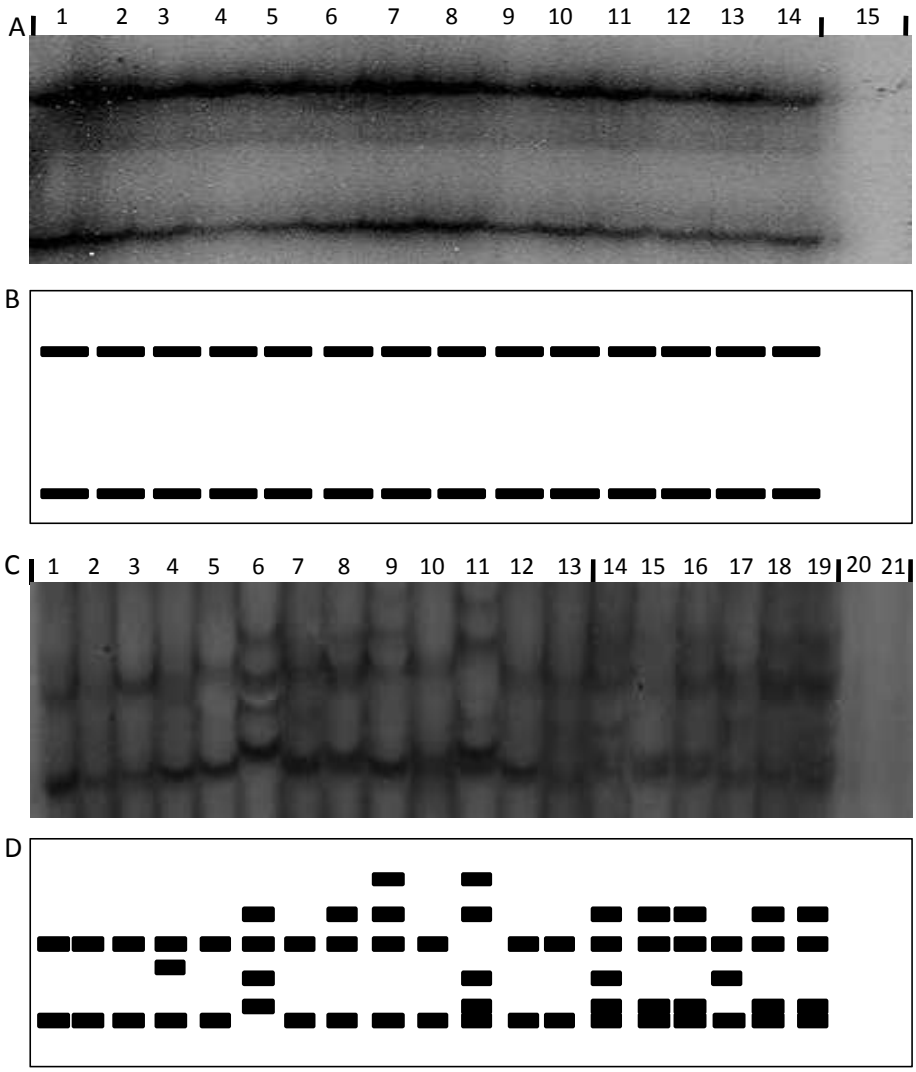

Figure S3. SSCP and MS-SSCA analysis. A) SSCP analysis performed on muscle genomic DNA from 14 individuals of seven populations (1-14) and PCR amplification control (15). B) Graphic representation of SSCP analysis. C) MS-SSCA analysis performed on bisulfite converted DNA from muscle of 13 individuals used in A (1-13), from eye of 6 individuals (14-19), contamination control for bisulfite conversion and PCR amplification are shown (20, 21). D) Graphic representation of the MS-SSCA analysis.

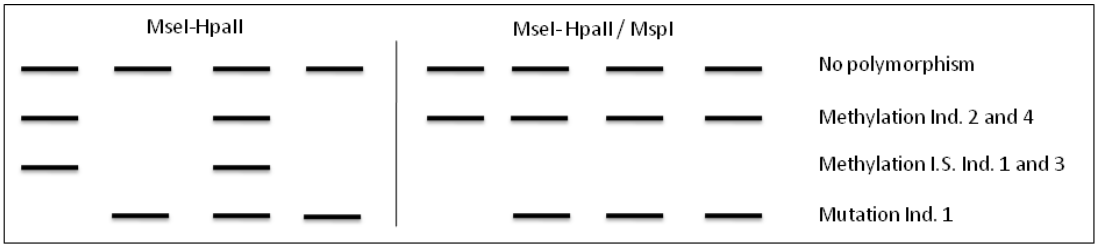

Figure S4. MSAP patterns expected from the comparison of four samples for the two treatments (MseI-HpaII *vs* MseI-HpaII/MspI). These patterns represent the four situations that enable the discrimination of the methylation states and presence of mutations.

Tableau S1. Epigenetic and genetic variation detected from the MSAP survey.
For each primer pair set (Mse – HpaII/Msp), the total number of fragments detected as well as the number of polymorphic fragments (epimutation or mutation) are given.

	MseI	MspI/HpaII	Fragments	Mutations	Epimutations
A	CTC	ATC	57	1	6
B	CCG	ATG	44	0	5
C	CTG	ATG	28	0	0
D	CGC	ACG	26	0	6
E	GCG	CTC	41	1	4
F	GGC	CTG	46	1	0
G	CGG	AGC	50	1	12
H	CCC	ACC	48	0	9
I	CAG	AGG	63	0	11
J	CAG	AGC	48	0	15
K	CGG	ACC	44	1	10
L	CGG	AGG	38	0	7
M	CCC	AGC	47	1	2
N	CTC	ACC	50	0	6
Total			630	6	93

Tableau S2. Primer details used for the two runs of nested amplification of I6.

Primer sequences	Details
Preamplification (run 1)	
AGTTTGAATGGAATTTTGTTTT	Forward primer
CCRAAAAACAACCATCAAAA	Reverse primer
Specific amplification (run 2)	
TTGAAAAAGGGATAGATAGTTG	Forward primer
CAACCATCAAAATTTTCACAC	Reverse primer

4. ARTICLE 3

Variable DNA methylation of transposable elements in *Chrosomus eos-neogaeus* hybrid genome

Rachel Massicotte et Bernard Angers

Article accepté pour publication (avec corrections) par *Genetica*.

Résumé

L'hybridation a des conséquences majeures sur la stabilité génomique des hybrides en occasionnant la perturbation de processus moléculaires importants tel que la méthylation de l'ADN. La perturbation de la méthylation de l'ADN a été associée à la mobilisation des éléments transposables dans les génomes hybrides. Des études précédentes portant sur l'évaluation des conséquences de l'hybridation sur la méthylation de l'ADN ont été réalisées sur des lignées hybrides de première génération (F1). Conséquemment, il était impossible de différencier les effets associés à la réunion de génomes interspécifiques des autres processus pouvant intervenir lors de la formation du zygote. L'objectif de la présente étude est de démêler l'influence que peut avoir le contexte génomique d'un génome hybride sur la méthylation des éléments transposables de celui des autres processus tel que les effets maternels et paternels. Nous avons tiré avantage d'un modèle biologique particulier, l'hybride diploïde *Chrosomus eos-neogaeus*, pour lequel une F1 se perpétue naturellement par reproduction asexuée. La variation de la méthylation de l'ADN de 14 groupes d'insertions détectée dans le génome hybride *C. eos-neogaeus* a été évaluée. Premièrement, la variation du niveau de méthylation de copies hautement similaires d'un retrotransposon DIRS1 reflète l'effet du site d'insertion. Bien que non significatifs, les résultats présentés indiquent un niveau de méthylation plus faible chez les hybrides *C. eos-neogaeus* en comparaison aux espèces parentales. Nous avons aussi observé un niveau de stochasticité considérable dans l'établissement des patrons de méthylation de ces loci. Dans leur ensemble, ces resultats suggèrent une réduction de la méthylation des éléments transposables après l'hybridation avec un

retour progressif à un niveau de méthylation élevé aux générations subséquentes. En conséquence, des processus tels que les effets maternels et paternels plutôt que la réunion de génomes interspécifiques seraient responsables de la perte intitiale du contrôle épigénétique des éléments transposables.

Abstract

Hybridization has profound consequences on hybrid genomic stability via the disruption of important molecular processes such as DNA methylation. Perturbation of DNA methylation has been associated with the mobilization of transposable elements in hybrid genomes. Previous studies investigating the consequences of hybridization on DNA methylation were all performed on newly formed F1 hybrid lineages. As a result, it was impossible to discriminate between the effects associated with the union of inter-specific genomes and other processes that have the potential to intervene at zygote formation. The aim of the present study was to disentangle the influence of the genomic landscape of a hybrid genome on transposable element methylation from other processes such as maternal and paternal effects. We took advantage of a particular biological model, the diploid *Chrosomus eos-neogaeus* hybrid, for which a F1 lineage is naturally perpetuating by asexual reproduction. DNA methylation variation of 14 groups of transposable element insertions detected in *C. eos-neogaeus* hybrid genome was investigated. First, the variation of the methylation level among highly similar copies of a DIRS1 retrotransposon reflects an effect of the insertion site. While not significant, the results presented here report a lower methylation level in *C. eos-neogaeus* hybrids in comparison to the parental species. We also observed a considerable level of stochasticity in the establishment of the methylation pattern over these loci. Altogether these results suggest a reduction of transposable element methylation after hybridization with a progressive return to a high level of DNA methylation over subsequent generations. Accordingly, processes such as maternal and paternal effects rather than combination of inter-specific

genomes may be responsible for the initial loss of epigenetic control of transposable elements.

4.1 Introduction

Inter-specific hybridization, the merging of two divergent genomes in one individual, leads to major genomic perturbations. In addition, maternal and/or paternal material inherited through the gametes may greatly influence hybrid genomic stability. Such genomic perturbations may have profound consequences for hybrid viability and fertility. Both the sequence divergence of incompatible alleles and the perturbation of chromatin integrity are important molecular determinants of hybrid dysfunctions (Michalak 2009).

One of the many molecular processes behind hybrid genomic instability is DNA methylation changes (O'Neill, O'Neill, and Graves 1998; Salmon, Ainouche, and Wendel 2005; Brown et al. 2008). It has been proposed that DNA methylation evolved as a mechanism to repress mobile elements (host defence model; (Bestor and Tycko 1996). Transcription and mobilization of transposable elements (TE) may cause extensive genome alterations, both in term of chromosomal rearrangements and chromatin integrity. In their study, O'Neill and coauthors (1998) have observed a global undermethylation for an interspecific mammalian hybrid (*Macropus eugenii* x *Wallabi bicolour*) (but see Roemer et al. 1999). The comparison of the parental and hybrid genomes resulted in the identification of hybrid-specific unmethylated TE. This indicates that a failure to maintain a proper level of DNA methylation leads to the activation of mobile elements which subsequently trigger chromosome remodelling. Thus, the epigenetic control of TE represents an important process that may prevent the disruption of gene regulation pathways and enable the expression of the intended individual phenotype (Rakyan et al. 2002).

Although TE are thought to be generally heavily methylated (Bird 2002), there can be variation of their methylation profile. Such variation is influenced by the TE sequences, the age and the genomic location of the insertions. Furthermore, both the level and pattern of methylation along a specific TE can vary among cells of the same tissue as well as individuals (Rakyan et al. 2002; Reiss et al. 2010; Massicotte et al. 2011). Although there is evident stochasticity in the establishment of TE methylation profiles, the influence of TE sequence has been highlighted by the similarity of the methylation status of homologous CpG dinucleotides among TE presenting little structural difference (Reiss et al. 2010). It also has been demonstrated that age has a positive influence on the TE methylation level but a negative effect on variance between cells and individuals (Reiss et al. 2010). Even more importantly, TE methylation can be modulated according to the genomic location of insertion (Park et al. 2006).

Previous studies investigating DNA methylation changes triggered by hybridization were performed on evolutionary recent (Salmon, Ainouche, and Wendel 2005) or newly formed first-generation (F1) hybrid lineages (O'Neill, O'Neill, and Graves 1998; Roemer et al. 1999; Brown, Golden, and O'Neill 2008). Thus, it was impossible to discriminate between the effects associated with the combination of inter-specific genomes and other processes that have the potential to intervene at the stage of zygote formation. The aim of the present study was to disentangle the influence of the genomic landscape of a hybrid genome on transposable element methylation from other processes such as maternal and paternal effects. The biological model used is the diploid *Chrosomus eos-neogaeus* hybrid for

which a F1 lineage is naturally perpetuating by asexual reproduction. The hybridization event that has lead to the formation of this hybrid lineage occurred ca. 30,000 years ago (Angers and Schlosser 2007). This long-lived naturally occurring F1 hybrid lineage is found in sympatry with either one or both parental species, *C. eos* and *C. neogaeus*, in many natural populations (Binet and Angers 2005; Angers and Schlosser 2007). We examined TE methylation of 14 paralogous insertions of a retrotransposon from the DIRS1 group and further investigated the variation of methylation profiles in *C. eos-neogaeus* hybrids and the parental species.

4.2 Material and methods

4.2.1 Biological model

The all-female *Chrosomus eos-neogaeus* (Cyprinidea, Pisces; formerly *Phoxinus*) (Strange and Mayden 2009) resulted from a few ancestral hybridization events between female finescale dace (*C. neogaeus*) and male northern redbelly dace (*C. eos*) (Dawley, Schultz, and Goddard 1987). The diploid *C. eos-neogaeus* hybrids are pseudogamous and reproduce clonally via gynogenesis (Goddard and Dawley 1990). Hybrids from the same lineage are genetically identical (i.e. clones) and hemizygous at each locus.

4.2.2 Genetic identification

The fish were identified using genetic markers. Briefly, the diploid hybrids, characterized by the presence of one set of chromosomes from *C. eos* and one set from *C. neogaeus,* were identified using species specific primers (Binet and Angers 2005). Diploid *C. eos-neogaeus* hybrids were then discriminated from triploid

hybrids according to the ploidy level of the nuclear genome by using eleven hypervariable microsatellites as detailed in (Binet and Angers 2005; Angers and Schlosser 2007). The microsatellite analysis also enabled the identification of the clonal lineage (clonal lineage B6; Angers and Schlosser 2007).

Tableau 2. Summary of samples analyzed and sequenced. Sample biotype, origin (Population, Individual, and Tissue), and number of clones sequenced for each primer pair (368pb, insertion groups 1, and 5 to 14; 268pb, insertion groups 2 to 4).

	Sample origin			Nb. Clones	
Biotype	Population	Individual	Tissue	(368 bp)	(268 bp)
Hybrid	LacTrois	L3#5	Brain	4	2
			Muscle	6	3
	LacQuatre	L4#1	Eye	8	
	Barbotte	B3	Brain	5	8
			Fin	1	
	Jonction	J13	Brain	8	13
			Fin	7	9
		J27	Brain	4	
			Muscle	5	
	Saad	S33	Brain	7	
			Muscle	7	
C. eos	Barbotte	B21	Brain		7
			Fin		6
		B42	Brain		5
			Fin		7
C. neogaeus	Saad	S27	Brain		7
			Fin		6
		S28	Brain		7
			Fin		8
			Total	62	88

4.2.3 Sample analyzed

We analyzed a total of 19 DNA samples extracted from four tissues of 10 individuals, six diploid *C. eos-neogeaus* hybrids, two *C. eos* and two *C. neogaeus*, belonging to five natural populations (Tableau 2). For more details on sampling and DNA handling see (Massicotte, Whitelaw, and Angers 2011).

4.2.4 Bisulfite conversion of DNA

Sodium bisulfite treatment of 200-500 ng of DNA was performed according to MethylCode™ Bisulfite Conversion Kit protocol (Invitrogen). A negative control was included for each bisulfite conversion protocol to make sure that no contamination occurred during treatment of genomic DNA.

Tableau 3. Details of primers used for TE amplification.

Primer sequences	Details
TE detection	
TTGAAAAAGGGATAGATAGTTG	Forward primer
ATATTTTCACATAAACATCTAC	Reverse primer
Specific amplification TE 2, 3, and 4	
ATAGTTGYGAATTGATTTGATT	Forward primer
CTTATCTTTAACTCCCAAAATT	Reverse primer

4.2.5 TE detection and amplification

We screened the *C. eos-neogaeus* hybrid genome for the identification of paralogous copies of DIRS1 retrotransposon using primer pair 1 (Figure 9A, Tableau 3). Primer pair 2 was used for the specific amplification of insertion groups 2, 3, and 4 exclusively (Figure 9A, Tableau 3).

A)

CTGAAAAAGGGATAGACAGCTGCG[1]AATTGACCTGATGCG[2]CTCTTCG[3]GACAGAAAAGCTTG
ATACAGGTCCG[4]AGTTCAGTCTGAGACCCAGGTAAATTATCTCTTGTGTAGGCACCAAGCAGCTT
CTTGTTTCG[5]TTTATCTTGAAACCTAAGCTCTCCAAATGAGACACG[6]AGCATATGTGTCTCTCTCT
CTGCCTGTGGCCATGACG[7]GCG[8]CG[9]CACAGCAGCAGATCG[10]TCCAAATATGCTGACACTCTCA
GACCCATTATTCTCAGCG[11]GTGTGAGCG[12]CTGCTTCCACACATTTGCTGAATATCCTGGGAGCT
AAACTCAAACCG[13]AACG[14]GGACTGTCAAAAACTCG[15]TAGGCTGTGTCTCG[16]ATAGGCAAACC
TGAGAAATTTTCTGTGTGCCG[17]GTAGATGCTTATGTGAAAATAT

B)

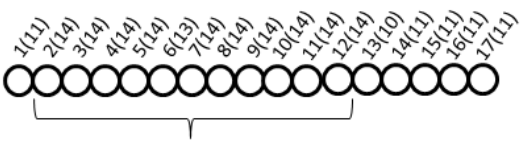

Figure 9. TE structure. A) Reference sequence with primer details (underlined) and location of the 17 CpG dinucleotides; B) Location of paralogous CpGs among the insertion groups. The number in parentheses indicates how many of the 14 groups bear these CpG dinucleotides. The horizontal parenthesis indicates the region covered by the two primer pairs.

4.2.6 Cloning and sequencing

The PCR products were cloned into the plasmid pGEM-T vector (Promega Corp.) followed by transformation in *E. coli* competent cells (strain JM109) according to the manufacturer's protocol. Clones from each sample were subsequently sequenced

(CEQ^{TM} 8000 Genetic Analysis System, Beckman Coulter). All sequences had a conversion rate >96%.

4.3 Results

We sequenced a total of 62 clones of PCR products amplified from extractions of bisulfite converted DNA from 11 *C. eos-neogaeus* hybrid samples (Tableau 2). This has lead to the detection of highly similar insertions that could be classified in 14 groups (Figure 10A). An insertion group was formed when at least two clones presented identical DNA sequences regardless of the methylation status of the CpGs. Among groups variation in sequence identity ranges from 96.7% to 99.7%. For the insertion groups 1, and 5 to 14, bisulfite-sequencing allowed a determination of the methylation polymorphism for a 368 bp fragment covering a total of 17 CpGs (Figure 9B). For the insertion groups 2, 3, and 4, we sequenced a total of 88 clones of PCR products amplified from extractions of bisulfite converted DNA from 13 samples including samples from both parental species (Tableau 2). For these three insertion groups, bisulfite-sequencing allowed a determination of the methylation polymorphism of a 268 bp fragment covering a total of 11 CpGs (Figure 9B).

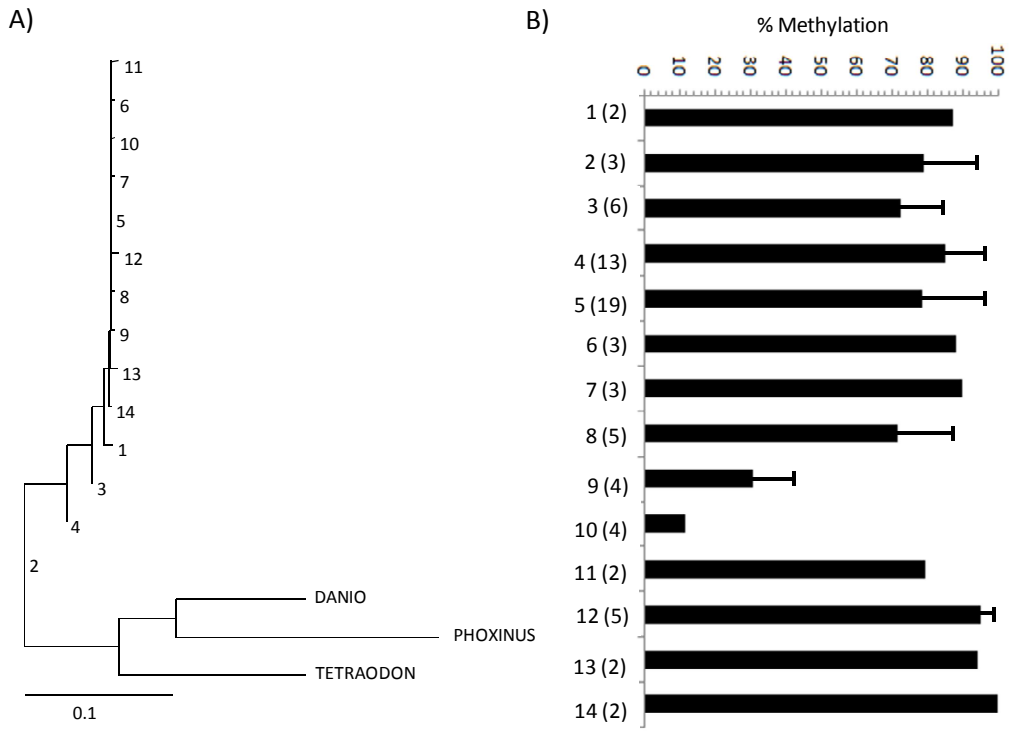

Figure 10. Relationships among insertion groups and insertion site effect on the methylation level. A) NJ of the 14 insertion groups detected in *C. eos-neogaeus* hybrid and three outgroups; *Tetraodon nigrivis*, *Danio rerio* and another *C. eos-neogaeus* DIRS1 sequence. The methylation status of CpG dinucleotides and the incomplete C to T conversions were removed prior to Neighbor Joining (NJ) analysis; B) Average percentage of methylated CpGs per insertion group. The number of clones sequenced for each of the 14 groups is given in parentheses.

4.3.1 Insertion site effect

In average, 92.5% of CpGs were methylated over the 14 insertion groups detected in the *C. eos-neogaeus* hybrid genome. Twelve insertions groups are heavily methylated (>70%) while two of them, insertions groups 9 and 10, have a lower level of methylation (30.9 and 11.8% respectively) (Figure 10B). We also detected epigenetic

variation among sequences from the same insertion group, i.e. in absence of genetic polymorphism (Figure 11). This result confirms that among groups, variation in the methylation level is not biased by the TE structure.

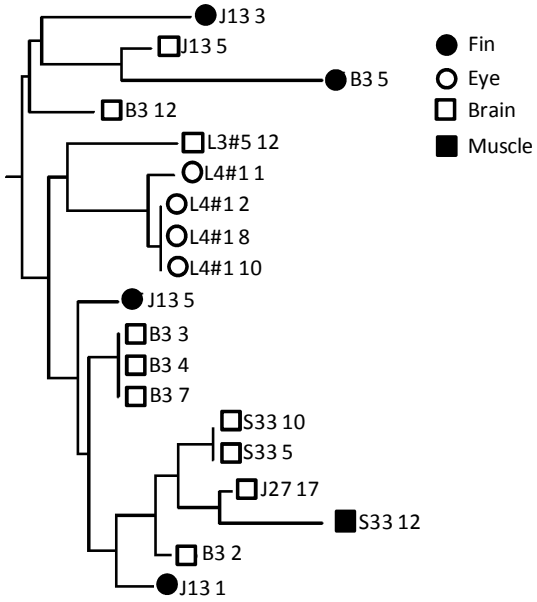

Figure 11. CpG dinucleotide methylation status relationships among samples analyzed for insertion group 5. There were no mutation among the sequences and the incomplete C to T conversion was removed prior to NJ analysis.

It is, however, worth noting that the 14 insertion groups investigated here were, in some instances, detected in different tissues from different individuals. Nevertheless, the observed variation in the methylation level among insertions belonging to different groups but detected in the same tissue and/or the same individual indicates that the variation in the methylation level is not biased by the

sample origin. As an example, the insertion groups 6 and 10 (Figure 10B) were detected in the same tissue (Brain) of the same individual (J13). Taken together, these results strongly support an influence of the genomic context of the *C. eos-neogaeus* hybrid genome on TE methylation level.

4.3.2 Effect of the genomic context of a hybrid genome

We further investigated the possible influence of the genomic context of a hybrid genome on the methylation level and pattern of three groups of insertions (insertions 2, 3 and 4; Figure 10B). Two individuals of each of the parental species, *C. eos* and *C. neogaeus*, from the same populations or nearby populations were used in comparison with the hybrids (Tableau 2). Insertion group 3 was present in both parental genomes while insertion groups 2 and 4 were exclusively detected in *C. eos* and *C. neogaeus* parental genome, respectively. It was, however, not possible to differentiate *C. eos* and *C. neogaeus* alleles for insertion group 3 in *C. eos-neogaeus* hybrids since there is no mutation between the parental genomes at this specific locus.

Of the three insertion groups analyzed, we observed less methylated CpGs in *C. eos-neogaeus* hybrids in comparison to their parental species (Figure 12A). However, the difference is not significant for any of the three insertion groups analyzed (insertion group 2: $p = 0.82$; insertion group 3: $p = 0.57$; insertion group 4: $p = 0.94$). In addition, there is also no significant difference when comparing tissue-specific methylation level between *C. eos* and the *C. eos-neogaeus* hybrids (insertion group 2 (Brain): $p = 0.64$; n (parental species) = 4 and n (hybrids) = 3, Figure 12B).

Also, we did not observe different methylation levels between the parental species for insertion group 3 (Figure 12C). Since we cannot identify the parental species alleles in the *C. eos-neogaeus* hybrids, it is impossible to differentiate between a loss of methylation between *C. eos*, *C. neogaeus* or even both parental allele in the hybrids. Such confounding effects of differentially methylated alleles in *C. eos-neogaeus* hybrids are not expected for insertion groups 2 and 4 since the TE copy was specific to only one parental species genome.

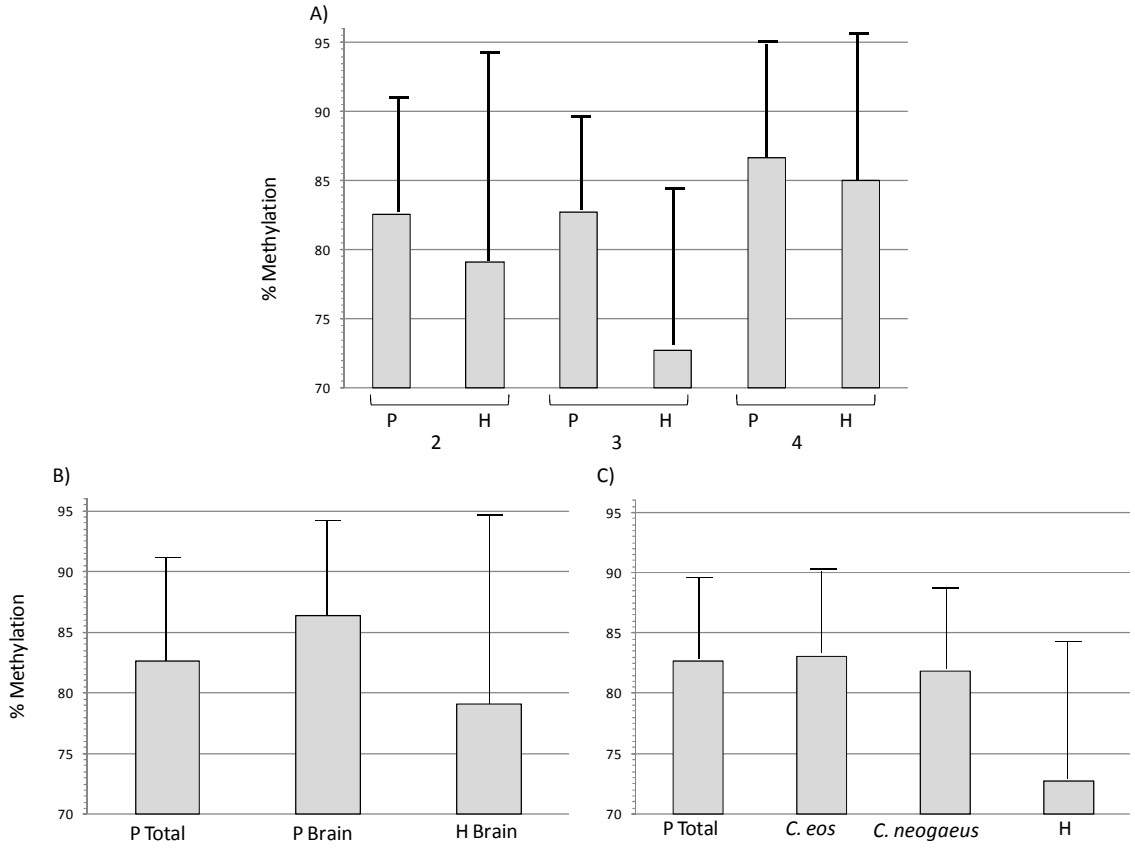

Figure 12. Average percentage of methylated CpGs. A) From left to right, insertion groups 2, 3, and 4 in parental species (P) and *C. eos-neogaeus* hybrids (H); B) insertion group 2, absence of tissue-specific effect; C) insertion group 3, absence of parent-specific effect.

Interestingly, the analysis of the methylation pattern revealed an important within-sample CpGs methylation variation (Figure 13). Insertion groups 2, 3, and 4 present 42.4%, 36.4% and 45% of polymorphic CpGs, respectively. Comparison of tissue-specific methylation pattern indicates that the parental species are as variable as the *C. eos-neogaeus* hybrids (insertion group 2 (Brain): $p = 0.17$; insertion group 3 (Brain): $p = 0.28$; insertion group 3 (Fin): $p = 0.38$; insertion group 4 (Brain): $p = 1$; insertion group 4 (Fin): $p = 0.05$). In addition, a NJ analysis did not identify any tissue, individual or biotype (*C. eos*, *C. neogaeus*, and *C. eos-neogaeus* hybrids) specific CpGs methylation patterns for any of the three loci analyzed (Figure 14). Altogether these results highlight a considerable level of stochasticity in the establishment of the methylation over these loci which make it difficult to observe significant difference among sample methylation profiles.

4.4 Discussion

Our study of the DNA methylation variation of TE in *C. eos-neogaeus* hybrids genome reveals an overall high level of CpG methylation. This is consistent with DNA methylation being an important epigenetic process in the control of TE movement in the genome (Suzuki and Bird 2008). Interestingly, the investigation of DNA methylation profiles of highly similar insertions of a single DIRS1 retrotransposon revealed a non-negligible amount of variation.

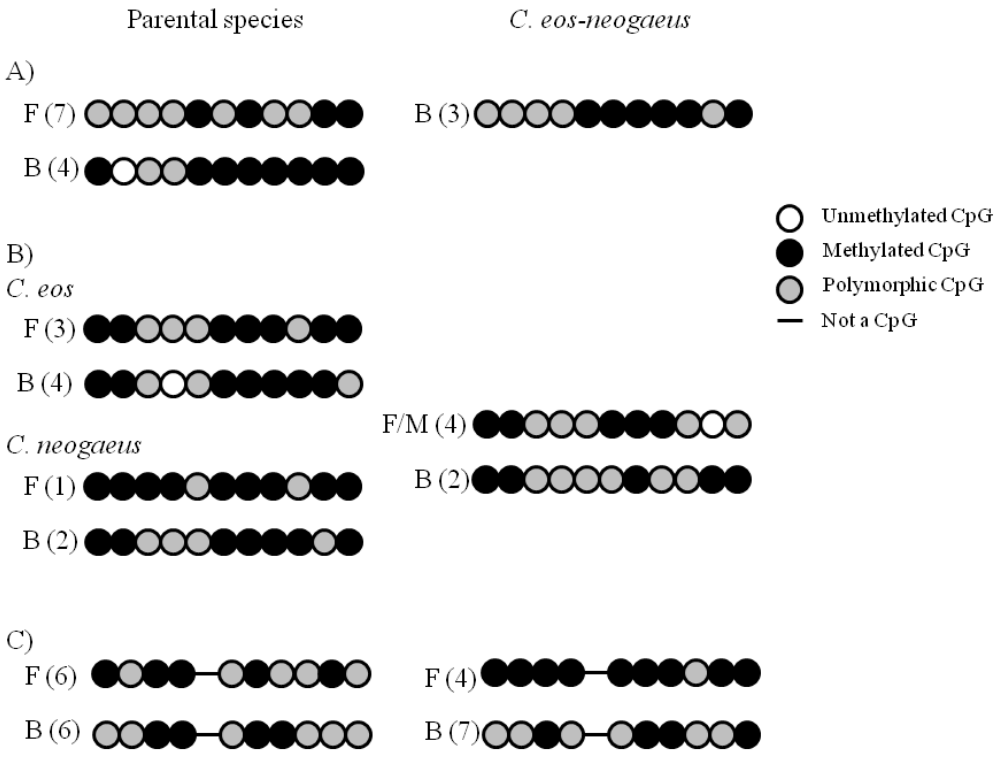

Figure 13. Methylation pattern polymorphism in parental species and *C. eos-neogaeus* hybrids. Location of invariant (unmethylated or methylated) and polymorphic CpGs. A) insertion group 2; B) insertion group 3; C) insertion group 4. Tissues analyzed: B = Brain, F = Fin and, M = Muscle. The number of clones sequenced for each sample is given in parentheses.

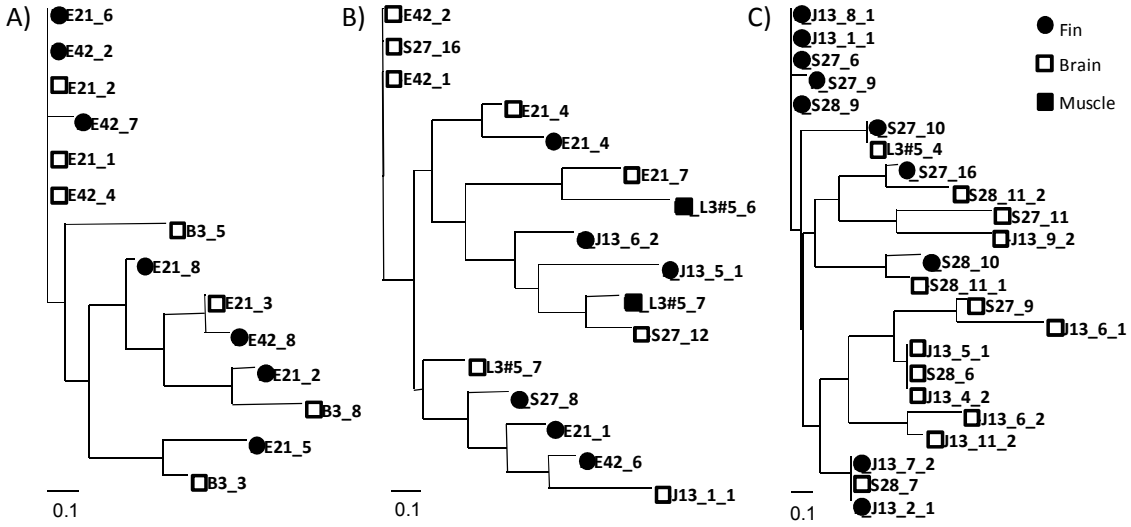

Figure 14. CpG dinucleotide methylation status relationships among samples analyzed. A) insertion group 2; B) insertion group 3 and; C) insertion group 4. The methylation status of the 11 CpGs was code as presence/absence data prior to pairwise distance calculation among samples and NJ analysis.

4.4.1 Insertion site effect

The comparison of highly similar insertions enables us to control for the effect of the structure of the TE sequence on the variation of the methylation profiles (Reiss et al. 2010). The fact that we detect differences in the methylation level among insertions of the same group, i.e. in absence of genetic polymorphism, reinforces the idea that the variation observed is not the result of an effect of the TE structure. It is also important to highlight that among groups, variation in the methylation level does not seem to be biased by the origin of the samples. Accordingly, the variation of the methylation level reflects the influence of the genomic context of TE insertion.

4.4.2 Effect of the genomic context of a hybrid genome

The comparison of TE methylation profiles shows no marked difference between *C. eos-neogaeus* hybrids and the parental species. Although not significant, the results presented here report a lower TE methylation level for *C. eos-neogaeus* hybrids in comparison to the parental species. The analysis of the methylation patterns also indicates a high level of stochasticity in the establishment of CpGs methylation over these insertions. However, we did not detect more polymorphic CpGs in the *C. eos-neogaeus* hybrids in comparison to the parental species. This is not surprising considering the high level of within sample epigenetic mosaicism observed over functionality important genomic regions (Flanagan et al. 2006; Schneider et al. 2010).

In sharp contrast with the other studies investigating the hybridization influence on the methylation level of F1 mammalian hybrid (O'Neill, O'Neill, and Graves 1998; Vrana et al. 1998; Roemer et al. 1999; Brown, Golden, and O'Neill 2008), the F1 hybrid lineage studied here has been perpetuated by asexual reproduction for approximately 30,000 years (Angers and Schlosser 2007). A recent study has demonstrated that the global methylation in *C. eos-neogaeus* hybrids from the same lineage is similar to what is observed in mice (Massicotte, Whitelaw, and Angers 2011). Also, a positive correlation between the insertion age and methylation level has been demonstrated in mice (Reiss et al. 2010). Coupled with the elevated level of TE methylation observed here, it indicates that the DNA methylation machinery is still effective in *C. eos-neogaeus* hybrids.

More importantly, the observation of a TE copy that was not detected in either of the parental species genomes provides direct evidence of a recent transposition that occurred after hybridization in this lineage (Massicotte, Whitelaw, and Angers 2011). This suggests an initial loss of the epigenetic control of TE in inter-specific hybrid genomes. An interesting idea is that newly formed hybrid lineages may only temporarily suffer from mobile elements genome invasion. DNA methylation may re-establish over time thus preventing unsustainable genomic instability and allowing the perpetuation of hybrid lineages. Accordingly, the reunion of inter-specific genomes may not be the process responsible for the perturbation of TE methylation and mobilization in hybrid genomes in the first place. Other processes such as dosage and specificity dependent interactions between maternally and/or paternally inherited factors (e.g., small RNAs) may lead to the activation and mobilization of TE at zygote formation (Josefsson et al. 2006; Michalak 2009).

DNA methylation is an essential epigenetic mechanism as it is closely implicated in the process of cellular differentiation of vertebrates (Meissner et al. 2008). As observed in the mice, the perturbation of the methylation machinery leads to an unviable zygote (Ashe et al. 2008). As long as hybridization does not affect the DNA methylation machinery itself, in some instances hybrids can potentially recover from the genomic instability initiated by the loss of epigenetic control of TE. Although the examples may be scarce, the long-lived *C. eos-neogaeus* hybrid studied here represents such an evolutionary successful lineage.

5. ARTICLE 4

General Purpose Genotype or how epigenetics extends the flexibility of a genotype

Rachel Massicotte et Bernard Angers

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Résumé

L'objectif de ce projet était de faire le lien entre la variabilité épigénétique (non liée à la variabilité génétique) entre les individus et la variation des conditions environnementales en milieu naturel. Nous avons étudié le polymorphisme de méthylation de l'ADN entre les individus d'une même lignée hybride du poisson clonal diploïde *Chrosomus eos-neogaeus* échantillonnés dans sept lacs séparés géographiquement. En dépit du nombre limité de fragments informatifs obtenus par une analyse de MSAP, les individus provenant d'un même lac ont des profils de méthylation généralement similaires et les lacs peuvent être regroupés en deux groupes de populations. Encore plus important, la variation significative du pH entre les lacs est corrélée aux deux groupes épigénétiques observés. Il semble donc que le genotype étudié a un potentiel de réponse différentiel aux variations environnemetnales par des modifications épigénétiques. Ce faisant, les processus épigénétiques représentent un mécanisme moléculaire d'intérêt contribuant à la plasticité phénotypique dans des environnements variables en accord avec le modèle GPG.

Abstract

This project aims at investigating the link between individual epigenetic variability (not related to genetic variability) and the variation due to natural environmental conditions. We studied DNA methylation polymorphisms of individuals belonging to a single hybrid lineage of the clonal diploid fish *Chrosomus eos-neogaeus* sampled in seven geographically distant lakes. In spite of a low number of informative fragments obtained from a MSAP analysis, individuals of a given lake are epigenetically similar and methylation profiles allow the clustering of individuals in two distinct groups of populations among lakes. More importantly, we observed a significant variation of pH among lakes that is consistent with the two epigenetic groups observed. It seems that the genotype studied has the potential to respond differentially via epigenetic modifications under variable environmental conditions. Thus, making epigenetic processes a relevant molecular mechanism contributing to phenotypic plasticity over variable environments in accordance with the GPG model.

5.1 Introduction

Over the years the debate about the evolutionary advantage of sexual over asexual reproduction has focused in part on the higher adaptive potential of populations with standing genetic variation (Neiman and Schwander 2011). Each generation the reproduction of amphimictic organisms results in genetic mixing thus creating a multitude of new genotypes (and potentially novel phenotypes) in natural populations. While in sexually reproducing organisms each individual possess a different genotype, asexually reproducing individuals from the same clonal lineage are presumed to be genetically identical.

On the other hand, asexuality has some advantages of its own; there is no need to produce males and asexual populations can double their size each generation (Maynard-Smith 1978). This twofold advantage of asexual reproduction is thought to be constrained by their limitation in colonizing new environments and/or when living in temporally unstable or heterogeneous environments. In such conditions, the survival, flexibility and adaptive potential of asexual lineages are aspects that are not well understood. The General Purpose Genotype (GPG) model (Baker 1965) (Figure 15(a)) proposed that evolutionary success of asexual organisms could be possible via generalist lineages selected for their flexible phenotypes utilizing wide ecological niches. Such phenotypic flexibility enables a given genotype to be successful in many different and variable environments (Schlutz 1977; Vrijenhoek 1994). Other models, such as the Frozen Niche Variation (FNV) model (Vrijenhoek 1978), rely on the existence of genetic diversity among multiple highly specialized clonal lineages within a population each having respective narrow ecological sub-niches to explain

the maintenance of asexual lineages. Each specialist lineage persists through time by partitioning of available ecological space so as to avoid clonal competition. However, micro-niche models do not provide explanations for how single clonal lineages can be successful across different and temporally variable environmental conditions.

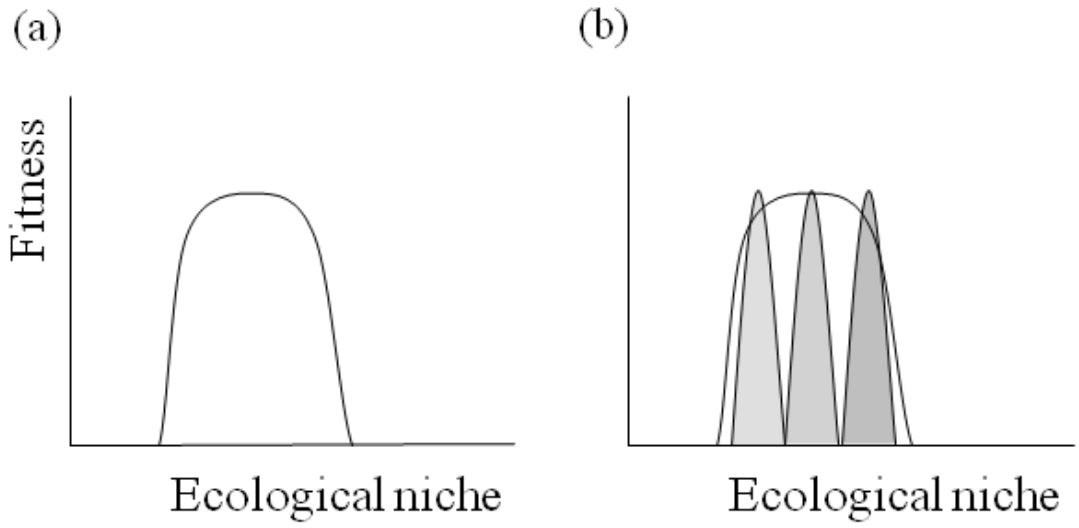

Figure 15. Graphic representation of the General Purpose Genotype (GPG) model and the flexibility hypothesis. (a) GPG model, a flexible genetic lineage (unfilled distribution) with a wide ecological niche and a high fitness under variable environmental conditions. (b) Epigenetic as a mechanism extending the flexibility of a genome, environmentally induced epigenotypes (grey distributions) from a single genetic lineage (unfilled distribution from (a)).

One of the processes underlying the GPG model is the concept of phenotypic plasticity, an environmentally-induced phenotypic difference that occurs within an organism's life-time in absence of genetic variation (Stearns 1989). Epigenetic

variation potentially represents a molecular mechanism that can generate phenotypic plasticity under natural environmental conditions (Angers, Castonguay, and Massicotte 2010). The modification of the epigenome of an organism by variable methylation of DNA sequences has been shown to play a role in the regulation of some genes expression (Bird 2002). There are now numerous examples of epigenetically driven phenotypic variations that are not related to DNA sequence encoded genetic polymorphisms (Rakyan et al. 2002; Manning et al. 2006; Kucharski et al. 2008). Such phenotypic variation can also be caused by an inability to maintain the original epigenetic state during embryogenesis (Jaenisch and Bird 2003). Environmental cues (extrinsic signal) such as diet (Waterland and Jirtle 2002; Kucharski et al. 2008), temperature (Sheldon et al. 1999), maternal behaviour (Weaver et al. 2004) and chemicals exposure (Crews et al. 2007) have been shown to influence the epigenetic profile of individuals.

The fact that the genome is able to integrate extrinsic signals from the environment to vary gene expression is a potentially important mechanism for producing phenotypic plasticity. This stands in sharp contrast with better understood mechanisms based in sequence encoded genetic variation. More importantly, some epigenetic variation has been shown to be unrelated to genetic polymorphism in natural populations (Massicotte, Whitelaw, and Angers 2011). While the genome provides the material to work upon, it is epigenetic regulation that in part enables genomic flexibility. Finally, recent studies have argued that some naturally occurring epimutations can be adaptive (Kucharski et al. 2008; Martin et al. 2009).

This project aims at investigating the link between among individual epigenetic variability (not related to genetic variability) and the variation of natural environmental conditions. In accordance with the General Purpose Genotype (GPG) model, a flexible genotype under different environmental conditions would exhibit distinct methylation patterns due to alternate gene expression profiles necessary to produce flexible phenotypes (Figure 15(b)). As a result, DNA methylation would represent a molecular mechanism extending the plasticity and flexibility of phenotypes produced by a given genotype. As a model we used the clonal fish hybrid *Chrosomus eos-neogaeus* (Cyprinidea, Pisces). We chose this system because a given clonal lineage of *C. eos-neogaeus* can be present over a large geographic distribution (Angers and Schlosser 2007), is found in many different types of habitats (Doeringsfeld et al. 2004), is thought to be generalist (Elder and Schlosser 1995; Mee and Rowe 2010) and more importantly, has been shown to be epigenetically variable (Massicotte, Whitelaw, and Angers 2011).

5.2 Materials and methods

5.2.1 Biological model and sampling

The all-female *C. eos-neogaeus* taxon resulted from hybridization events between female finescale dace (*C. neogaeus*) and male northern redbelly dace (*C. eos*) (Dawley, Schultz, and Goddard 1987). The diploid hybrids reproduce clonally via gynogenesis (Goddard and Dawley 1990; Goddard et al. 1998). Sperm from one of the parental species is thus required, but only to trigger embryogenesis: the resulting offspring are generally genetically identical to the mother (Goddard et al. 1998). In

this complex, the paternal genome can be incorporated into the zygote (Goddard and Dawley 1990; Doeringsfeld et al. 2004; Binet and Angers 2005) resulting in triploid or mosaic hybrids which differ in the proportion of diploid-triploid cell lineages (Dawley, Schultz, and Goddard 1987).

Fish from seven lakes belonging to different watersheds of the St Lawrence River, Quebec, Canada (Tableau 4; Figure 16(a)) were sampled during the reproduction season and over a short period of approximately two weeks. Total DNA from muscle tissue of parental species, three *C. eos* and three *C. neogaeus*, and 26 gynogenetic hybrids belonging to seven different lakes was extracted by proteinase K digestion followed by phenol-chloroform purification and ethanol precipitation (Orkin 1990). The lakes sampled were each classified as one of the four different types of environment according to a characterization previously used to describe *C. eos-neogaeus* populations (Schlosser et al. 1998), water pH and temperature were also measured. Total body length, total body weight and gonads weight were measured for each individual in order to estimate the Gonadosomatic index (GSI) and the Fulton's K condition factor index (K) (Lambert and Dutil 1997). The lakes sampled are known to contain either one or both parental species (*C. eos* and *C. neogaeus*) as well as gynogens and triploids hybrids (Binet and Angers 2005; Angers and Schlosser 2007).

5.2.2 Genetic identification

The gynogenetic hybrids were identified according to (Binet and Angers 2005). Briefly, *C. eos-neogaeus* hybrids were identified using diagnostic markers designed

on two genes. Primers of each marker were designed to provide PCR products of different sizes for *C. eos* and *C. neogaeus*, allowing chromosome identification. Individuals that displayed alleles of both parental species were classified as gynogenetic hybrids.

Gynogenetic hybrids (diploid) were then discriminated from triploid hybrids according to the ploidy level of the nuclear genome by using nine hypervariable microsatellites as detailed in Binet and Angers (2005) and Angers and Schlosser (2007). Gynogens are expected to be hemizygous at every species-specific locus while triploid hybrids (*C. eos-neogaeus* x *eos*) are expected to be heterozygous at loci specific for *C. eos* species. The microsatellite analysis also enabled the identification of the clonal lineage (Angers and Schlosser 2007) and the discrimination of derived mutations. Only gynogenetic hybrids (diploid) were used for further analysis.

5.2.3 MSAP analysis

We investigated epigenetic polymorphism at CCGG motif via a MSAP analysis (Xiong et al. 1999) performed on parental species, three *C. eos* and three *C. neogaeus*, and the 26 *C. eos-neogaeus* gynogenetic hybrids identified in the procedure mentioned above. Each DNA sample was respectively digested with MseI/HpaII and MseI/MspI to allow the detection of differentially methylated sequences. Aliquots (4 µl) of each sample for each primer combinations were loaded on 6% polyacrylamide gels (19:1 acrylamide to bisacrylamide) containing 8 M urea and 1X TBE gels. Fragments that displayed methylation polymorphism among samples at restriction sites were identified by the presence/absence banding pattern

between the two treatments. Full methylation of both cytosines and hemi-methylation of the internal cytosines cannot be investigated by MSAP. As a consequence, it was impossible to distinguish these fragments from unmethylated sequences.

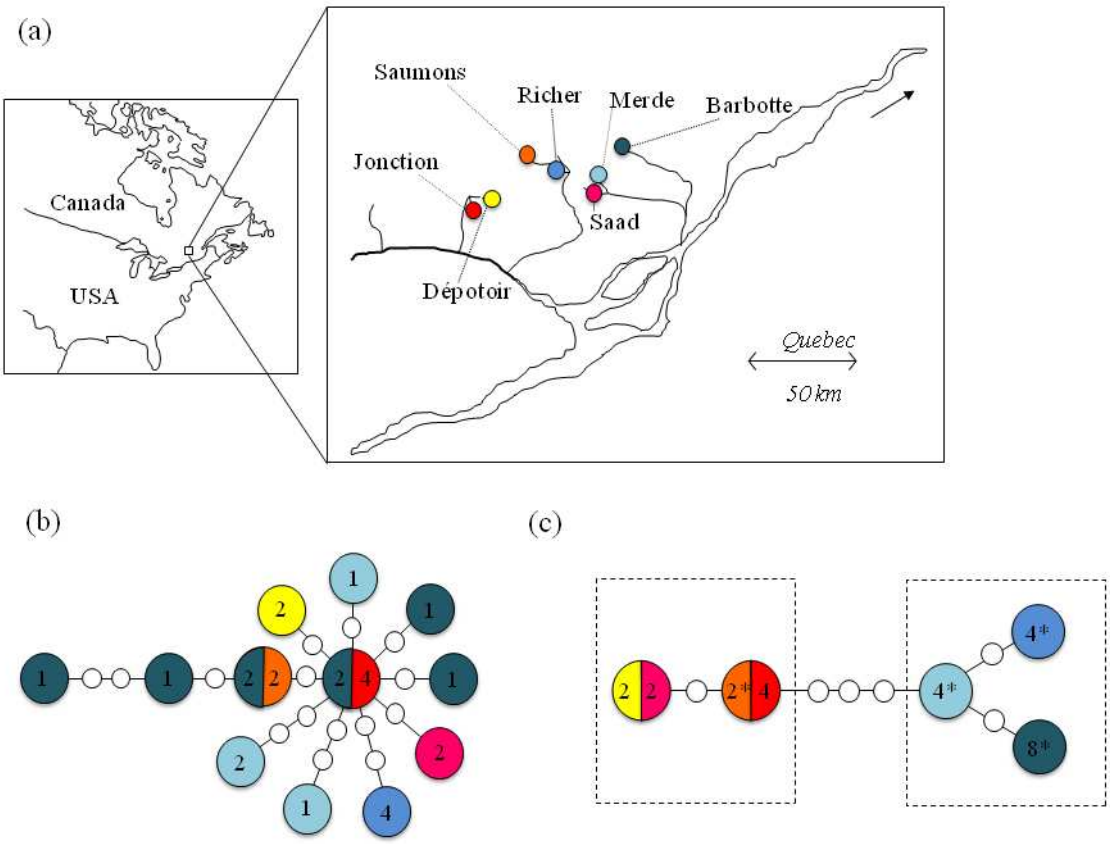

Figure 16. Details of sampling, genotypes and epigenotypes diversity. (a) Sampled lakes in the Laurentian region, Quebec, Canada. (b) Minimum spanning network of the 12 genotypes identified by scoring nine microsatellite loci. The number of gynogenetic hybrids of each genotype per lake is indicated. (c) Minimum spanning network of the five main epigenotypes identified by the MSAP analysis. The number of gynogens of each epigenotype per lake is indicated. * refers to intra-population variation.

Tableau 4. Summary of ecological and molecular data. Lake environmental characteristics, individual morphometric characteristics, gynogenetic hybrids abundance, genetic diversity (number of genotypes) and epigenetic diversity (number of epigenotypes).

Lakes	Geographic coordinates	Date of sampling	Habitat type*	Drainage	Altitude (m)	T (°C)	pH	Weight (g)	Length (cm)	K	GSI	Gynogens	Genotype	Epigenotype
Richer	45°50'35'' N 74°11'39''W	2007-05-29	C	Nord	360	24	6.4	3.32 ± 0.94	6.93 ± 0.71	0.97 ± 0.05	7.33 ± 2.27	4	1	2
Jonction	45°46'37'' N 74°34'29''W	2007-06-01	C	Rouge	340	24	7.1	2.38 ± 0.56	6.08 ± 0.54	1.05 ± 0.07	9.74 ± 0.71	4	1	1
Barbotte	46°5'36'' N 73°52'7''W	2007-05-30	C	L'Assomption	280	22	6.5	2.16 ± 0.4	6.04 ± 0.41	0.97 ± 0.05	7.19 ± 2.5	8	6	2
Merde	45°57'55.9'' N 74°1'41,8''W	2007-05-28	B	L'Assomption	360	23	6.2	3.44 ± 1.36	6.78 ± 0.77	1.04 ± 0.07	11.37 ± 2.7	4	3	4
Dépotoir	45°50'41.6'' N 74°33'20.9''W	2007-05-31	B	Rouge	320	25	7.1	1.85 ± 0.38	5.65 ± 0.45	1.01 ± 0.03	10.73 ± 0.53	2	1	1
Saumons	45°59'38'' N 74°18'21''W	2007-06-16	A	Nord	490	22	7.0	3.2 ± 0.26	6.8 ± 0	1.01 ± 0.08	6.51 ± 1.31	2	1	2
Saad	45°54'51.4'' N 74°1'41.3''W	2007-06-16	D	L'Assomption	320	24	6.8	1.89 ± 0.18	5.9 ± 0.2	0.92 ± 0.008	4.48 ± 2.54	2	1	1

* Habitats characterization according to Schlosser et al. (1998): A- pond of moderate depth, B- a shallow beaver pond, C- a moderately deep area of open water upstream from a beaver dam, D- pond of moderate depth with flooded standing and fallen trees.

5.3 Results

5.3.1 Genetic polymorphism: microsatellites loci analysis

The analysis of nine highly variable microsatellites loci indicates that all samples belong to the same clonal lineage (lineage B6, Angers and Schlosser 2007). Microsatellite variation detected 14 mutations over nine loci and twelve multi locus mutant genotypes were identified within the clonal lineage (Figure 16(b)). These genotypes display very little divergence since all but one genotype differ by only one or two mutations from the putative ancestral clone, with an average of 2.3 mutations among genotypes. The number of sub-lineages carrying derived mutations per lake varied from one to six (Tableau 4).

5.3.2 Epigenetic polymorphism: MSAP analysis

A total of 257 reproducible fragments detected between 150 and 600 bp were assessed with a set of six primer pairs. Over the 257 fragments detected in *C. eos-neogaeus* hybrids, 60 were exclusive to *C. neogaeus*, 67 to *C. eos* and 114 were present in both parental species genomes. The remaining 16 fragments detected could not be associated to either of the parental species genomes. Eight fragments (3.11%) revealed informative methylation polymorphism among populations. Three fragments exclusive to *C. eos*, three fragments exclusive to *C. neogaeus* and two fragments that were present in both parental species genomes were differently methylated for some *C. eos-neogaeus* hybrids. The number of epigenotypes per lake varied from one to four (Tableau 4) and is not correlated with the number of samples ($R^2 = 0.07$, $p = 0.56$).

Two of the eight fragments are variable within populations while the others are only variable among populations. For the 6 fragments that varied among populations, five main epigenotypes were detected. Although the sample size is lower for some populations, individuals from a given population consistently shared the same methylation profile (Figure 16(c)). In most instances, individuals could be regrouped according to the lake of origin based on their unique methylation profile.

Contrasting with genetic relationships among clones where variants are descendents of an ancestral genotype (Figure 16(b)), populations clustered in two distinct epigenetic groups separated by three epimutations (Figure 16(c)). No significant relationship was detected between genetic and epigenetic variation (Figure 17). For instance, individuals from two distinct lakes and harbouring the same genotype clustered in distinct epigenetic groups. Similarly, there is no relationship between genetic intra-population variability and epigenotypes. As an example, the six different genotypes from Barbotte Lake (Figure 16(b)) clustered into the same epigenetic group (Figure 16(c)).

There is no indication that epigenetic profile is related to geographic position, hydrologic network (Figure 16(a)) or date of sampling (Tableau 4). Also, no difference in individual body size length ($p = 0.26$), body weight ($p = 0.28$), Fulton's K ($p = 0.91$) and GSI ($p = 0.72$) were detected among populations. In addition, the shared epigenetic profiles among populations are not correlated with the habitats characterization of lakes (Tableau 4). While there is no important temperature fluctuation among lakes, we observed a significant variation of pH between the two

epigenetics groups (Tableau 4). This is a particularly important result since it correlates the clustering of populations in two epigenetic groups to the variation of a local environmental condition.

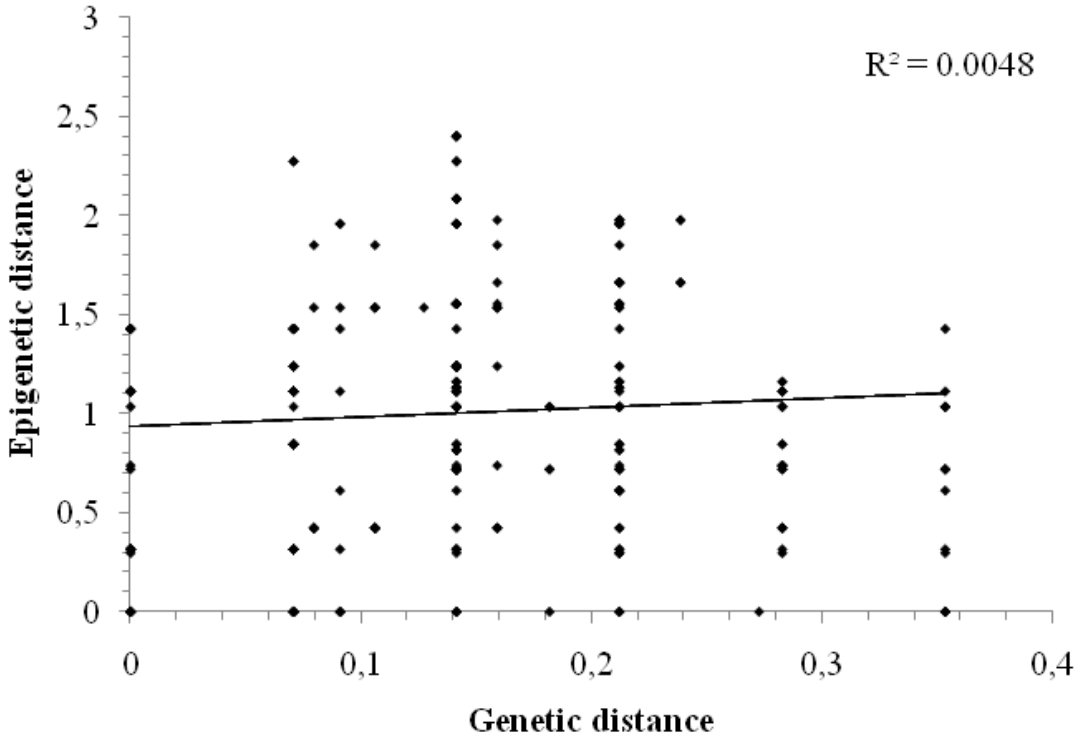

Figure 17. Relationship between the genotypes (genetic variation, microsatellite analysis) and the epigenotypes (methylation profiles difference, MSAP analysis).

5.4 Discussion

The present study reports an effect of the local environmental conditions on the variation of the methylation profile among genetically identical individuals belonging to different natural populations. This is a particularly important result considering that most studies investigating the influence of the integration of the extrinsic signal of the environment on epigenetic variation were performed under control conditions

(Sheldon et al. 1999; Waterland and Jirtle 2002; Weaver et al. 2004) (but see Crews et al. 2007). This indicates that the variation of natural environmental conditions can lead to DNA methylation polymorphism at the population level.

5.4.1 A successful generalist lineage

The *C. eos-neogaeus* hybrid lineage studied here (lineage B6) is widespread in the south-western part of Quebec and is abundant in many populations from numerous watersheds (Angers and Schlosser 2007). The seven lakes under investigation are thought to be characterized by different environmental conditions of a variety of abiotic and biotic conditions (e.g. oxygen concentration, diet, predation level, presence of competitors) (Schlosser et al. 1998). Accordingly, each of the different lakes can be thought of as a different ecological niche. As a result, clonal lineage B6 can be characterized as a generalist lineage that is able to adjust in order to persist among many ecological niches. This situation has already been reported in for lakes in Minnesota (USA) and Algonquin Park (Ontario) (Schlosser et al. 1998; Mee and Rowe 2010).

5.4.2 Environmentally induced epigenotypes

First, we did not detect any relationship between genotype and epigenotype. This is in accordance with a previous study that demonstrated pure (or facilitated) epigenetic variation in natural populations of *C. eos-neogaeus* hybrids (Massicotte, Whitelaw, and Angers 2011). More importantly, the genomic mutations detected are restricted to highly variable microstallites loci, there is no mutation at mtDNA (Angers and Schlosser 2007) and very few mutations were detected on AFLP loci (Massicotte,

Whitelaw, and Angers 2011). This supports the view that the fragment variation detected with MSAP analysis is due to differences in methylation, not to DNA mutation.

Interestingly, the epigenetic polymorphism observed is shared among individuals of the same population in most instances. This suggests an influence of common environmental factors on the resulting epigenetic profiles or a long-term inheritance of epigenetic variation (modifications that could have been acquired before postglacial colonization). This later can be discounted considering the low probability of the inheritance of epigenetic variation across generations (Niemitz and Feinberg 2004) and the absence of correlation between genetic and epigenetic polymorphism. Accordingly, the observation of epigenetic variation among lakes suggests that current environmental conditions influence the DNA methylation profiles among genetically identical individuals from different populations. Furthermore, and in contrast to previous observations, the detection of the same epigenotype in different lakes indicates that the epigenetic polymorphisms observed are not the result of random variation (Massicotte, Whitelaw, and Angers 2011). More importantly, the correlation between the two epigenetic groups with the pH of the lakes strongly supports an effect of the local environmental conditions on the variation in the methylation profile. The variations in the pH may be caused by and/or will result in the variation of many other environmental factors potentially having respective or conjoint effects on the methylation polymorphism.

5.4.3 Revisiting the importance of heritability for epigenetic variation

Previous reports in the literature suggest that in order to be of importance in evolution, epigenetic changes must be heritable across generations (Richards 2006; Bossdorf, Richards, and Pigliucci 2008; Richards 2008). In the situation for which an epimutation leading to a beneficial phenotypic modification appears in one generation and that the environmental conditions do not change in subsequent generations, the heritability of the new epigenetic mark may represent a transient step leading to genetic assimilation (Pal and Miklos 1999). Although epimutations potentially represent a fast pathway toward adaptation (Richards 2008), we do not believe that the main interest of epigenetic mechanisms is to mimic what is occurring at the adaptive genomic level. If heritable, both genetic and epigenetic polymorphisms are frozen. In temporally unstable or heterogeneous environments, such canalisation of the phenotype does not seem beneficial (Young and Badyaev 2007). Furthermore, heritability of epigenetic changes in vertebrates is not expected to be frequent considering the two phases of erasure prior to the initiation of zygote development (Niemitz and Feinberg 2004). (Angers, Castonguay, and Massicotte 2010) have recently identified some of the beneficial aspects of epigenetic mechanisms in that these processes may enable rapid and reversible changes in response to environmental perturbations. For instance, such is observed for the influence of the maternal behaviour on a glucocorticoid receptor gene promoter in the rat hippocampus (Weaver et al. 2004). Rather than passing on to the next generation epimutations that may not be adaptive under new environmental regimes, selection might favour individuals with a plastic genome that easily adjusts epigenetically to environmental variables. Thus, the hard wired genetic variation and the flexible

epigenetic variation may be complementing each other by respectively leading to long term and short term adaptation.

5.5 Conclusion

The epigenetic regulation of the genome when under variable environmental conditions leads to the formation of different epigenotypes. Each population presenting different epigenetic profiles can be seen as acclimated epigenotypes from a single genetic lineage. It thus seems that this lineage has the potential to respond via epigenetic modifications such as DNA methylation when under variable environmental conditions. Even more importantly, this lineage potentially has the ability to adjust following a perturbation in the environment and/or the capacity to colonize different environments. Thus epigenetic processes may represent a molecular mechanism sustaining the GPG model.

6. CONCLUSION

L'objectif de cette thèse de doctorat était d'évaluer l'étendue de la variabilité épigénétique, plus particulièrement du polymorphisme de méthylation de l'ADN non liée à la variabilité génétique dans les populations en milieu naturel. Les sujets traités ainsi que les résultats obtenus nous auront permis de faire la lumière sur certaines questions fondamentales du domaine de l'épigénétique des populations. Dans cette conclusion de thèse, je m'attarderai à faire la synthèse des principaux résultats obtenus ainsi que de leurs implications en écologie et en évolution.

6.1 Les aspects d'intérêt de la recherche effectuée

Il est tout d'abord intéressant de relever les aspects novateurs de cette thèse qui ont permis de mieux cerner l'impact que peuvent avoir les processus épigénétiques. Une première particularité de la recherche effectuée est que contrairement à la grande majorité de la recherche en épigénétique qui s'effectue sur des modèles végétaux (Cubas, Vincent, and Coen 1999; Kalisz and Purugganan 2004; Manning et al. 2006; Verhoeven, Van Dijk, and Biere 2010), cette étude porte sur un modèle animal. Malgré certaines ressemblances entre l'épigénome des plantes et des animaux, plusieurs caractéristiques ne sont pas partagées entre ces deux groupes (Suzuki and Bird 2008). En prenant en considération les distinctions majeures entre ces deux épigénomes, il apparaît primordial d'étudier les processus épigénétiques impliqués dans la régulation de l'expression des gènes afin d'être en mesure de comprendre les effets potentiels de la variabilité épigénétique chez les animaux.

Dans un deuxième temps, l'utilisation d'un poisson à reproduction clonale pour modèle biologique aura permis de contrôler l'effet de la variabilité génétique sur

la variabilité épigénétique (Richards 2006). Cet aspect est particulièrement important considérant qu'il rend possible l'observation de la variabilité épigénétique non liée à la variabilité génétique et par le fait même, d'identifier les autres sources potentielles menant à la variabilité épigénétique entre les individus. De plus, l'hybride clonal du complexe *Chrosomus eos-neogaeus* est un organisme se retrouvant dans différentes populations naturelles (Binet and Angers 2005; Angers and Schlosser 2007). Ces hybrides sont donc nécessairement plus représentatifs de la réalité que les organismes maintenus en milieu contrôlé. Il aura aussi été possible de comparer les profils épigénétiques de plusieurs individus génétiquement identiques mais se retrouvant dans différentes populations naturelles caractérisées par des conditions environnementales variables. Puisqu'il est particulièrement difficile d'identifier les différents biotypes du complexe lors de l'échantillonnage sur le terrain, nous disposions malheureusement d'un nombre limité d'hybrides clonaux pour l'analyse de quelques-unes des populations. Enfin, l'approche globale utilisée afin de répondre à l'objectif de la thèse n'aura pas permis d'associer la variation épigénétique observée a un phénotype alternatif. Dans l'ensemble, les caractéristiques de ce système auront toutefois rendu possible la réalisation d'une première étude de ce genre sur un modèle vertébré.

6.2 L'épigénétique des populations: un domaine de recherche d'actualité en science

Le premier chapitre de la thèse est une revue de littérature en épigénétique. Nous avons été invités à rédiger cet article afin d'informer les écologistes et les évolutionnistes sur ce sujet d'actualité en science. Dans cet article, nous dressons un

portrait détaillé de la méthylation de l'ADN, des causes et des conséquences potentielles du polymorphisme de méthylation sur l'expression différentielle des gènes et conséquemment, la modification du phénotype. Un des aspects les plus importants relevé dans cet article est sans aucun doute l'effet direct que l'environnement peut avoir sur les profils de méthylation. Finalement, nous avons discuté de comment la variabilité épigénétique, héritable ou non héritable, peut ultimement influencer l'évolution des organismes en milieu naturel. Le scénario proposé découle de la réflexion suivante: différents génotypes peuvent présenter un niveau de sensibilité variable de leur capacité à répondre aux changements de l'environnement. Dans la mesure où les épimutations environnementales occasionnent l'apparition de phénotypes alternatifs favorables, les individus portant ce(s) génotype(s) sensible(s) aux changements de l'environnement auront une plus grande chance de survie et de reproduction. En ce sens, il apparaît moins crucial que les épimutations environnementales soient héritables puisque le(s) génotype(s) sensible(s) augmentera(ont) en fréquence permettant ainsi de faire face aux conditions de l'environnement à la génération suivante. Enfin, ce scénario n'entre pas en contradiction mais vient plutôt compléter les discussions concernant l'importance et le destin de la variabilité épigénétique au niveau des populations (Richards 2008).

6.3 Les hybrides *C. eos-neogaeus*: un modèle biologique représentatif

Bien que le modèle biologique utilisé présente des particularités intéressantes pour les fins de cette étude, il a tout d'abord été primordial de s'assurer de son potentiel de représentativité considérant qu'il s'agit d'un poisson hybride à reproduction clonale. La grande majorité des études concernant les effets de l'hybridation inter-spécifique

concluent en une perturbation de la méthylation globale des génomes hybrides de première génération (F1) (O'Neill, O'Neill, and Graves 1998; Vrana et al. 1998; Salmon, Ainouche, and Wendel 2005; Brown, Golden, and O'Neill 2008) (mais voir (Roemer et al. 1999). En premier lieu, nous avons donc comparé le niveau de méthylation entre les hybrides *C. eos-neogaeus*, les espèces parentales, *C. eos* et *C. neogaeus*, ainsi qu'un autre modèle vertébré. Les résultats démontrent que le génome des hybrides n'est pas moins globalement méthylé en comparaison au génome de la souris. De plus, une étude détaillée des profils de méthylation indique un niveau de méthylation moins élevé pour les hybrides en comparaison aux espèces parentales mais cette tendance s'est avérée non significative. Il semble qu'à long terme l'hybridation n'influence pas significativement les profils de méthylation. Malgré leur statut hybride et leur mode de reproduction clonale, les hybrides *C. eos-neogaeus* peuvent être considérés comme un modèle biologique représentatif des vertébrés.

6.4 La variabilité épigénétique en absence de variabilité génétique

Un premier point majeur qui ressort des résultats de cette thèse est la détection de variabilité épigénétique en absence de variabilité génétique chez un modèle vertébré en milieu naturel. En omettant les études effectuées sur les jumeaux monozygotes pour lesquelles seulement deux individus génétiquement identiques peuvent être comparés (Fraga et al. 2005; Petronis 2006), cette étude représente une première détection de variabilité épigénétique en absence de variabilité génétique chez les vertébrés. Afin de mettre en évidence ce type de variabilité épigénétique, nous avons d'abord étudié l'impact de l'intégration des signaux intrinsèques du programme développemental sur les profils de méthylation entre les différents tissus d'un même

individu. En comparant les profils de méthylation d'un même tissu pour plusieurs individus, nous avons par la suite évalué le polymorphisme de méthylation. Cet aspect est capital considérant que seule la variabilité épigénétique non liée à la variabilité génétique représente un niveau d'information moléculaire additionnel car indépendant du génome (Richards 2006). La variabilité épigénétique non liée à la variabilité génétique pourrait donc mener à la production de toute une gamme de phénotypes alternatifs à partir d'un unique génotype. Ces résultats permettent de faire le point sur un aspect longtemps considérée comme hypothétique en épigénétique.

6.5 Les sources de la variabilité épigénétique entre les individus

Un autre aspect d'intérêt des résultats concerne les sources de la variabilité épigénétique observée entre les individus. Les résultats présentés dans cette thèse reflètent d'un effet conjoint des processus stochastiques et de l'environnement sur le polymorphisme de méthylation entre les individus. Premièrement, une étude détaillée des profils de méthylation suggère une importante stochasticité dans l'établissement des patrons de méthylation entre les individus et ce, à deux niveaux. Nous avons d'abord observé un nombre élevé de dinucléotides CpG pour lesquels l'état de méthylation est variable entre les cellules d'un même tissu (variégation) (Rakyan et al. 2002). Cette variation de l'état de méthylation des dinucléotides CpG homologues occasionne une mosaïque de patrons de méthylation; chaque cellule d'un même tissu peut potentiellement présenter un patron de méthylation différent (Flanagan et al. 2006; Schneider et al. 2010).

Dans un deuxième temps, le polymorphisme de méthylation entre les individus suggère que la fixation locus spécifique des épiallèles se fait aléatoirement. Tout comme pour les mutations, certaines épimutations apparaissent et se fixent de façon aléatoire entre les individus. En contrepartie, les épimutations représentent un potentiel de variation plus important car celles-ci seraient 100 fois plus fréquentes que les mutations somatiques (Bennett-Baker, Wilkowski, and Burke 2003). La comparaison de ces deux sources de variation chez les hybrides *C. eos-neogaeus* indique un taux d'épimutations de presque deux ordres de magnitude plus élevé que le taux de mutations. Il est par contre plus difficile de prévoir de l'effet ponctuel des épimutations sur le résultat de l'expression des gènes (phénotype). Cependant, la variation de l'état de méthylation d'un seul dinucléotide CpG (Weaver et al. 2004) tout autant que la densité de méthylation au niveau du promoteur d'un gène peut avoir une influence déterminante sur son expression.

À l'opposé du génotype, l'épigénotype peut quant à lui être directement modifié par l'environnement (Jaenisch and Bird 2003). Cette caractéristique de la variabilité épigénétique est supportée dans nos résultats par l'observation de polymorphisme de méthylation entre les individus de différentes populations. De surcroît, les populations étudiées ont pu être réunies en deux groupes en fonction des différents épigénotypes observés entre les individus. Les données montrent aussi une association entre le profil épigénétique et le pH des lacs où sont prélevés les poissons. Ces observations font un lien direct entre les profils épigénétiques et une condition physico-chimique de l'environnement local. De plus, cette différence du pH est en elle-même ou peut subséquemment entraîner la variation d'une multitude de facteurs

abiotiques et biotiques. Cela suggère que les variations naturelles des conditions environnementales peuvent mener à du polymorphisme de méthylation entre les individus. Ce résultat est déterminant puisqu'à ce jour, les exemples de l'effet de l'environnement sur le façonnement de l'épigénome découle presqu'entièrement d'expérimentations réalisées en milieu contrôlé (Waterland and Jirtle 2002; Weaver et al. 2004; Dolinoy, Huang, and Jirtle 2007; Kaminen-Ahola et al. 2010; Verhoeven, Jansen et al. 2010).

6.6 Les implications de la variabilité épigénétique entre les individus

Il est impossible de conclure cette thèse de doctorat sans discuter des implications de la variabilité épigénétique observée entre les individus. Bien que codé par le génome, le phénotype d'un individu ne se réalise pas sans l'intervention des processus épigénétiques (Bird 2002). En plus des marques épigénétiques qui s'établissent en fonction du programme de développement cellulaire, les processus stochastiques et l'environnement occasionnent l'apparition d'épimutations (Richards 2008). Les épimutations représentent la source de la variabilité épigénétique non liée à la variabilité génétique entre les individus (Richards 2006). En plus de la variabilité génétique et de l'environnement, la variabilité épigénétique non liée à la variabilité génétique est une composante à considérer dans le partitionnement de la variabilité phénotypique. Les processus épigénétiques représentent donc un mécanisme moléculaire qui permet l'interaction entre le génome et l'environnement dans la détermination du phénotype (Gorelick 2005).

Cette interaction possible entre le génome et l'environnement suivant l'action des processus épigénétiques souligne le potentiel de réponse des organismes face aux changements de leur environnement. Les processus épigénétiques représentent donc un mécanisme moléculaire qui sous-tend la plasticité phénotypique, c'est-à-dire une variation du phénotype pour un même génotype en réponse à l'environnement (norme de réaction) (Stearns 1989). Considérant la plus faible probabilité de l'héritabilité des épimutations entre les générations chez les vertébrés, nous suggérons que la sélection pourrait favoriser les génomes épigénétiquement flexibles. Conséquemment, les processus épigénétiques, tel que la méthylation de l'ADN, mèneraient à l'adaptation à court terme en permettant la flexibilité génomique.

6.7 Perspective

Les résultats présentés dans cette thèse de doctorat ouvrent la porte à une multitude d'autres questions qui seront des plus pertinentes à investiguer dans un futur rapproché. Il sera entre autre intéressant de faire le lien entre les variations de divers facteurs environnementaux (comme par exemple la diète, différentes conditions physico-chimiques et divers contaminants) et la variabilité épigénétique. L'évaluation du succès de différents génotypes (différentes lignées clonales) face aux variations de ces facteurs environnementaux viendrait de plus étayer l'hypothèse de la flexibilité génomique par l'action des processus épigénétiques. Bien que plus difficile à prévoir, ces différentes observations effectuées en milieu contrôlé pourraient potentiellement permettre de dresser un portrait global du lien direct entre les effets de l'environnement, la méthylation de l'ADN, l'expression génique et la variabilité phénotypique.

En conclusion, la réalisation de ce projet de thèse permet d'entrevoir l'impact majeur que les processus épigénétiques, tel que la méthylation de l'ADN, peuvent avoir sur l'écologie et l'évolution des organismes en milieu naturel. Les discussions concernant la nécessité d'actualiser la théorie synthétique de l'évolution sont entamées depuis quelques années (Pigliucci 2007). Bien que les résultats présentés et les sujets traités n'entrent pas en conflit avec la théorie synthétique de l'évolution actuelle, il semble évident qu'il serait pertinent d'étendre celle-ci afin d'inclure les processus nouvellement découverts qui permettent de détailler des phénomènes biologiques d'importance.

7. ANNEXES

Annexe 1. Protocole de MSAP

1) Digestion double (MseI – HpaII) de l'ADN génomique, 1h00 à 37°C.

(N.B. Un maximum ADN est digéré en 1h00 mais la digestion peut être laissée toute la nuit à 37°C afin de maximiser celle-ci. Les numéros des tampons peuvent varier en fonction de la marque des enzymes utilisés.)

Recette pour chaque échantillon:
MseI 0,5
HpaII 0,5
Tampon (#1) 2,0
BSA 10x 2,0
Eau 5,0
ADN 10,0
Total: 20,0 µl

2) Diviser le produit de la digestion obtenu précédemment en deux volumes égaux de 10 µl.

3) Digestion simple (MspI) de 10 µl de produit de la digestion simple, 1h00 à 37°C.

Recette pour chaque échantillon:
MspI 0,25
Tampon (#2) 1,0
BSA 10x 1,0
Eau 2,75
ADN de la digestion double 5,0
Total: 10,0 µl

4) Préparation des adaptateurs

Adaptateur MseI: 50 µl MseIfor (GACGATGAGTCCTGA)
50 µl TAMseligrev (TACTCAGGACTCAT)

Adaptateur Taq: 50 µl MseIfor (GACGATGAGTCCTGA)
50 µl CGligrev (CGCTCAGGACTCATC)

Incuber 2 min. À 60°C et laisser 15 min. à température de la pièce.

5) Ligation des adaptateurs, 1h00 à température de la pièce et ensuite toute la nuit à 4°C

Recette pour chaque échantillon:
Adaptateur MseI 1,0
Adaptateur Taq 1,0

Tampon 5x 3,0
T4Ligase 0,2
Eau 3,3
Total: 8,5 µl

6) Ajouter 15 µl d'eau par échantillon et incuber 20 min. à 65°C.

7) PCR pré-sélective de chaque échantillon obtenu suite aux deux traitements de digestion (MseI-HpaII et MseI-HpaII/ MspI) (N.B. L'ajout de $MgSo_4$ (et du $MgCl_2$ dans certains cas) est fonction de la marque de la Taq polymérase utilisée.)

Combinaison d'amorces pré-sélectives (1pb en plus à la fin de l'amorce, MseI C ou G et HpaII/MspI A ou C):
MseI: GATGAGTCCTGAGTAAN
HpaII/MspI: GATGAGTCCTGAGCGGN

Recette pour chaque échantillon:
Eau 12,42
Tampon 10x 2,5
$MgSo_4$ 2,5
DNTP 1,5
Amorce 2,0
Amorce 2,0
Taq polymérase 0,08
ADN 2,0
Total: 25,0 µl

Programme PCR:
92°C - 30 s.
92°C - 20 s.
56°C - 10 s.
68°C - 1 min.
30 cycles
68°C - 2 min.
15°C - …

8) Ajouter 200 µl d'eau par échantillon et faire une dilution supplémentaire 1:1 (10 µl de produit amplifié à l'étape 7 et le même volume d'eau).

9) PCR sélective pour chaque échantillon dilué à l'étape 8.

Combinaisons d'amorces sélectives (3pb en plus à la fin des amorces voir les combinaisons A à N Tableau S1 du chapitre 3)
MseI: GATGAGTCCTGAGTAAN(C ou G) NN
HpaII / MspI: GATGAGTCCTGAGCGGN(A ou C) NN

Recette pour chaque échantillon:
Eau 5,46
Buffer 10x 1,25
$MgSo_4$ 1,25
DNTP 0,5
Amorce 1,0
Amorce 1,0
Taq polymerase 0,04
ADN 2,0
Total: 12,5 µl

Programme PCR:
92°C - 15 s.
92°C - 15 s.
65 à 56°C - 15 s.
68°C - 1 min. 30 s.
15 cycles
92°C - 15 s.
56°C - 15 s.
68°C - 1 min. 30 s.
30 cycles
68°C - 2 min.
15°C - …

10) Pour chaque échantillon, ajouter 6,3 µl de bleu de bromophénol au produit de l'amplification sélective obtenu à l'étape 9.

11) Dénaturer 2 min. à 95°C et mettre sur glace.

12) Charger de 4 à 5 µl de chaque échantillon sur un gel de polyacrylamide 6% contenant 8M d'urée et TBE 1X.

13) Migration d'environ 2h30 (300mA, 1700V et 200W).

14) Procéder à la révélation au nitrate d'argent.

8. RÉFÉRENCES

Agrawal, A. A. 2001. Ecology - Phenotypic plasticity in the interactions and evolution of species. *Science* 294 (5541):321-326.

Alleman, M., Sidorenko, L., McGinnis, K., Seshadri, V., Dorweiler, J. E., White, J., Sikkink, K., and Chandler, V. L. 2006. An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature* 442 (7100):295-298.

Angers, B., Castonguay, E., and Massicotte, R. 2010. Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Molecular Ecology* 19 (7):1283-1295.

Angers, B. and Schlosser, I. J. 2007. The origin of Phoxinus eos-neogaeus unisexual hybrids. *Molecular Ecology* 16 (21):4562-4571.

Anway, M. D., Cupp, A. S., Uzumcu, M., and Skinner, M. K. 2005. Epigenetic transgenerational actions of endocrine disruptors and mate fertility. *Science* 308 (5727):1466-1469.

Ashe, A., Morgan, D. K., Whitelaw, N. C., Bruxner, T. J., Vickaryous, N. K., Cox, L. L., Butterfield, N. C., Wicking, C., Blewitt, M. E., Wilkins, S. J., Anderson, G. J., Cox, T. C., and Whitelaw, E. 2008. A genome-wide screen for modifiers of transgene variegation identifies genes with critical roles in development. *Genome Biology* 9 (12):16.

Avner, P. and Heard, E. 2001. X-chromosome inactivation: Counting, choice and initiation. *Nature Reviews Genetics* 2 (1):59-67.

Baker, H. 1965. Characteristics and modes of origin of weeds. In *The genetics of colonizing species*, edited by G. Stebbins. New York: Academic Press.

Bennett-Baker, P. E., Wilkowski, J., and Burke, D. T. 2003. Age-associated activation of epigenetically repressed genes in the mouse. *Genetics* 165 (4):2055-2062.

Bestor, T. H. and Tycko, B. 1996. Creation of genomic methylation patterns. *Nature Genetics* 12 (4):363-367.

Binet, M. C. and Angers, B. 2005. Genetic identification of members of the Phoxinus eos-neogaeus hybrid complex. *Journal of Fish Biology* 67 (4):1169-1177.

Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes & Development* 16 (1):6-21.

———. 2007. Perceptions of epigenetics. *Nature* 447 (7143):396-398.

Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature* 321 (6067):209-213.

———. 1995. GENE NUMBER, NOISE-REDUCTION AND BIOLOGICAL COMPLEXITY. *Trends in Genetics* 11 (3):94-100.

Blewitt, M. E., Vickaryous, N. K., Paldi, A., Koseki, H., and Whitelaw, E. 2006. Dynamic reprogramming of DNA methylation at an epigenetically sensitive allele in mice. *Plos Genetics* 2 (4):399-405.

Bonduriansky, R. and Day, T. 2009. Nongenetic Inheritance and Its Evolutionary Implications. In *Annual Review of Ecology Evolution and Systematics*.

Boss, P. K., Bastow, R. M., Mylne, J. S., and Dean, C. 2004. Multiple pathways in the decision to flower: Enabling, promoting, and resetting. *Plant Cell* 16:S18-S31.

Bossdorf, O., Richards, C. L., and Pigliucci, M. 2008. Epigenetics for ecologists. *Ecology Letters* 11 (2):106-115.

Boyes, J. and Bird, A. 1991. DNA methylation inhibits transcription indirectly via a methyl-CpG binding-protein. *Cell* 64 (6):1123-1134.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics* 13:115-155.

Brown, J. D., Golden, D., and O'Neill, R. J. 2008. Methylation perturbations in retroelements within the genome of a Mus interspecific hybrid correlate with double minute chromosome formation. *Genomics* 91 (3):267-273.

Cai, Q. and Chinnappa, C. C. 1999. Cytosine methylation levels in the genome of Stellaria longipes. *Journal of Biosciences* 24 (1):27-33.

Callahan, H. S., Pigliucci, M., and Schlichting, C. D. 1997. Developmental phenotypic plasticity: Where ecology and evolution meet molecular biology. *Bioessays* 19 (6):519-525.

Cervera, M. T., Ruiz-Garcia, L., and Martinez-Zapater, J. M. 2002. Analysis of DNA methylation in Arabidopsis thaliana based on methylation-sensitive AFLP markers. *Molecular Genetics and Genomics* 268 (4):543-552.

Chan, S. W. L., Henderson, I. R., and Jacobsen, S. E. 2005. Gardening the genome: DNA methylation in Arabidopsis thaliana (vol 6, pg 351, 2005). *Nature Reviews Genetics* 6 (7):590-590.

Chandler, V. L. 2007. Paramutation: From maize to mice. *Cell* 128 (4):641-645.

Chandler, V. L., Eggleston, W. B., and Dorweiler, J. E. 2000. Paramutation in maize. *Plant Molecular Biology* 43 (2-3):121-145.

Chong, S. Y. and Whitelaw, E. 2004. Epigenetic germline inheritance. *Current Opinion in Genetics & Development* 14 (6):692-696.

Colot, V. and Rossignol, J. L. 1999. Eukaryotic DNA methylation as an evolutionary device. *Bioessays* 21 (5):402-411.

Crews, D. 2008. Epigenetics and its implications for behavioral neuroendocrinologry. *Frontiers in Neuroendocrinology* 29 (3):344-357.

Crews, D., Gore, A. C., Hsu, T. S., Dangleben, N. L., Spinetta, M., Schallert, T., Anway, M. D., and Skinner, M. K. 2007. Transgenerational epigenetic imprints on mate preference. *Proceedings of the National Academy of Sciences of the United States of America* 104 (14):5942-5946.

Crispo, E. 2007. The Baldwin effect and genetic assimilation: Revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* 61 (11):2469-2479.

Cubas, P., Vincent, C., and Coen, E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* 401 (6749):157-161.

Dawley, R. M., Schultz, R. J., and Goddard, K. A. 1987. Clonal reproduction and polyploidy in unisexual hybrids of Phoxinus eos and Phoxinus neogaeus (Pisces, Cyprinidae). *Copeia* (2):275-283.

de Jong, G. 2005. Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. *New Phytologist* 166 (1):101-117.

Debat, V. and David, P. 2001. Mapping phenotypes: canalization, plasticity and developmental stability. *Trends in Ecology & Evolution* 16 (10):555-561.

Derome, N., Duchesne, P., and Bernatchez, L. 2006. Parallelism in gene transcription among sympatric lake whitefish (Coregonus clupeaformis Mitchill) ecotypes. *Molecular Ecology* 15 (5):1239-1249.

Dobrovic, A., Bianco, T., Tan, L. W., Sanders, T., and Hussey, D. 2002. Screening for and analysis of methylation differences using methylation-sensitive single-strand conformation analysis. *Methods* 27 (2):134-138.

Doeringsfeld, M. R., Schlosser, I. J., Elder, J. F., and Evenson, D. P. 2004. Phenotypic consequences of genetic variation in a gynogenetic complex of Phoxinus eos-neogaeus clonal fish (Pisces : Cyprinidae) inhabiting a heterogeneous environment. *Evolution* 58 (6):1261-1273.

Dolinoy, D. C., Huang, D., and Jirtle, R. L. 2007. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proceedings of the National Academy of Sciences of the United States of America* 104 (32):13056-13061.

Dudley, S. A. and Schmitt, J. 1996. Testing the adaptive plasticity hypothesis: Density-dependent selection on manipulated stem length in Impatiens capensis. *American Naturalist* 147 (3):445-465.

Duhl, D. M. J., Vrieling, H., Miller, K. A., Wolff, G. L., and Barsh, G. S. 1994. NEOMORPHIC AGOUTI MUTATIONS IN OBESE YELLOW MICE. *Nature Genetics* 8 (1):59-65.

Elder, J. F. and Schlosser, I. J. 1995. Extreme Clonal Uniformity of Phoxinus Eos Neogaeus Gynogens (Pisces, Cyprinidae) among Variable Habitats in Northern Minnesota Beaver Ponds. *Proceedings of the National Academy of Sciences of the United States of America* 92 (11):5001-5005.

Feil, R. 2006. Environmental and nutritional effects on the epigenetic regulation of genes. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* 600 (1-2):46-57.

Fieldes, M. A. and Amyot, L. M. 1999. Evaluating the potential of using 5-azacytidine as an epimutagen. *Canadian Journal of Botany-Revue Canadienne De Botanique* 77 (11):1617-1622.

Flanagan, J. M., Popendikyte, V., Pozdniakovaite, N., Sobolev, M., Assadzadeh, A., Schumacher, A., Zangeneh, M., Lau, L., Virtanen, C., Wang, S. C., and Petronis, A. 2006. Intra- and interindividual epigenetic variation in human germ cells. *American Journal of Human Genetics* 79 (1):67-84.

Fraga, M. F., Ballestar, E., Paz, M. F., Ropero, S., Setien, F., Ballestart, M. L., Heine-Suner, D., Cigudosa, J. C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T. D., Wu, Y. Z., Plass, C., and Esteller, M. 2005. Epigenetic differences arise during the lifetime of monozygotic twins. *Proceedings of the National Academy of Sciences of the United States of America* 102 (30):10604-10609.

Francis, D., Diorio, J., Liu, D., and Meaney, M. J. 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* 286 (5442):1155-1158.

Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L., and Paul, C. L. 1992. A GENOMIC SEQUENCING PROTOCOL THAT YIELDS A POSITIVE DISPLAY OF 5-METHYLCYTOSINE RESIDUES IN INDIVIDUAL DNA STRANDS. *Proceedings of the National Academy of Sciences of the United States of America* 89 (5):1827-1831.

Gagnaire, P. A., Albert, V., Jonsson, B., and Bernatchez, L. 2009. Natural selection influences AFLP intraspecific genetic variability and introgression patterns in Atlantic eels. *Molecular Ecology* 18 (8):1678-1691.

Goddard, K. A. and Dawley, R. M. 1990. Clonal inheritance of a diploid nuclear genome by a hybrid fresh-water minnow (Phoxinus eos-neogaeus, Pisces, Cyprinidae). *Evolution* 44 (4):1052-1065.

Goddard, K. A., Megwinoff, O., Wessner, L. L., and Giaimo, F. 1998. Confirmation of gynogenesis in Phoxinus eos-neogaeus (Pisces : Cyprinidae). *Journal of Heredity* 89 (2):151-157.

Goodwin, T. J. D. and Poulter, R. T. M. 2001. The DIRS1 group of retrotransposons. *Molecular Biology and Evolution* 18 (11):2067-2082.

Gorelick, R. 2004. Neo-Lamarckian medicine. *Medical Hypotheses* 62 (2):299-303.

———. 2005. Environmentally alterable additive genetic effects. *Evolutionary Ecology Research* 7 (3):371-379.

Gorelick, R. and Laubichler, M. D. 2008. Genetic = Heritable (Genetic ≠ DNA). *Biological Theory* 3:79-84.

Gorelick, R., Laubichler, M. and Massicotte, R. 2010. Asexuality and epigenetic variation. In *Epigenetics: Linking Genotype and Phenotype in Development and Evolution*, edited by B. a. H. Hallgrímsson, B.K. San Francisco: University of California Press.

Hattman, S. 2005. DNA-[adenine] methylation in lower eukaryotes. *Biochemistry-Moscow* 70 (5):550-558.

Hauenschild, A., Ringrose, L., Altmutter, C., Paro, R., and Rehmsmeier, M. 2008. Evolutionary Plasticity of Polycomb/Trithorax Response Elements in Drosophila Species. *Plos Biology* 6 (10):2130-2143.

Hedrick, P. W. 1998. Balancing selection and MHC. *Genetica* 104 (3):207-214.

Hellman, A. and Chess, A. 2007. Gene body-specific methylation on the active X chromosome. *Science* 315 (5815):1141-1143.

Hitchins, M. P., Wong, J. J. L., Suthers, G., Suter, C. M., Martin, D. I. K., Hawkins, N. J., and Ward, R. L. 2007. Brief report: Inheritance of a cancer-associated MLH1 germ-line epimutation. *New England Journal of Medicine* 356 (7):697-705.

Hotz, H., Semlitsch, R. D., Gutmann, E., Guex, G. D., and Beerli, P. 1999. Spontaneous heterosis in larval life-history traits of hemiclonal frog hybrids. *Proceedings of the National Academy of Sciences of the United States of America* 96 (5):2171-2176.

Ito, M., Koike, A., Koizumi, N., and Sano, H. 2003. Methylated DNA-binding proteins from arabidopsis. *Plant Physiology* 133 (4):1747-1754.

Jablonka, E. and Lamb, M. J. 1991. SEX-CHROMOSOMES AND SPECIATION. *Proceedings of the Royal Society of London Series B-Biological Sciences* 243 (1308):203-208.

———. 1995. *Epigenetic inheritance and evolution. The Lamarkian dimension.* Oxford: Oxford University Press.

———. 2008. Soft inheritance: Challenging the Modern Synthesis. *Genetics and Molecular Biology* 31 (2):389-395.

Jablonka, E., Oborny, B., Molnar, I., Kisdi, E., Hofbauer, J., and Czaran, T. 1995. The adaptive advantage of phenotypic memory in changing environments.

Philosophical Transactions of the Royal Society B-Biological Sciences 350 (1332):133-141.

Jablonka, E. and Raz, G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for teh study of heredity and evolution. *Quarterly Review of Biology* 84 (2):131-176.

———. 2009. Transgenerational epigenetic inheritance: prevalence, mechnaisms, and implications for the study of heredity and evolution. *Quarterly Review of Biology* 84 (2):131-176.

Jaenisch, R. and Bird, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* 33:245-254.

Jeon, T. J. 2008. DNA Adenine Methylation of sams1 Gene in Symbiont-Bearing Amoeba proteus. *Journal of Microbiology* 46 (5):564-570.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. 2000. Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. *Science* 290 (5490):344-347.

Josefsson, C., Dilkes, B., and Comai, L. 2006. Parent-dependent loss of gene silencing during interspecies hybridization. *Current Biology* 16 (13):1322-1328.

Kalisz, S. and Purugganan, M. D. 2004. Epialleles via DNA methylation: consequences for plant evolution. *TRENDS in Ecology and Evolution* 19 (6):309-314.

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K. A., Fahey, P., Cox, T. C., Whitelaw, E., and Chong, S. Y. 2010. Maternal Ethanol Consumption Alters the Epigenotype and the Phenotype of Offspring in a Mouse Model. *Plos Genetics* 6 (1).

Kinoshita, H., Shi, Y., Sandefur, C., and Jarrard, D. F. 2000. Screening hypermethylated regions by methylation-sensitive single-strand conformational polymorphism. *Analytical Biochemistry* 278 (2):165-169.

Kucharski, R., Maleszka, J., Foret, S., and Maleszka, R. 2008. Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 319 (5871):1827-1830.

Lachmann, M. and Jablonka, E. 1996. The inheritance of phenotypes: An adaptation to fluctuating environments. *Journal of Theoretical Biology* 181 (1):1-9.

Lambert, Y. and Dutil, J. D. 1997. Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of cod (Gadus morhua)? *Canadian Journal of Fisheries and Aquatic Sciences* 54:104-112.

Letting, D. L., Fecteau, D. A., Haws, T. F., Reed, S. L., Hopkins, R. O., Coleman, R. D., and Goddard, K. A. 1999. Unexpected ratio of allozyme expression in diploid and triploid individuals of the clonal hybrid fish *Phoxinus eos-neogaeus*. *Journal of Experimental Zoology* 284:663-674.

Li, E., Beard, C., and Jaenisch, R. 1993. Role for DNA methylation in genomic imprinting. *Nature* 366 (6453):362-365.

Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H., and Ecker, J. R. 2008. Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell* 133 (3):523-536.

Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q.-M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R., Ruotti, V., Millar, A. H., Thomson, J. A., Ren, B., and Ecker, J. R. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462 (7271):315-322.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P. M., and Meaney, M. J. 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 277 (5332):1659-1662.

Liu, J., He, Y. H., Amasino, R., and Chen, X. M. 2004. siRNAs targeting an intronic transposon in the regulation of natural flowering behavior in Arabidopsis. *Genes & Development* 18 (23):2873-2878.

Liu, Z. J. and Maekawa, M. 2003. Polymerase chain reaction-based methods of DNA methylation analysis. *Analytical Biochemistry* 317 (2):259-265.

Lynch, M. 1984. Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Quarterly Review of Biology* 59 (3):257-290.

MacKay, A. B., Mhanni, A. A., McGowan, R. A., and Krone, P. H. 2007. Immunological detection of changes in genomic DNA methylation during early zebrafish development. *Genome* 50 (8):778-785.

Maekawa, M., Sugano, K., Kashiwabara, H., Ushiama, M., Fujita, S., Yoshimori, M., and Kakizoe, T. 1999. DNA methylation analysis using bisulfite treatment and PCR-single-strand conformation polymorphism in colorectal cancer showing microsatellite instability. *Biochemical and Biophysical Research Communications* 262 (3):671-676.

Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A. J., King, G. J., Giovannoni, J. J., and Seymour, G. B. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nature Genetics* 38 (8):948-952.

Martienssen, R. A. and Colot, V. 2001. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* 293 (5532):1070-1074.

Martin, A., Troadec, C., Boualem, A., Rajab, M., Fernandez, R., Morin, H., Pitrat, M., Dogimont, C., and Bendahmane, A. 2009. A transposon-induced epigenetic change leads to sex determination in melon. *Nature* 461 (7267):1135-U237.

Massicotte, R., Magnan, P., and Angers, B. 2008. Intralacustrine site fidelity and nonrandom mating in the littoral-spawning northern redbelly dace (Phoxinus eos). *Canadian Journal of Fisheries and Aquatic Sciences* 65 (9):2016-2025.

Massicotte, R., Whitelaw, E., and Angers, B. 2011. DNA methylation A source of random variation in natural populations. *Epigenetics* 6 (4):422-428.

Maynard-Smith, J. 1978. *The Evolution of sex*. Cambridge, U.K. : Cambridge University Press.

Meaney, M. J. 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annual Review of Neuroscience* 24:1161-1192.

Mee, J. A. and Rowe, L. 2010. Distribution of Phoxinus eos, Phoxinus neogaeus, and their asexually-reproducing hybrids (Pisces: Cyprinidae) in Algonquin Provincial Park, Ontario. *PLoS One* 5 (10).

Meissner, A., Mikkelsen, T. S., Gu, H. C., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X. L., Bernstein, B. E., Nusbaum, C., Jaffe, D. B., Gnirke, A., Jaenisch, R., and Lander, E. S. 2008. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454 (7205):766-U91.

Meksem, K., Ruben, E., Hyten, D., Triwitayakorn, K., and Lightfoot, D. A. 2001. Conversion of AFLP bands into high-throughput DNA markers. *Molecular Genetics and Genomics* 265 (2):207-214.

Michaels, S. D. and Amasino, R. M. 1999. FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* 11 (5):949-956.

Michaels, S. D., He, Y. H., Scortecci, K. C., and Amasino, R. M. 2003. Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 100 (17):10102-10107.

Michalak, P. 2009. Epigenetic, transposon and small RNA determinants of hybrid dysfunctions. *Heredity* 102 (1):45-50.

Millar, S. E., Miller, M. W., Stevens, M. E., and Barsh, G. S. 1995. Expression and transgenic studies of the mouse agouti gene provide insight into the mechanisms by which mammalian coat patterns are generated. *Development* 121 (10):3223-3232.

Morgan, H. D., Santos, F., Green, K., Dean, W., and Reik, W. 2005. Epigenetic reprogramming in mammals. *Human Molecular Genetics* 14:R47-R58.

Morgan, H. D., Sutherland, H. G. E., Martin, D. I. K., and Whitelaw, E. 1999. Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics* 23 (3):314-318.

Mori, T., Hiraka, I., Kurata, Y., Kawachi, H., Kishida, O., and Nishimura, K. 2005. Genetic basis of phenotypic plasticity for predator-induced morphological defenses in anuran tadpole, Rana pirica, using cDNA subtraction and microarray analysis. *Biochemical and Biophysical Research Communications* 330 (4):1138-1145.

Neiman, M. and Schwander, T. 2011. Using Parthenogenetic Lineages to Identify Advantages of Sex. *Evolutionary Biology* 38 (2):115-123.

Niemitz, E. L. and Feinberg, A. P. 2004. Epigenetics and assisted reproductive technology: A call for investigation. *American Journal of Human Genetics* 74 (4):599-609.

O'Neill, R. J. W., O'Neill, M. J., and Graves, J. A. M. 1998. Undermethylation associated with retroelement activation and chromosome remodelling in an interspecific mammalian hybrid. *Nature* 393 (6680):68-72.

Okano, M., Xie, S. P., and Li, E. 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature Genetics* 19 (3):219-220.

Orkin, S. 1990. Molecular cloning - A laboratory manual, 2nd edition - Sambrook,J, Fritsch,EF, Maniatis,T. *Nature* 343 (6259):604-605.

Pal, C. 1998. Plasticity, memory and the adaptive landscape of the genotype. *Proceedings of the Royal Society B-Biological Sciences* 265 (1403):1319-1323.

Pal, C. and Miklos, I. 1999. Epigenetic inheritance, genetic assimilation and speciation. *Journal of Theoretical Biology* 200 (1):19-37.

Park, C. W., Park, J., Kren, B. T., and Steer, C. J. 2006. Sleeping Beauty transposition in the mouse genome is associated with changes in DNA methylation at the site of insertion. *Genomics* 88 (2):204-213.

Perera, F., Tang, W.-y., Herbstman, J., Tang, D., Levin, L., Miller, R., and Ho, S.-m. 2009. Relation of DNA Methylation of 5 '-CpG Island of ACSL3 to Transplacental Exposure to Airborne Polycyclic Aromatic Hydrocarbons and Childhood Asthma. *PLoS One* 4 (2).

Petronis, A. 2006. Epigenetics and twins: three variations on the theme. *Trends in Genetics* 22 (7):347-350.

Pigliucci, M. 1996. How organisms respond to environmental changes: From phenotypes to molecules (and vice versa). *Trends in Ecology & Evolution* 11 (4):168-173.

———. 2007. Do we need an extended evolutionary synthesis? *Evolution* 61:2743-2749.

Pigliucci, M. and Murren, C. J. 2003. Perspective: Genetic assimilation and a possible evolutionary paradox: Can macroevolution sometimes be so fast as to pass us by? *Evolution* 57 (7):1455-1464.

Pigliucci, M., Murren, C. J., and Schlichting, C. D. 2006. Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology* 209 (12):2362-2367.

Ponger, L. and Li, W. H. 2005. Evolutionary diversification of DNA Methyltransferases in eukaryotic Genomes. *Molecular Biology and Evolution* 22 (4):1119-1128.

Price, T. D., Qvarnstrom, A., and Irwin, D. E. 2003. The role of phenotypic plasticity in driving genetic evolution. *Proceedings of the Royal Society of London Series B-Biological Sciences* 270 (1523):1433-1440.

Rakyan, V. K., Blewitt, M. E., Druker, R., Preis, J. I., and Whitelaw, E. 2002. Metastable epialleles in mammals. *Trends in Genetics* 18 (7):348-351.

Rakyan, V. K., Chong, S., Champ, M. E., Cuthbert, P. C., Morgan, H. D., Luu, K. V. K., and Whitelaw, E. 2003. Transgenerational inheritance of epigenetic states at the murine Axin(Fu) allele occurs after maternal and paternal transmission. *Proceedings of the National Academy of Sciences of the United States of America* 100 (5):2538-2543.

———. 2003. Transgenerational inheritance of epigenetic states at the murine *Axin*^{Fu} allele occurs after maternal and paternal transmission. *Proceedings of the National Academy of Sciences of the United States of America* 100 (5):2538-2543.

Rakyan, V. K., Hildmann, T., Novik, K. L., Lewin, J., Tost, J., Cox, A. V., Andrews, T. D., Howe, K. L., Otto, T., Olek, A., Fischer, J., Gut, I. G., Berlin, K., and Beck, S. 2004. DNA methylation profiling of the human major histocompatibility complex: A pilot study for the Human Epigenome Project. *Plos Biology* 2 (12):2170-2182.

Rando, O. J. and Verstrepen, K. J. 2007. Timescales of genetic and epigenetic inheritance. *Cell* 128 (4):655-668.

Rassoulzadegan, M., Grandjean, V., Gounon, P., Vincent, S., Gillot, I., and Cuzin, F. 2006. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* 441 (7092):469-474.

Razin, A. and Riggs, A. D. 1980. DNA methylation and gene-function. *Science* 210 (4470):604-610.

Reiss, D., Zhang, Y., Rouhi, A., Reuter, M., and Mager, D. L. 2010. Variable DNA methylation of transposable elements The case study of mouse Early Transposons. *Epigenetics* 5 (1):68-79.

Richards, E. J. 2006. Inherited epigenetic variation: revisiting soft inheritance. *Nature Reviews Genetics* 7:395-401.

———. 2006. Opinion - Inherited epigenetic variation - revisiting soft inheritance. *Nature Reviews Genetics* 7 (5):395-U2.

———. 2008. Population epigenetics. *Current Opinion in Genetics & Development* 18 (2):221-226.

Riggs, A. D. 1975. X inactivation, differentiation and DNA methylation. *Cytogenet. Cell Genet.* 14:9-25.

Riggs, A. D., Xiong, Z. G., Wang, L. J., and LeBon, J. M. 1998. Methylation dynamics, epigenetic fidelity and X chromosome structure. In *Epigenetics*, edited by D. J. C. G. Chadwick.

Roemer, I., Grutzner, F., Winking, H., Haaf, T., Orth, A., Skidmore, L., Antczak, D., and Fundele, R. 1999. Genome evolution - Global methylation in eutherian hybrids. *Nature* 401 (6749):131-132.

Rutherford, S. L. and Lindquist, S. 1998. Hsp90 as a capacitor for morphological evolution. *Nature* 396 (6709):336-342.

Salmon, A., Ainouche, M. L., and Wendel, J. F. 2005. Genetic and epigenetic consequences of recent hybridization and polyploidy in Spartina (Poaceae). *Molecular Ecology* 14 (4):1163-1175.

Schlichting, C. D. 2004. The role of phenotypic plasticity in diversification. In *Phenotypic plasticity: functional and conceptual approaches*, edited by T. J. DeWitt and S. M. Scheiner. Oxford: Oxford University Press.

Schlichting, C. D. and Pigliucci, M. 1993. CONTROL OF PHENOTYPIC PLASTICITY VIA REGULATORY GENES. *American Naturalist* 142 (2):366-370.

Schlosser, I. J., Doeringsfeld, M. R., Elder, J. F., and Arzayus, L. F. 1998. Niche relationships of clonal and sexual fish in a heterogeneous landscape. *Ecology* 79 (3):953-968.

Schluter, D. 1993. Adaptative radiation in sticklebacks-size, shape, and habitat use efficiency. *Ecology* 74 (3):699-709.

Schlutz, R. J. 1977. Evolution and ecology of unisexual fishes. *Evol Ecol* 10:277-231.

Schneider, E., Pliushch, G., El Hajj, N., Galetzka, D., Puhl, A., Schorsch, M., Frauenknecht, K., Riepert, T., Tresch, A., Muller, A. M., Coerdt, W., Zechner, U., and Haaf, T. 2010. Spatial, temporal and interindividual epigenetic variation of functionally important DNA methylation patterns. *Nucleic Acids Research* 38 (12):3880-3890.

Scott, W. B. and Crossman, E. J. 1973. *Freswater fishes of Canada*. Edited by O. d. r. s. l. p. d. Canada. Vol. Bulletin 184. Ottawa.

Sheldon, C. C., Burn, J. E., Perez, P. P., Metzger, J., Edwards, J. A., Peacock, W. J., and Dennis, E. S. 1999. The FLF MADS box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell* 11 (3):445-458.

Sheldon, C. C., Conn, A. B., Dennis, E. S., and Peacock, W. J. 2002. Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. *Plant Cell* 14 (10):2527-2537.

Sheldon, C. C., Finnegan, E. J., Rouse, D. T., Tadege, M., Bagnall, D. J., Helliwell, C. A., Peacock, W. J., and Dennis, E. S. 2000. The control of flowering by vernalization. *Current Opinion in Plant Biology* 3 (5):418-422.

Simpson, G. G. 1953. The Baldwin effect. *Evolution* 7:110-117.

Singh, A., Negi, M. S., Moses, V. K., Venkateswarlu, B., Srivastava, P. S., and Lakshmikumaran, M. 2002. Molecular analysis of micropropagated neem plants using aflp markers for ascertaining clonal fidelity. *In Vitro Cellular & Developmental Biology-Plant* 38 (5):519-524.

Sollars, V., Lu, X. Y., Xiao, L., Wang, X. Y., Garfinkel, M. D., and Ruden, D. M. 2003. Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. *Nature Genetics* 33 (1):70-74.

Stam, M., Belele, C., Dorweiler, J. E., and Chandler, V. L. 2002. Differential chromatin structure within a tandem array 100 kb upstream of the maize b1 locus is associated with paramutation. *Genes & Development* 16 (15):1906-1918.

Stearns, S. C. 1989. The evolutionary significance of phenotypic plasticity - phenotypic sources of variation among organisms can be described by developmental switches and reaction norms. *Bioscience* 39 (7):436-445.

Strange, R. M. and Mayden, R. L. 2009. Phylogenetic Relationships and a Revised Taxonomy for North American Cyprinids Currently Assigned to Phoxinus (Actinopterygii: Cyprinidae). *Copeia* (3):494-501.

Sumner, S., Pereboom, J. J. M., and Jordan, W. C. 2006. Differential gene expression and phenotypic plasticity in behavioural castes of the primitively eusocial wasp, Polistes canadensis. *Proceedings of the Royal Society B-Biological Sciences* 273 (1582):19-26.

Suzuki, M. M. and Bird, A. 2008. DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics* 9 (6):465-476.

Thoday, J. M. 1953. Components of fitness. *Symposia of the Society for Experimental Biology* 7:96-113.

Vandegehuchte, M. B., Lemiere, F., and Janssen, C. R. 2009. Quantitative DNA-methylation in Daphnia magna and effects of multigeneration Zn exposure. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* 150 (3):343-348.

Vaughn, M. W., Tanurdzic, M., Lippman, Z., Jiang, H., Carrasquillo, R., Rabinowicz, P. D., Dedhia, N., McCombie, W. R., Agier, N., Bulski, A., Colot, V., Doerge, R. W., and Martienssen, R. A. 2007. Epigenetic natural variation in *Arabidopsis thaliana*. *Plos Biology* 5 (7):1617-1629.

Verhoeven, K. J. F., Jansen, J. J., van Dijk, P. J., and Biere, A. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist* 185 (4):1108-1118.

Verhoeven, K. J. F., Van Dijk, P. J., and Biere, A. 2010. Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. *Molecular Ecology* 19 (2):315-324.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP - A NEW TECHNIQUE FOR DNA-FINGERPRINTING. *Nucleic Acids Research* 23 (21):4407-4414.

Vrana, P. B., Guan, X. J., Ingram, R. S., and Tilghman, S. M. 1998. Genomic imprinting is disrupted in interspecific Peromyscus hybrids. *Nature Genetics* 20 (4):362-365.

Vrijenhoek, R. C. 1978. Coexistence of clones in a heterogeneous environment. *Science* 199:549-552.

———. 1994. Unisexual fish-model systems for studying ecology and evolution. *Annual Review of Ecology and Systematics* 25:71-96.

Waddington, C. H. 1953. Epigenetics and evolution. *Symp. Soc. exp. Biol.* 7.

———. 1961. Genetic assimilation. *Advances in Genetics* 10:257-293.

Waterland, R. A. and Jirtle, R. L. 2002. Maternal dietary methyl donor supplementation affects offspring phenotype by increasing cytosine methylation at the agouti locus in Avy mice. *Faseb Journal* 16 (4):A228-A228.

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., and Meaney, M. J. 2004. Epigenetic programming by maternal behavior. *Nature Neuroscience* 7 (8):847-854.

Weber, M., Davies, J. J., Wittig, D., Oakeley, E. J., Haase, M., Lam, W. L., and Schubeler, D. 2005. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nature Genetics* 37 (8):853-862.

West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford, U.K.: Oxford University Press.

Whitelaw, N. C. and Whitelaw, E. 2006. How lifetimes shape epigenotype within and across generations. *Human Molecular Genetics* 15:R131-R137.

Wilkins, J. F. 2005. Genomic imprinting and methylation: epigenetic canalization and conflict. *Trends in Genetics* 21 (6):356-365.

Wolff, G. L., Kodell, R. L., Moore, S. R., and Cooney, C. A. 1998. Maternal epigenetics and methyl supplements affect agouti gene expression in A(vy)/a mice. *Faseb Journal* 12 (11):949-957.

Wright, S. 1932. The roles of mutation, inbreeding, crossbreeding and selection in evolution. *Proceedings of the Sixth International Congress of Genetics* 1:356-366.

Xiong, L. Z., Xu, C. G., Maroof, M. A. S., and Zhang, Q. F. 1999. Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. *Molecular and General Genetics* 261 (3):439-446.

Yamamoto, F. and Yamamoto, M. 2004. A DNA microarray-based methylation-sensitive (MS)-AFLP hybridization method for genetic and epigenetic analyses. *Molecular Genetics & Genomics: MGG* 271 (6):678-686.

Young, R. L. and Badyaev, A. V. 2007. Evolution of ontogeny: linking epigenetic remodeling and genetic adaptation in skeletal structures. *Integrative and Comparative Biology* 47 (2):234-244.

Zhao, X. X., Chai, Y., and Liu, B. 2007. Epigenetic inheritance and variation of DNA methylation level and pattern in maize intra-specific hybrids. *Plant Science* 172 (5):930-938.

Zilberman, D., Gehring, M., Tran, R. K., Ballinger, T., and Henikoff, S. 2007. Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. *Nature Genetics* 39 (1):61-69.