Hybrid Incompatibility in *Drosophila*: An Updated Genetic and Evolutionary Analysis

Advanced article

Article Contents

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Negative interactions between independently evolved genes in two species are responsible of incompatibility of their hybrid, manifested by sterility and inviability. The heterogametic sex (XY males in *Drosophila*) is the most affected and the X chromosome has the largest effect on hybrid incompatibility (HI). These rules of speciation depend on the genetic architecture of HI. Albeit some speciation genes have a major effect, this architecture shows a complex polygenic structure of multiple interactions. HI genes are frequently associated to genetic factors that evolved selfishly by favouring their preferential transmission. Genetic analyses show signatures of positive selection in speciation genes that may favour the role of adaptive evolution. Whether these signatures are compatible with evolution of selfish factors – an idea that is gaining support – still remains a contentious issue. Finally, there is also a current upsurge of evidences in favour of the importance of genetic regulation in the evolution of hybrid incompatibilities.

Introduction: The Pioneers

Darwin has often been criticised that, despite the title of his magnum opus *The origin of species*, he never tackled the biological basis of this origin. This criticism is unfair because he not only devoted a whole chapter to this topic but also discussed in it the problem of hybrid sterility in relation to natural selection. His view was that hybrid sterility cannot arise by adaptation, but rather must be 'incidental on other acquired differences'. Darwin also discussed the relative role of gene flow and adaptation in the origin of species, undermining the necessity of strong isolation in front of the action of natural selection in the origin of species, a view that is now being vindicated (see Fontdevila, 2011, Chapter 4, p. 116, for a detailed discussion). He also recognised several genetic facts of hybridisation, among them that sterility is the result 'from the organisation (the hybrid genome, in modern parlance) having been disturbed by two organisations (genomes) having been compounded into one'. However, lacking a general knowledge of Mendelian inheritance at the time, Darwin was not able to produce a solid theory on the genetic basis of hybrid incompatibility (HI).

More than 50 years had to pass until the Morgan's fly lab in Columbia University pioneered genetic studies of HI. How much divergence must two populations show to be considered different species still is a matter of controversy. However, intuitively, population thinking requires that, regardless of what mechanism is at work, two species lineages must keep their differential genetic identity by avoiding the conflating effect of introgression. Since the most ubiquitous mechanism of gene exchange, at least in sexual organisms, is sex, there can be no question that reproductive isolation occurred to the first evolutionists as a mechanism to keep species identity. Alfred Sturtevant and Theodosius Dobzhansky are credited for their visionary genetic experiments with hybrids between pairs of closely related *Drosophila* species: *D. melanogaster–D. simulans* by Sturtevant (1920) and *D. pseudoobscura–D. persimilis* by Dobzhansky (1936). Both found that genetic factors, rather than chromosomal incompatibilities to pair in meiosis, were responsible for HI. Crosses between *D. melanogaster* and *D. simulans* yield hybrids that are either sterile or inviable, and the incompatibility of hybrids is sex-specific depending on the direction of the cross. This observation and further crosses with flies modified for their X chromosome content (e.g. XXY *D. melanogaster* females) prompted Sturtevant to posit that hybrid inviability was caused by factors on the X chromosome. Unfortunately, the inviability or sterility of these hybrids prevented introgression crosses to be followed up, and no further dissection of the genetic of HI could be performed. This difficulty could be overcome using crosses between the more closely related species pair *D. pseudoobscura–D. persimilis*, which produce fertile females and sterile males. Aware of this fact, Dobzhansky (1936) was able to map the hybrid sterility

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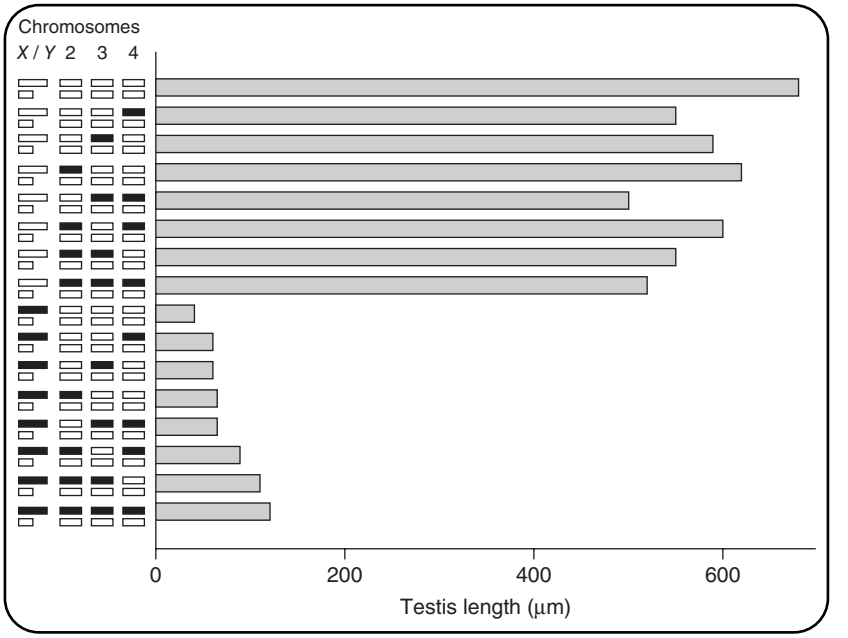


Figure 1 Depiction of results of the first genetic analysis of male hybrid sterility carried out by Dobzhansky (1936). Bar length denotes the testis length (a proxy for male hybrid fertility) of hybrid male genotypes obtained by backcrossing female F_1 hybrids between *Drosophila pseudoobscura* and *Drosophila persimilis* to parental *D. pseudoobscura* males. Males with a *D. pseudoobscura X* chromosome (top half) often are fertile, but males carrying a *D. persimilis X* chromosome (bottom half) are almost completely sterile. These results show the disproportionately large effect of the X chromosome substitution on hybrid male sterility when compared to autosome substitution effects. Solid bar chromosomes denote *D. persimilis*, and open bar chromosomes denote *D. pseudoobscura*. Redrawn with permission from Dobzhansky, 1936 © Genetics Society of America.

factors to particular chromosomal positions (**Figure 1**). His work gave an experimental basis to the first plausible, and most publicised, genetic model of postzygotic HI, posited independently by Bateson (1909), Dobzhansky (1937) and Muller (1942), and named after them as the BDM model. It predicts that HIs are the product of epistatic interactions in the hybrid between alleles of complementary loci that have independently evolved in populations that never coexisted previously (**Figure 2**). Since then, much work has been done to advance our knowledge on the genetic and evolutionary bases of HIs. Here I will discuss an updated analysis of this topic, centering on *Drosophila* studies but occasionally diverging to other species when necessary for a more comprehensive understanding. **See also**: **Genetic Analysis of Hybrid Incompatibilities in *Drosophila***

The Search for 'Speciation Genes'

After these pioneering studies, it is not surprising that the biological species concept, which defines species as groups of actually or potentially interbreeding natural populations that are reproductively isolated from one another (Dobzhansky, 1935; Mayr, 1942), arose, and remains as one of the most widely used criteria for species definition. Among the isolation mechanisms underlying the identity of biological species, postzygotic isolation mechanisms (predominantly hybrid inviability and sterility) have long captured the attention of evolutionists (Dobzhansky, 1936), particularly when other important prezygotic barriers, including habitat, seasonal, ethological, mechanical and gametic isolation, fail to maintain species isolation in specific contexts. **See also**: **Isolating Mechanisms**

This idea of postzygotic isolation unleashed a series of research projects, predominantly using *Drosophila* species, to find 'speciation genes' with the aim of identifying those interacting genes responsible for speciation. Despite decades of research, however, current knowledge of the genetic architecture of postzygotic isolation mechanisms, though significantly advanced, remains contentious. The classic approach of using recombination with species-specific markers to map HI factors (**Figure 3**A) required a large number of them to reduce the undetected recombination between genomes to a minimum. Thus, the number of markers sets the maximum detectable HI factors. Early experiments often used morphological markers, and generally always identified an HI locus with a significant effect linked to each of them (Coyne and Charlesworth, 1989). Therefore, these experiments suggested to early evolutionists that several genes were likely responsible for HIs. However, the question remained as to whether the underlying architecture contained a set of a few major genes with each having a large effect on HI, or it consists of many minor interacting factors whose individual effect on HI was low but cumulative.

Until the 1990s, the prevailing view, at least implicitly, was that few major genes were responsible for HI in *Drosophila* (Coyne and Charlesworth, 1989; Charlesworth *et al*., 1987; Orr, 1989). Backcross (BC) hybrids between *Drosophila buzzatii* and *Drosophila koepferae*, however, demonstrated that, in general, hybrid male sterility (HMS) was not due, at least not exclusively,

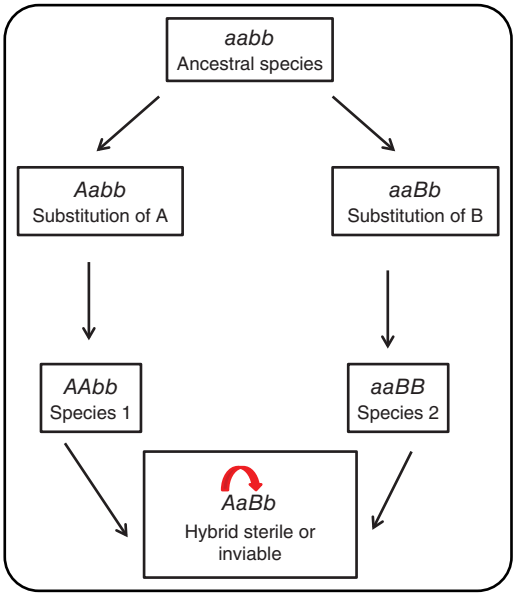
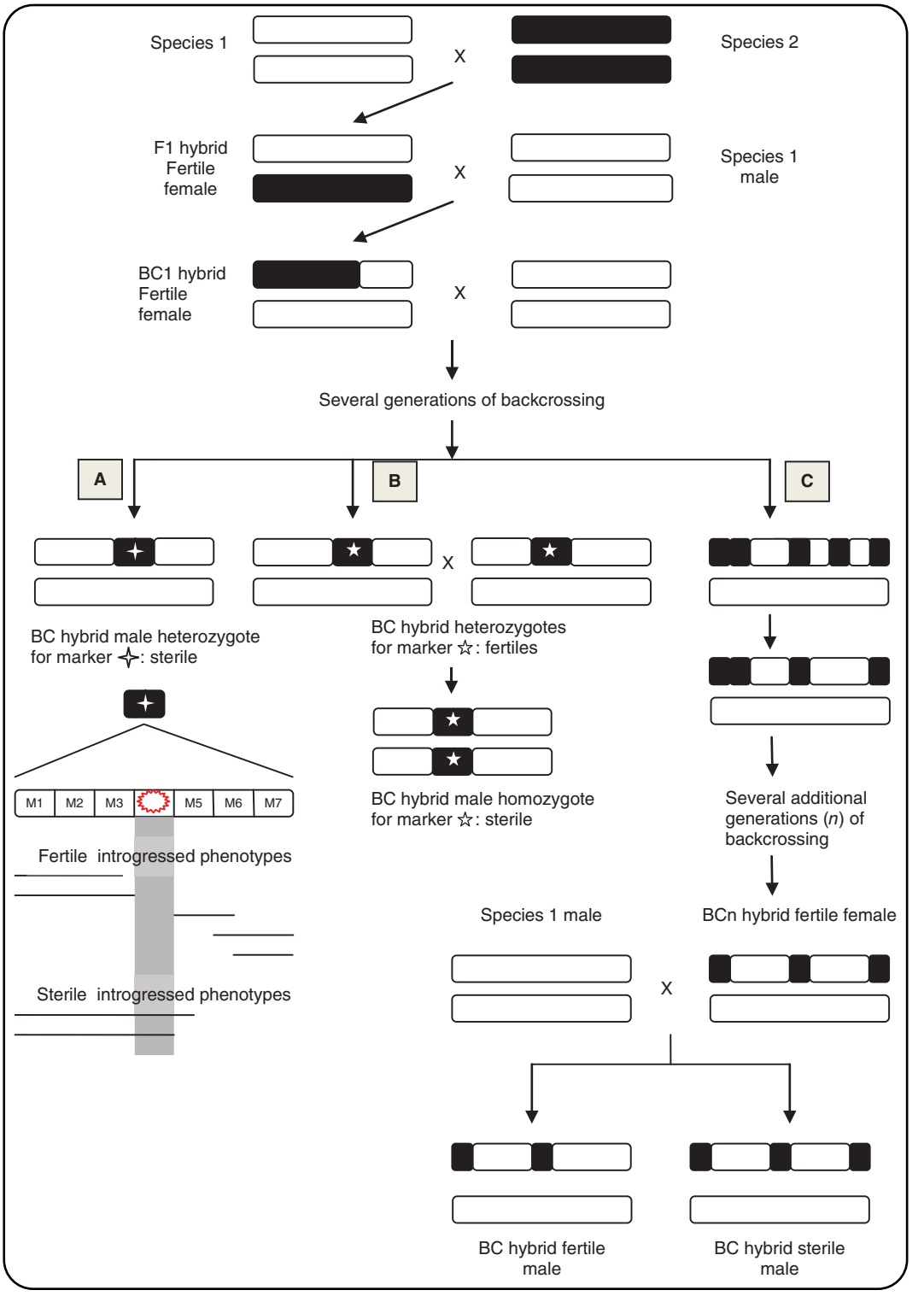


Figure 2 Flowchart of the evolution of hybrid incompatibility following the BDM model. The ancestral species (*aabb*) is split into two populations, originating two lineages isolated from each other. In one lineage, allele *a* is substituted by allele *A* in two steps until fixation of genotype *AA*. Analogously, in the other lineage a similar substitution occurs for the allele B. During this evolutionary process, neither intermediate nor final genotype (*Aabb* → *AAbb*; or *aaBb* → *aaBB*) is sterile or inviable. If in a later stage, both evolved populations meet together and cross; the hybrid genotype (*AaBb*) may be sterile or inviable, depending on the strength of the negative epistatic interaction between *A* and *B* alleles. Notice that these alleles have never been together in the same genotype throughout their evolutionary substitution and natural selection had no chance to test their compatibility. In the hybrid, however, they co-exist and may be incompatible with one another. The BDM model circumvents the impossibility of developing HI by one-locus evolution, because in that case the intermediate stage (*Aa* or *Bb*) would already be inviable or sterile.

to few individual genes of large effect (Naveira and Fontdevila, 1986). The fact that no autosomal region, singly introgressed or combined with other regions, could produce HMS unless the total added introgressed region exceeded a minimum size (about 30% of the autosomal chromosome length) prompted these investigators to postulate a large number of minor HI factors dispersed in the genome, whose incompatibility manifests only when a minimum of them interacts epistatically. Most interesting, both short and long X-chromosome segments of *D. koepferae* introgressed into *D. buzzatii* background always produced sterile hybrid males. This might be interpreted as due to each short X segment at least containing one major gene, but this was later contradicted because sterile hybrid males were obtained by simultaneously introgressing several smaller X-chromosome segments of *Drosophila mauritiana* into *D. simulans* that singly did not produce sterility (Naveira, 1992). The lower size of introgressed X segment for sterility is rather the consequence of a higher density of HMS factors in the X chromosome, as has also been shown in later studies (Masly and Presgraves, 2007; Tao *et al*., 2003a). However, this polygenic threshold model (the PT model), corroborated by further research (Naveira and Fontdevila, 1991a, 1991b; Naveira, 1992), was received with skepticism.

During the 1990s and the 2000s, several researchers carried out a series of experiments that culminated in the mapping and characterisation of a number of genes that had a significant major effect on interspecies hybrid sterility (**Table 1**). The paradigmatic example concerns the study of the *Odysseus* (*Ods*) gene, which contributes to the sterility in hybrids between *D. simulans* and *D. mauritiana*. Initially identified using classical mapping methods (Coyne and Charlesworth, 1989; Perez *et al*., 1993), it was later submitted to molecular manipulation assays (Ting *et al*., 1998). Then, it was concluded that *Ods* may contribute to hybrid sterility but not in isolation, requiring the cooperation of other genes. The same conclusion has been reached whenever the individual effect of any putative major 'speciation' gene has been tested using gene manipulations (Tang and Presgraves, 2009). This cooperative interactive behaviour was formerly dubbed the 'weak allele–strong interaction' by Wu and Hollocher (1998). Perhaps the finding of the so-called hybrid rescue mutations has also been much influential in favour of major effect genes. Briefly, the inviability of hybrid males from the cross between *D. melanogaster* females and *D. simulans* males can be avoided (rescued) by loss-of-function mutations on just two genes: *Hmr* on the X chromosome of *D. melanogaster*, and *Lhr* on chromosome 2 of *D. simulans* (**Table 1**). A large criticism to the general meaning of these rescue genes, however, relies on that the approach to detect them applies only to HIs of a simple genetic basis and not to reveal complex multi-locus HIs. Moreover, the *Hmr–Lhr* interaction does not cause hybrid inviability in a full *D. melanogaster* background, suggesting that this incompatibility needs the cooperation of more genetic factors from *D. simulans* to produce complete inviability. More recently, several genetic studies using animal and plant species have revealed a complex interaction of many loci underlying HI (Cabot *et al*., 1994; Chang *et al*., 2010; Nosil and Schluter, 2011). So far, the majority of documented architectures of hybrid sterility suggest that several to many genetic factors are involved and that a minimum number are necessary to drive hybrids to complete sterility or inviability (reviewed in Maheshwari and Barbash, 2011; Presgraves, 2010).

The minimum number of factors that must interact to produce a significant HI, however, is still viewed differently by diverse authors, ranging from those who assign a large importance to some genes of large effect ('major' genes) (Phadnis and Orr, 2009) to others who consider that the accumulated small effects of many 'minor' genes are most relevant for the HI (Naveira and Fontdevila, 1986). To demonstrate that there is a minimum of sterility factors necessary for generating HI, Naveira and Maside (1998) performed insertions of small restriction fragments of *D. melanogaster* into a fixed fertile hybrid strain comprising three X-chromosome sections from *D. mauritiana* introgressed into a *D. simulans* background, and demonstrated that hybrid sterility is induced by slightly increasing the size of the inserted fragment (from 1.9 to 6.7 kbp), irrespective of its coding potential. This study provides strong evidence for the threshold nature of the PT model. However, many researchers do not agree that exchangeability among genes is the most relevant factor, inferring that their identity is more important. For instance, Chang and Noor (2010) showed that the introgression from *D. persimilis* into a *D. pseudoobscura bogotana* background of a chromosomal



fragment (QTL) larger than one that does produce HMS in combination with two other QTL failed to induce HMS. These authors claimed that this result supports that the size of the introgressed fragment is not responsible of HMS and discredits the extreme PT model. Even so, in that study it is possible that the introgressed region, whose location and characterization was not described by the authors, failed to produce sterility just because it is devoid of interacting sterility factors and its introgression generates outlier sterility phenotypes, sometimes found, not following the PT model (**Figure 4**). Obviously, only genome-wide studies can assess the overall validity of the PT model.

The PT model has been recently assessed by Morán and Fontdevila (2014) using a genome-wide association study (GWAS). By combining molecular, cytogenetic and bioinformatic approaches, they aimed to detect, map, and characterise a representative set of hybrid sterility-associated AFLP (amplified fragment length polymorphism) markers in the genome fraction of *D. koepferae* introgressed into *D. buzzatii* by serial backcrossings (**Figure 3**C). In sum, this work supports the PT model. First, a set of introgressed genome fragments (about 26% of the genome) could be associated with hybrid sterility and a minimum of them (a threshold) were required to induce significant male sterility (**Figure 4**). This minimum consists of a non-specific set in which polygenes can be exchangeable so far as they interact sufficiently to impair fertility. Second, these genome fragments mapped with a tendency to cluster in chromosomal regions of low recombination. Third, using bioinformatics it was demonstrated that the marker surroundings are enriched with genes whose functions are implicated in development and reproductive routes.

This fine-scale genome-wide analysis agrees with other analyses in that a complex set of epistatic interactions between genetic factors underlies the HMS. Recently, several GWAS performed in house mouse also revealed complex interactions among sterility loci, which suggests multiple, non-independent genetic incompatibilities that contribute to reproductive barriers (Dzur-Gejdosova *et al*., 2012; Turner *et al*., 2014; Turner and Harr, 2014). Even in *Drosophila*, when new fine-scale approaches are used, the complexity of interaction networks is revealed in the action of large-effect speciation genes such as *Ovd* (Phadnis, 2011). The importance of the incompatibility networks notwithstanding, not a single case of BDM incompatibility is known for which all partner loci of a HI speciation gene have been described, as stated by Phadnis (2011).

We are beginning to realise that the genetic complexity of HI is large, implicating many interactions mostly among partially recessive genes of small effect, but we cannot exclude the sporadic participation of some genes of 'major' effect. How frequent are these 'major' genes one cannot tell yet, but after more than 20 years of intensive *Drosophila* work, it is amazing that the number of them that have been characterised is small, totaling in *Drosophila* three for HMS and four for HMI (hybrid male inviability) (**Table 1**), and a similar low number in yeast, mice and plants (Maheshwari and Barbash, 2011; Presgraves, 2010). This could be due to their paucity in the genome, but others have attributed it to the difficulty of genetic manipulation when dealing with a character like reproductive isolation because of intrinsic problems to perform serial crosses when hybrid individuals are sterile or inviable. **See also**: **Speciation Genes**

The Rules of HI

Since the pioneering ideas on HI advanced by early evolutionists, much work has been devoted to empirically bolster the rules that govern these ideas. Though not much has changed of the HI concepts posited by the pioneers, a lot of evidences have been produced, which also reveal unanticipated new insights. Following is a summary of the present knowledge of these rules.

The Haldane's rule: its genetic bases

'When in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex'. This sentence, stated by J. B. S. Haldane in 1922 and largely ignored for 50 years, launched one of the most ubiquitous and

Figure 3 Flowchart of three strategies for mapping genetic factors (loci) associated to hybrid male sterility in *Drosophila*. The process starts by crossing two species (1 and 2) to generate hybrid fertile females, which are individually backcrossed (BC) to males of the host species (1) to yield a progeny of recombinant hybrids. In this first BC_1 generation, male recombinant hybrids are sterile whereas hybrid females are fertile. Then individual hybrid females are backcrossed for several generations to host males (species 1) until introgressed sterile male phenotypes (BC) show to be associated to genetic markers of donor species 2 (strategies A and B) or progenies consists of both BC sterile and fertile males (strategy C). In strategy A, a set of morphological markers are used to detect the associated sterility factor by recombinant analysis, but usually the recombinant marker (represented by a four peak star) maps a wide region that must be further dissected using more specific markers (M1–M7) flanking the putative sterility factor depicted by a stared circle at M4 (in red). The figure shows (by a shaded bar column) how the introgressed fragments that yield fertile phenotypes do not contain the M4 marker, whereas those that yield sterile phenotypes do (see Perez *et al*., 1993 who used this strategy to map *Ods* gene). Strategy B applies to detect partially recessive autosomal alleles whose full effect on hybrid male sterility can be detected only in homozygosity. Using different techniques to introgress individual marker regions (five peak star) after many bakcrossings, crosses between BC hybrid heterozygotes for the same marker can yield a set of hybrid male homozygotes that can be tested for sterility. This procedure was used by Masly and Presgraves (2007) to show that most sterility factors are recessive. Strategy C uses natural markers detected by dissecting the genome by means of DNA techniques such as AFLP. Markers specific to each species allows locating regions that are introgressed by the donor species in the BC_n hybrid males. When the progeny of a BC_n hybrid fertile female consists of both fertile and sterile males, one can detect the markers associated to sterility by comparing the marker patterns of siblings. Note that in the figure the chromosome of the BC hybrid sterile male contains a marker specific of species 2 (in the right extreme side) that is absent in its fertile sibling. By genome-wide association studies for many markers, the genetic architecture of hybrid male sterility can be assessed as in Morán and Fontdevila's (2014) work. Homologous chromosomes are depicted by bars: white and black bars for species 1 and 2, respectively. By recombination through backcrossing, chromosome fragments of species 2 (in black) are introgressed in homologous chromosomes of species 1 (in white). Note that the amount of introgressed fragments decreases with the number of backcrossing generations, until only one marked fragment is selected for its association to male sterility (strategies A and B) or until the combined introgressed fragments yield a progeny with fertile and sterile males (strategy C).

Table 1 Examples of hybrid incompatibility speciation genes in *Drosophila* and their evolutionary properties

Gene	Name	Species cross	Hybrid incompatibity	Positive selection	Proposed evolutionary basis	Complex BDM interaction[a]
Hmr	Hybrid male recue	*D. melamogaster*/ *D. simulans*	F_1 male inviability; F_1 female inviability and sterility	Yes	Genetic conflict[b]/ mutational heterochromatic turnover	Yes
Lhr	Lethal hybrid rescue	*D. simulans*/ *D. melanogaster*	F_1 male inviabiity	Yes	Genetic conflict[b]/ mutational heterochromatic turnover	Yes
Nup 96	Nucleoporin 96	*D. simulans*/ *D. melanogaster*	BC-like male inviability	Yes	Genetic conflict/ host-patogen response	Yes
Nup 160	Nucleoporin 160	*D. simulans*/ *D. melanogaster*	BC-like male inviability and female sterility	Yes	Genetic conflict/ host-patogen response	Yes
Ods	Odysseus	*D. mauritiana*/ *D. simulans*	BC-like male sterility	Yes	Genetic conflict[b]	Yes
Ovd	Overdrive	*D. pseudoobscura bogotana*/ *D. pseudoobscura pseudoobscura*	F_1 male sterility	Yes	Genetic conflict[c]	Yes
JYalpha	JYalpha	*D. simulans*/ *D. melanogaster*	Introgression hybrid male sterility	No	Gene transposition[d]	No
Zhr	Zygotic hybrid rescue	*D. melanogaster*/ *D. simulans*	F_1 female lethality	?	Genetic conflict[b]/ mutational heterochromatic turnover	Unknown

Source: After Presgraves (2010); Maheshwari and Barbash (2011), and Nosil and Schluter (2011).
[a]Complex BDM interaction means that the number of BDM genes that epistatically interact is large, often unknown but usually more than 3.
[b]This conflict involves heterochromatin-mediated distortion or drive.
[c]This conflict involves sex-ratio distortion.
[d]In this case the *Drosophila* male fertility-essential gene *JYalpha*, located on chromosome 4 in *Drosophila melanogaster*, has moved to chromosome 3 in the *Drosophila simulans* clade species. As a result, recombinant hybrid males homozygous for the *D. melanogaster* third chromosome and *D. simulans* fourth chromosome are sterile because they completely lack *JYalpha* (Masly *et al*., 2006).

solid rules in the field of speciation. Haldane's rule has been supported by classic surveys (Coyne, 1992) and also by new ones on taxa ranging from vertebrates to insects (**Table 2**), and also to crustacea, gastropoda and nematodes (Schilthuizen *et al*., 2011). Muller, in his 1942 illuminating and seminal paper titled 'Isolating mechanisms, evolution, and temperature', was first to propose that hybrid sterility is the result of many alleles that act as partial recessives. Thus, only in the heterogametic sex (XY males or ZW females) the X(Z)-linked recessives are completely expressed and can produce BDM interactions, but are masked in the homogametic sex (XX females and ZZ males). This hypothesis, known as 'the dominance hypothesis', was later formalised by Turelli and Orr (1995), showing under which conditions of dominance the Haldane's rule holds true.

Though genetic and comparative studies support the dominance theory (Masly and Presgraves, 2007, and references therein) and even confirm its generality (Schilthuizen *et al*., 2011), this theory cannot by itself explain why often there are more documented cases of Haldane's rule for HMS than for HMI in male-heterogametic taxa (**Table 2**) (but this does not always apply to female heterogametic taxa, see Schilthuizen *et al*., 2011 for Aves, Reptilia and Lepidoptera). To cope with this difficulty, the theory of faster male evolution was advanced. Male-expressed genes are under control of two kinds of sexual selection: female choice and male competition (Wu and Davis, 1993; Wu *et al*., 1996), but female-expressed genes are only subject to female choice. This excess of sexual selection in male-expressed genes would lead to faster accumulation of incompatibilities of male sterility than of those causing HMI or hybrid female sterility and inviability (Wu and Davis, 1993). Another putative cause for faster male evolution has also been suggested, namely that spermatogenesis is a highly sensitive process that is easily disrupted in hybrids. The problem with these male-related theories is that neither can explain the presence of the Haldane's rule nor the excess of hybrid female inviability in some female heterogametic taxa. Moreover, in male heterogametic Teleostei, the pattern often follows that of female heterogametic taxa (Schilthuizen *et al*., 2011).

It is known for a long time that different parts of the genome, both nuclear and mitochondrial, do not share the same interest in their transmission. Some elements behave selfishly, that is, they attempt to be transmitted preferentially hindering the

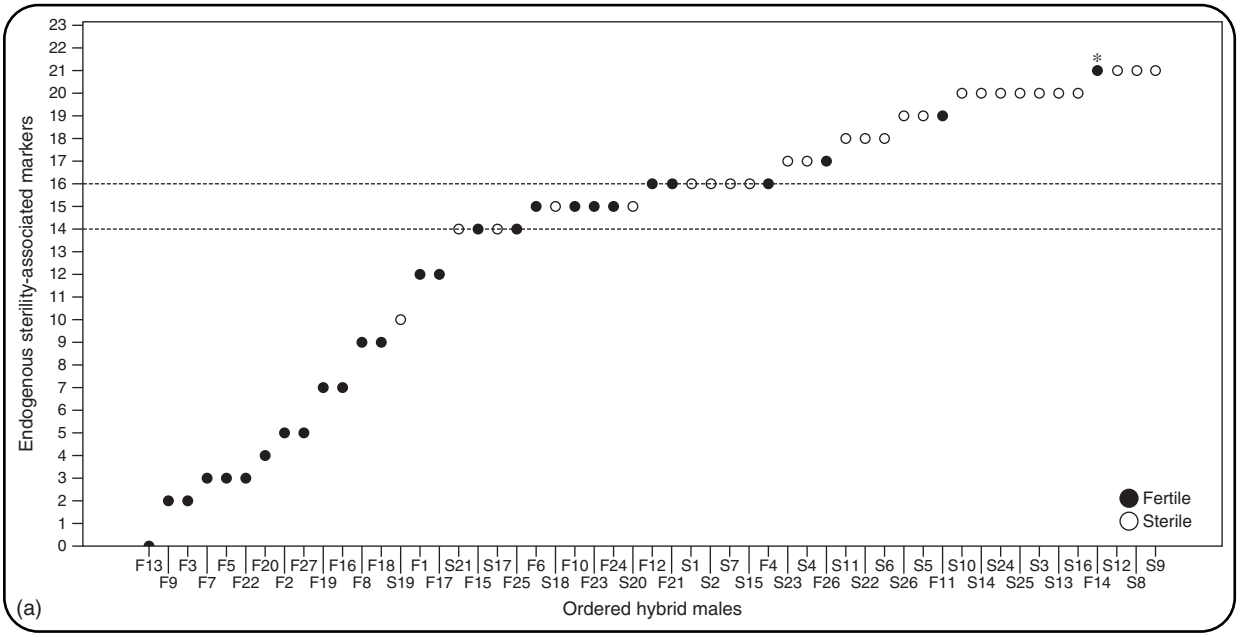


Figure 4 Ordered representations of sterility-associated markers present in each hybrid introgressed male obtained by backcrossing hybrid *Drosophila buzzatii*/*Drosophila koepferae* females to *D. buzzatii* males for three generations. (a) Ordered depiction of the fertile (F) and sterile (S) hybrid males in relation to the increasing number of sterility-associated markers present in each hybrid male supports the cumulative action, with a threshold, of genes surrounding these sterility markers. The threshold number of sterility-associated markers required to elicit sterility is approximately 14–16, whose limits are represented by two dotted lines that correspond to the transition from fertile to sterile hybrid phenotypes. Some outlier individuals exist, which shows that the effect on sterility of genes linked to markers is variable, and in some instances a particular mixture of a number of low-effect (high-effect) markers over (under) the threshold can produce a fertile (sterile) phenotype (see hybrid males F_{26}, F_{11}, and F_{14} over, and S_{19} under the threshold). Some of fertile outliers show a reduced fertility, such as F_{14} fertile individual (*) with an observed reduced offspring of only three adult flies. (b) Segregation matrix of sterility-associated markers. Sterility-associated markers (shown in columns) were scored in a binary matrix as 0 (white cells) when absent or 1 (grey cells) when present. To facilitate the matrix inspection all the fertile (F) males are grouped in the upper rows of the matrix and separated from the sterile (S) males by a straight black line. Inside each fertility class, (F, S) males (left margin) were organised by their increasing content of sterility-associated markers (right margin). The figure shows that presence of a specific marker to produce sterility is not a necessity; rather, the presence of a minimum number of markers over a threshold induces sterility with high probability. In fact, some ubiquitous or highly present markers in sterile hybrids are also present in many fertile ones (e.g. GCGGG17, CAGCG8, TGCAT13 markers are present in all sterile males and in many fertile ones) and no marker exclusively occurs in sterile males. These data support the ample exchangeability of sterility-associated markers. Reproduced with permission from Oxford University Press after Morán and Fontdevila (2014).

transmission of other elements. These 'selfish elements' include transposable elements, repetitive DNA (deoxyribonucleic acid), segregation distorters, cytoplasm male sterility factors, and the like. The genome of every species has evolved mechanisms to suppress this genetic conflict (GC) towards the benefit of the organism, but in the hybrids the interaction between selfish genes and their suppressors may be disrupted leading to HI (Johnson, 2010). This drive theory, as it became known, states that GC drives the success of transmission of sex chromosomes leading to an arms race between gene driver factors and their suppressors, causing faster evolution of fertility genes in both male and female heterogametic hybrids (Hurst and Pomiankowski, 1991; Tao and Hartl, 2003; Presgraves, 2010; Garrigan *et al*., 2014 and references therein). The drive theory, if true, would solve the problem of the faster male evolution and other male-related theories to explain the presence of Haldane's rule in both male and female hybrid heterogametic sexes (see below for an extended discussion). Finally, a significant number of recent studies (~20%) have reported patterns not envisaged by the Haldane's rule (Schilthuizen *et al*., 2011) that could mainly be explained by cytonuclear incompatibilities.

The large X effect

A ubiquitous observation in backcross experiments of hybrid sterility, referred to as the large X effect, is that substitution of an X chromosome of one species in the genetic background of the other has a larger effect on hybrid sterility or inviability in the heterogametic sex (hemizygous for the X chromosome in *Drosophila* XY males) than a similar substitution of a foreign autosome. This has been observed across a range of species such as *Drosophila* and mice, and also in butterflies and birds, where females are the heterogametic sex (see references in Garrigan *et al*., 2014). This ubiquity makes the large X effect a main rule of speciation. The dominance theory explains, by itself, that the recessive incompatibility genes of the X chromosome could express themselves in hemizygosity, whereas those on autosomes, in heterozygosity, could not. But, another possibility when the heterogametic sex is the male could be that male sterility factors accumulate faster on the X chromosome than on autosomes, as was suggested by the faster X theories. They include at least three mechanisms. First, it is known that when a new favourable mutation is recessive, its probability of fixation

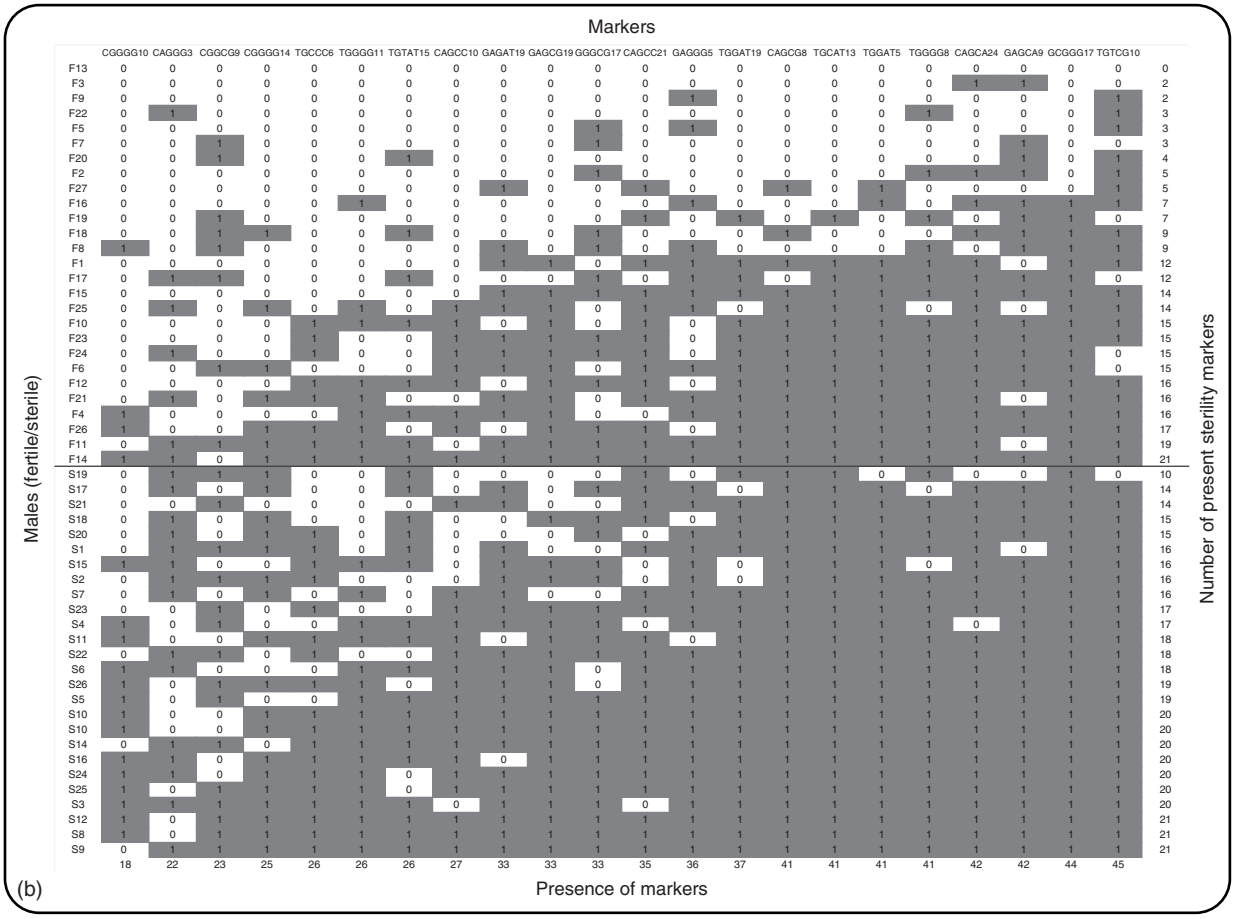


Figure 4 (*Continued*)

is higher if it occurs on the X chromosome (Charlesworth *et al*., 1987; Vicoso and Charlesworth, 2009; Mank *et al*., 2010) and will diverge between species faster than autosomal ones, generating more X-associated BDM incompatibilities. Second, the same drive mechanism described for the Haldane's rule may favour the accumulation of selfish elements in the X chromosome and their autosomal suppressors, causing higher divergence at X-linked genes (Hurst and Pomiankowski, 1991; Tao and Hartl, 2003; Llopart, 2012). Third, the X chromosome evolved dosage compensation and gene inactivation in the male germ line to correct its lower gene content relative to autosomes and its lack of synapsis along a large part of its length, respectively. A lot of X-linked gene divergence between species may be produced by either of these adjusting mechanisms, leading to hybrid sterility (Llopart, 2012, and see below for an extended discussion).

Some empirical foundations of the HI rules

Any of the explanatory mechanisms stated above have empirical evidence, yet their relative importance is still a matter of controversy. The old controversy between the large X effect due simply to its hemizygous status (the dominance theory) versus its higher density of X-linked incompatibility loci is currently settled. Some of the early experiments were objected based either on the lack of length precision of the introgressed segments (Hollocher and Wu, 1996; True *et al*., 1996) or because they compared X-introgressed segments in hemizygosis with the autosomal introgressed segments in heterozygosis, as ensues from the backcross experiments (Wu and Davis, 1993). Since heterozygosis may obscure or change the effect of incompatibility factors, the comparison should be done with autosomal introgressed segments in homozygosis rather than in heterozygosis. These criticisms have later been corrected by more precise experiments. Though experiments by True *et al*. (1996) – in which the autosomal segments of *D. mauritiana* introgressed into *D. simulans* were made homozygous (**Figure 3**B) – showed that X introgressions caused higher sterility than autosomal introgressions, there still were doubts about the precise estimates of introgression sizes. Namely, if X chromosome segments were larger than autosomal ones, large X effect had no empirical support. In order to avoid the size problem, Masly and Presgraves (2007) performed a similar introgression analysis of chromosomal segments of *D. mauritiana* into *Drosophila sechellia*, two island species that diverged only about 240 000 years ago, but now they estimated the introgression sizes more

Table 2 Summary of studies of Haldane's rule

Taxa, sex determination and sterility/inviability	Following Haldane's rule[a]	Not following Haldane's rule[a]	Following Haldane's rule[b]	Not following Haldane's rule[b]	Totals following	Totals not following	Proportion following
Drosophila XY/XX							
Sterility	199	3	11	0	210	3	0.99
Inviability	14	9	–	–	14	9	0.61
Mammals XY/XX							
Sterility	25	0	9	0	34	0	1.00
Inviability	0	1	4	0	4	1	0.80
Lepidoptera ZW/ZZ							
Sterility	29	1	6	1	35	2	0.95
Inviability	56	1	4	1	60	2	0.97
Aves (Birds) ZW/ZZ							
Sterility	72	3	3	1	75	4	0.95
Inviability	21	2	215	2	236	4	0.98
Other Diptera (*Anopheles* XY/XX and *Aedes* X′X/XX)							
Sterility	67	0	2	0	69	0	1.00
Inviability	22	4	–	–	22	4	0.85
Reptilia ZW/ZZ							
Sterility			0	1	0	1	0
Inviability			1	0	1	0	1.00
Amphibia (multiple sex determination)							
Sterility			0	1	0	1	0
Inviability			66	28	66	28	0.70
Teleostei (fish) (multiple sex determination)							
Sterility			5	0	5	0	1.00
Inviability			6	37	6	37	0.14
Totals							
Sterility	392	7	36	4	428	11	0.97
Inviability	113	17	296	68	409	85	0.83

Source: [a]Presgraves (2010); [b]Schilthuizen *et al*. (2011), data since 1996 not overlapping with those of previous source.

precisely with molecular markers (**Figure 5**). Using X-linked and autosomal segments of similar size, they found not only that recessive hybrid incompatibilities outnumber dominant ones and introgressions producing HMS were the majority by far, in agreement with the dominance and faster male theories, but also that 60% of X-linked introgressions versus only 18% of autosomal introgressions caused HMS. These results confirmed, in a more controlled way, previous results that HI factors are more abundant on the X chromosome than on the autosomes and gave support to the large X effect hypothesis. **See also**: **Genetic Analysis of Hybrid Incompatibilities in *Drosophila***

It seems clear that the large X effect plays a fundamental role in the evolution of HI, but some of the responsible evolutionary mechanisms still remain contentious. For instance, the faster X molecular evolution of X-linked loci based on recessive favourable mutations in the X chromosome has been subjected to different experimental results, ranging from positive (Mank *et al*., 2010) to negative acceptance (Thornton *et al*., 2006). It was not clear that substitution rates of X-linked and autosomal loci in *Drosophila* are different, which has led researchers to turn to other more plausible explanations. Fortunately, the whole-genome population analyses currently available may be used to test the hypotheses stated above. Using a de novo genome assembly of *D. mauritiana* annotated with RNAseq data, Garrigan *et al*. (2014) performed a whole-genome analysis from ten individuals, showing that X chromosome has lower diversity and greater divergence than the autosomes, and also an excess of recent selective sweeps. Interestingly, the X chromosome contains more signatures of genes that show a history of recurrent adaptive protein evolution than the autosomes and they are enriched for functions implicated in gametogenesis, chromosome dynamics and satellite DNA. I will further discuss this below but, in sum, these results strongly suggest to these researchers that GC and positive natural selection on the X chromosome are main subjects of the faster evolution of X chromosome in *D. mauritiana*.

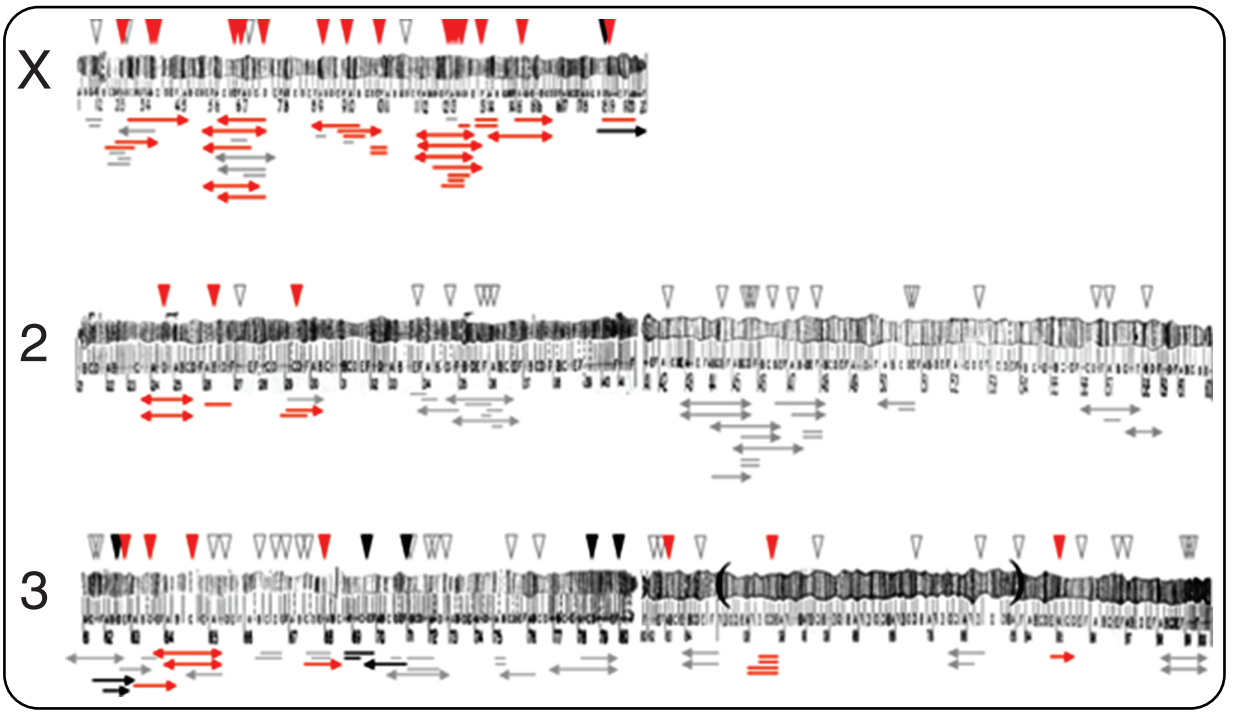


Figure 5 Distribution of hybrid incompatibilities of *Drosophila mauritiana* introgressions in the *Drosophila sechellia* genome. Above the X, second and third chromosomes, triangles show the insertion sites of different P-elements used for introgressions: black indicates hybrid inviable; red indicates hybrid male-sterile; white indicates hybrid fertile or untested. Below the chromosomes, arrows depict the estimated sizes of many introgressions. Black indicates hybrid inviable introgressions; red indicates hybrid male-sterile; grey indicates hybrid fertile. This figure graphically depicts that the density of hybrid male sterility factors is >4 times higher on the X than on an average (and similarly sized) autosomal arm. These numbers are minimum estimates because each introgression might contain more than one incompatibility gene, hybrid inviable introgressions may mask tightly linked hybrid male steriles, and some regions of the genome that may contain hybrid incompatibility loci were not screened. Reproduced from Masly and Presgraves (2007) © US National Library of Medicine, National Institutes of Health licensed under Creative Commons Attribution.

While some experiments using molecular genetics and genomic techniques support the importance of the individuality of genes in the generation of HI, more work had to be done to acquire an overview of the architecture and the functionality of HI at the genome level. Evolutionists agree that discriminating between the genic and the genomic views requires high-resolution GWAS of the architecture and the function of HMS (Chang *et al*., 2010; Chang and Noor, 2010; Maheshwari and Barbash, 2011). Yet, there still are few GWAS of HMS factors. Perhaps the most complete high-resolution genome-wide screens for HMS come from studies with a set of species belonging to the *Drosophila simulans* clade, which comprises *D. simulans*, *D. sechellia*, and *D. mauritiana*. Several introgression studies reported that about 15 *D. mauritiana* regions cause total HMS when homozygous in the background of the other species (True *et al*., 1996; Masly and Presgraves, 2007; Tao *et al*., 2003a,b). In other studies, no case of dominant HMS single introgression has been reported (Tao and Hartl, 2003) and the large majority of BDM interactions are between recessive alleles (Presgraves, 2003). This confirms the complexity of HI architecture but seems to contradict Haldane's rule, which postulates incompatibilities between recessive X-linked factors and dominant-acting autosomal factors. However, Chang and Noor (2010) demonstrated that individually recessive autosomal HMS factors become partially dominant when co-introgressed, reinforcing the view that epistatic interaction between several HMS factors is a necessary condition for total HMS. These results agree with Muller's (1942) statement that there must be 'a large class of recessive complementary incapacitating genes, a class much larger than the dominants, which usually escapes observation'. Besides, these genome-wide studies likely underestimate the number of HMS loci in the genome because each introgression may contain more than one HMS locus and some regions of the genome could not be explored.

Evolving Hybrid Incompatibilities

While association patterns suggest a causal view of the underlying genetic pattern of HIs, they do not unveil how these incompatibilities evolved. Traditionally, ever since Darwin, HI has been attributed to by-products of ecological adaptation in speciation (Sobel *et al*., 2010), including pleiotropic and hitchhiking effects as the most likely. It is not surprising that the finding of signatures of selection in speciation genes has often been taken as proof of the prime role of ecological adaptation in speciation. Although this adaptive hypothesis has its appeal, there are few well-documented cases that HI evolved as a

by-product of ecological adaptation (but see Wright *et al*., 2013). On the contrary, however, the rapid evolution and functional divergence of speciation-related DNA sequences often shows no relationship to environmental traits. Accordingly, the present molecular analyses of speciation suggest that internal genomic mechanisms, including several genetic arms race processes, may be playing a prime role (Johnson, 2010; Presgraves, 2010). **See also**: **Speciation: Genetics**

The early steps of the evolutionary origin of HIs

When studying the evolutionary basis of HI, one must distinguish between its present genetic architecture from the process that produced it. The fact that the present genetic architecture reveals a large complexity of BDM interactions does not necessarily imply that they should be crucial for the initial steps of species divergence. In fact, most of them might have accumulated after speciation had been completed (Orr and Turelli, 2001). By the same token, the finding of genetic factors of great effect on HI does not guarantee their implication in the initial steps of the divergence process. Thus, most evolutionists agree that studies with pairs of recently diverged species are crucial for revealing the initial steps of the genetic evolution of HI.

Despite some authors' early claim that the genes responsible for the first steps in postzygotic reproductive isolation basically are identical in nature and performance to the genes incorporated further in species divergence (Wu and Hollocher, 1998), the final proof will not emerge until the HI between recently diverged species has been studied. These studies have shown that there is no reason to think that the initial genetic architecture of HMS is of a different kind than that found in later stages of HMS (Orr *et al*., 2007). However, while these studies shed light on the first stages of speciation, they do this in a limited way. This is so because they do not tell us much about the putative intraspecific variability for the speciation genes which later evolutionary forces may act upon. This has thoroughly been investigated in *Drosophila* (Reed and Markow, 2004), mice (White *et al*., 2012) and *Tribolium* (Demuth and Wade, 2007), and a high variability for HMS was found to be already present within and between populations of incipient species or races when they still show a high degree of interspecies crossability. Interestingly, the genetic architecture of this incipient HMS is of the same kind as that found in later stages of reproductive isolation, namely a complex polygenic epistatic system. These results bolster the view that much of the variability for genetic factors is already present in the normal polymorphisms of original populations and does not appear de novo in later divergence times.

The role of GC in speciation

Despite the fact that the number of HI studies suggesting the role of GC in speciation has steadily increased in recent years, most of them proved only to be circumstantial. The evidence of GC is often hidden in extant species (but see McDermott and Noor, 2012) and only revealed in hybrids. Such is the case of segregation distortion in *D. pseudoobscura*, a condition by which only one allele in the heterozygote is transmitted to the detriment of its partner. Two subspecies of *D. pseudoobscura* exist. Neither subspecies shows any evidence for segregation distortion, but when the X chromosome of *D. pseudoobscura bogotana* is introgressed in the *D. pseudoobscura pseudoobscura* genome, the partially fertile hybrid males yield almost only females. In fact, the Y-bearing sperms are eliminated by the action of some X-linked distorter genes that evolved in *D. pseudoobscura bogotana* and were silenced by suppressors absent in *D. pseudoobscura pseudoobscura* (Orr and Irving, 2005). Similar evidence of this mechanism exists for other species of *Drosophila* and other animals and plants (Presgraves, 2010; Meiklejohn and Tao, 2010). Interestingly, the distorter locus often maps to the same site as the sterility locus in these hybrids (Phadnis and Orr, 2009; Orr and Irving, 2005; Garrigan *et al*., 2014). Both segregation distortion and male sterility are caused by interactions among X-linked *D. pseudoobscura bogotana* distorter factors and other factors on *D. pseudoobscura pseudoobscura* autosomes. Phadnis and Orr (2009) discovered one of the X-linked distorters, *Ovd* (overdrive), that acts in hybrid sterility and segregation distortion. Despite the fact that *Ovd* is necessary for hybrid sterility, it needs the cooperation of other interacting loci to produce a strong HI. As mentioned above, these complex interactive systems seem to be a general rule for the speciation genes to fully accomplish their role as generators of postzygotic isolation. In a more recent study, Phadnis (2011) could map some of these cooperating loci using different mapping strategies with molecular markers. He found at least seven interacting loci: two of large effect, including *Ovd*, three of small effect in *the D. pseudoobscura bogotana* X chromosome, and two dominant loci in the *D. pseudoobscura pseudoobscura* autosomes (**Figure 6**). Moreover, this study confirmed the partial overlapping of the hybrid sterility genetic architecture and the genetic basis of hybrid segregation distortion.

The frequent finding that speciation genes are also associated with processes of GC has prompted to many evolutionists to posit the idea that hybrid incompatibilities may be the outcome of a kind of 'arms race' that evolved independently in each splitting species. A classic example is the finding that the *D. simulans* gene *Nup96* is incompatible with one (or several) gene on the *D. melanogaster* X chromosome, generating hybrid inviability (Presgraves *et al*., 2003; Presgraves, 2010). This gene belongs to a family that encodes nucleoporins (Nups), which are proteins that are associated with the nuclear pore complex involved in the selective control of materials in and out of the nucleus. They are also involved in the proper segregation in the meiosis, so their malfunction is one way for selfish elements to gain a segregation advantage, also known as meiotic drive, a form of segregation distortion. The hypothesis states that, in each species, different episodes of arms race between selfish *Nup* genes and suppressor genes to establish correct meiosis have produced the divergence incompatibility that we observe as hybrid inviability.

The role of adaptive evolution in speciation

Many genes coding for proteins involved in GC, and also in HI, show signatures of positive selection. This is the case of the *Nup* complex (Presgraves and Stephan, 2007). In fact, these signatures

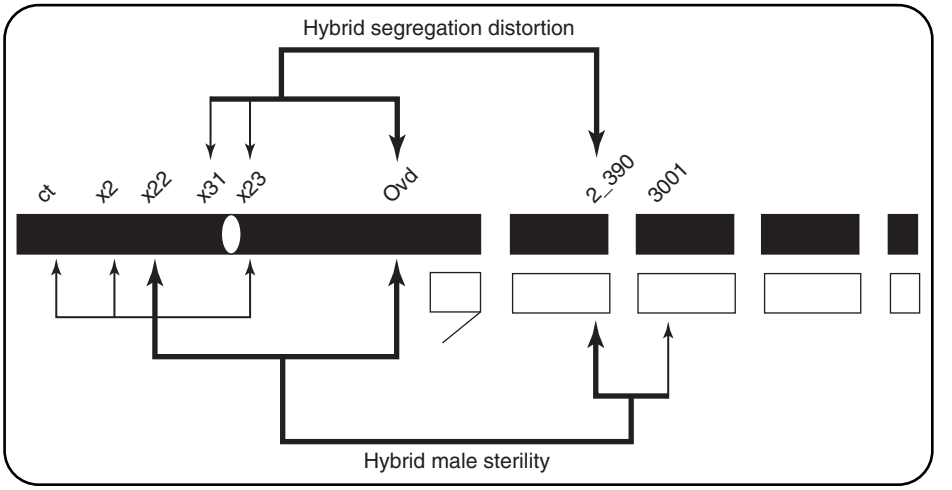


Figure 6 Genetic architecture of male sterility and segregation distortion in hybrid F_1 males between *D. pseudoobscura bogotana* and *D. pseudoobscura pseudoobscura*. Hybrid male sterility is caused by loci (QTL regions) on the *bogotana X* chromosome, which interact with dominantly acting loci (QTL regions) on the *pseudoobscura* autosomes. Segregation distortion is caused by three loci on the *bogotana* X chromosome; *bogotana* 2_390 acts as an almost fully recessive suppressor of segregation distortion. The full expression of both phenotypes, however, requires 2_390 and 3001 regions (QTLs) from *pseudoobscura* sub-species to be present simultaneously. Hybrid male sterility and segregation distortion show a partial overlap in their genetic bases (e.g. regions *X23* and 2_390 act on both phenotypes). Solid (open) bars depict *bogotana* (*pseudoobscura*) chromosomes. The large metacentric *X* chromosome is denoted as a long bar with its centromere as an open oval, the Y chromosome as a hooked bar, and the autosomes as short bars. Thick arrow lines denote large-effect regions, and thin arrow lines denote small-effect regions. Republished with permission from Phadnis (2011) © Genetics Society of America.

have become very common in genome-wide association analyses of postzygotic isolation. For instance, the evolution of *Ovd* is shown by the abundance of non-synonymous substitutions in the bogotana lineage (Presgraves, 2010; Phadnis and Orr, 2009). This scenario is perfectly compatible with a GC because these substitutions were likely selected for their gene transmission advantage rather than for the benefit of the organism. Positive selection, however, should not be confused with adaptive selection, because signatures of positive selection can arise not only through ecological adaptation but also by non-adaptive mutational neutral episodes and internal GCs (Maheshwari and Barbash, 2011). Yet, ecological selection cannot be readily dismissed, because in most cases the natural history of these species is largely unknown. In particular, speciation genes, like the *Nup* complex, have often been associated to pathogen defense (Cronshaw and Matunis, 2004), and very recently a similar relationship between plant genes implicated in hybrid necroses and host-pathogen arms race has been found in plants (Chae *et al*., 2014). **See also**: **Speciation Genes**

The role of regulatory divergence in BDM incompatibilities

Originally, BDM incompatibilities were mainly attributed to polymorphic gene products, such as proteins and RNAs. Therefore, the divergence in coding sequences has been the focus of most evolutionary studies of HIs. Current increasing evidence, however, bolsters the prime role of regulatory processes in HI, including incompatibilities between trans-acting factors and cis-acting elements (Haerty and Singh, 2006). The importance of gene regulation in evolution, suggested a long time ago (Britten and Davidson, 1969; King and Wilson, 1975), has been substantiated by multiple studies showing that regulatory divergence may be an important component among species differentiation.

The role of regulatory divergence acquires a special importance in the regulation of the X chromosome in the male germline of *Drosophila*. Sex chromosomes in heterogametic species evolved from autosomes by a complex process of differentiation involving accumulation of alleles that influence chromosome sex determination. This selects for reduction of recombination between the proto-Y (W) and the X (Z) chromosome, which leads to gene degradation of the non-recombining region of proto-Y (W) chromosome due to several well-known mechanisms. Eventually, the loss of genes in the Y or W sex chromosomes leads to hemizygosity in the heterogametic sex, which results in a reduction in fitness. This fitness cost is mainly attributed to (1) the halving of gene dose and (2) the expression of recessive deleterious alleles in the hemizygous individuals (XY males or ZW females). In *Drosophila*, the evolution of increased expression in the X chromosome of hemizygous individuals ensues to counteract this cost, a phenomenon named 'dosage compensation' (DC). The benefit of DC is two-fold: first it restores the full dose of hemizygous alleles in the X chromosome, and, second, it increases the expression of hypomorphic alleles (i.e. alleles in which the protein function is reduced as a result of low expression or efficiency) that are unmasked in hemizygotes. As stated above, the evolution of DC generates much species divergence in X-linked regulatory factors, which produces the incompatibility in hybrids mostly through the large X effect (Llopart, 2012).

Transcriptional inactivation of the X chromosome in the male germline during the initial stages of spermatogenesis in meiosis

provides another example of the role of regulation in HI. Most of the evidence of this inactivation comes from studies in a large range of groups including mammals, nematodes and grasshoppers. Despite that studies in *Drosophila* have been sometimes contradictory, currently there is sufficient evidence that this mechanism is also present in *Drosophila*. It is not clear why this inactivation takes place, but among the several hypotheses proposed, the meiotic silencing of unsynapsed chromatin is the most favoured. Since sex chromosome evolution involves loss of genes and lack of synapsis in a large fraction of the X chromosome with its reduced Y chromosome partner, inactivation of the sex chromosome occurs (Meiklejohn and Tao, 2010). The mechanisms underlying inactivation are complex and involve molecular components at checkpoints that detect when meiosis go wrong. In fact, both transcriptional inactivation and DC require that the X chromosome should be recognised as distinct from autosomes, probably by transcription factors that bind to specific cis-regulatory elements. Species divergence in the molecular basis of this recognition may be responsible of HMS mapping mainly to the X chromosome. Actually, the X chromosome is enriched for genes, normally silenced in spermatogenesis, that are overexpressed in hybrid testis, impairing the gametogenesis and leading to male sterility. **See also** **Meiotic Sex Chromosome Inactivation**

Regulatory incompatibilities are not only restricted to sex chromosomes; in fact they are pervasive throughout the whole genome. A recent example of characterizing the genetic basis of HMS in detail by using a systems genetics approach combining mapping of gene expression traits with sterility phenotypes and genomic regions (e.g. quantitative trait locus, QTL) has been performed in a hybrid zone of two mouse subspecies *Mus musculus musculus* and *Mus musculus domesticus* (Turner *et al*., 2014; Turner and Harr, 2014). The authors identified several thousands of cis- and trans-acting QTLs contributing to expression (eQTL) associated with sterility phenotypes. Thus, a large number of hybrid incompatibilities were detected, allowing the identification of known genomic regions, and also unknown ones, involved in hybrid sterility. This approach, in their own view, provides a way to reveal general features of incompatibility networks such as the number and dominance of loci, types of interactions and developmental/regulatory pathways more likely to be shared among taxa than specific speciation genes.

Most studies aim to understand regulatory divergence by comparing the F1 hybrid expression of specific alleles to expression in their parental species. This approach was performed mainly in mouse and in *Drosophila*. Gene expression is regulated by interaction between trans-acting factors with cis-regulatory DNA sequences, such as promoters and enhancers. Previous studies in *Drosophila* using F1 hybrids reported that cis-regulatory divergence could explain at least half of differences in gene expression between species. A recent genome-wide analysis, however, using introgression hybrids between two sister species of *Drosophila*, namely a strain of *D. simulans* introgressed with a small region in homozygosis on the third chromosome from *D. mauritiana* (Meiklejohn *et al*., 2014), reported that divergent gene expression caused by the introgressed segments results mainly from trans-acting regulation. Thus about 90% of divergently regulated genes change their expression as a result of trans-factor divergence in these introgressed hybrids.

The relative contribution of cis- and trans-regulation to the evolution of gene expression is of paramount importance to the building up of HI. Meiklejohn *et al*. (2014) suggest that misexpression, that is, hybrid expression outside of the normal range in parental species, normally results from interspecific interactions between alleles at trans-regulatory factors rather than between trans-factors and cis-regulatory sequences. Thus, expression phenotypes of lower fitness in introgression hybrids would be highly present among misexpressed genes. These hybrid incompatibilities would be mostly underlain by trans-regulatory divergences, and may result from trans–trans interactions, which bolster the idea that cis-regulatory sequences and their interacting proteins may contribute less to the evolution of HIs than trans-regulatory factors in the expression of single-copy genes.

Coda: Final Remarks and Future Perspectives

The understanding of speciation owes much of its advances to studies of HI. Though speciation is a complex process involving the build-up of many reproductive barriers between diverging populations, nowhere like in hybrids these gene flow barriers are more evident. Yet, HI is a kind of postzygotic isolation, but not the only one. It underlies intrinsic mechanisms of isolation via sterility and inviability of hybrids, but hybrids may also interact with the environment and suffer from low fitness relative to their parental species. These extrinsic postzygotic mechanisms of isolation have been documented on numerous occasions and even some authors suggested them to be more important than intrinsic mechanisms in the early stages of divergence (Schluter, 2009). Here I have only reviewed the intrinsic postzygotic mechanisms, but this does not allow us to disregard the importance of extrinsic postzygotic mechanisms and even those mechanisms that act prezygotically in the species formation (see Wolf *et al*., 2010 and Butlin *et al*., 2012 for extensive reviews on speciation genetics). **See also**: **Isolating Mechanisms**

Though studies on HI have traditionally represented the stronghold of speciation knowledge, this review shows that, though much has been accomplished, there is still a long way to go for a complete understanding of the underlying causes of postzygotic isolation mechanisms. Some bases like Haldane's rule and the large X effect are well established, but even their underlying causes are still subject to controversy. For example, the faster male theory does not seem sufficient to explain Haldane's rule, and other mechanisms like the GC may underlie this rule. Likewise, the hemizygosity of the X chromosome does not fully explain the disproportionate fixation of X-linked favourable genes to explain the large X effect in HI. Then, other empirical works in *Drosophila* using advanced genomic techniques suggest that positive selection driven by GC may also explain this large X effect. Moreover, GC and even gene translocation (see the gene JYalpha in **Table 1**) have been advanced as new mechanisms, distinct from the BDM model, of great importance to understand HI (Orr *et al*., 2007).

Another contentious issue concerns the architecture of reproductive isolation. In *Drosophila*, several 'speciation genes' of

major effect have posited the view that HI is caused by one or few genes. Despite the existence of these major genes, there is growing evidence that they do not act alone and need the cooperation of many other loci to fully accomplish intrinsic isolation mechanisms. Other empirical evidence points to a polygenic architecture underlain by multiple loci. For example, the PT model posits that these polygenes act cumulatively and their effect will be fully accomplished only when a minimum of them surpasses a threshold number. Much recent empirical work in several organisms, including *Drosophila*, mice and yeasts, supports this model. Interestingly, recent work in hybrid zones of mice (Turner and Harr, 2014; Turner *et al*., 2014) identifies complex interactions among sterility loci, each encompassing hundreds of genes, suggesting multiple, non-independent genetic incompatibilities that contribute to barriers to gene flow in the hybrid zone. These studies also suggest that BDM interactions are complex and synergistic, and that network models of incompatibilities may describe more accurately the evolution of reproductive barriers.

Early interest in coding genes has shifted to regulatory loci as the subject to understand population divergence of importance to HI. Though initially cis-regulation was mostly considered the prime mechanism of divergence, lately trans-regulation has gained support from introgression studies.

All these new advances and controversies show how the field of HI is fully alive. New technologies and analytical developments feasible at the genome, transcriptome and proteome levels are producing new insights in this field. Namely, these technical advances can use non-model organisms and natural populations of both model and non-model species to enrich our already vast knowledge of HI mainly obtained from *Drosophila*. Questions like: What are the first steps in the genetic divergence of populations? Are they related to pleiotropic effects of some adaptive genes or to close linkage between adaptive genes and genes that promote HI through BDM incompatibilities? Or, is speciation a by-product of ecological adaptation by natural selection or the outcome of other internal mechanisms like GC, as some authors strongly propose (Crespi and Nosil, 2013)?, may be answered in the few years to come. As a recent example of the finesse of new methodologies applied to deciding between pleiotropy and linkage, Wright *et al*. (2013) showed, using high-resolution genome mapping, that copper tolerance and hybrid lethality in *Mimulus guttatus* are not controlled by the same pleiotropic gene, as was hypothesised from studies performed 30 years ago; rather both characters are caused by two closely linked but different genes. In this case, selection on the copper tolerance gene caused the high frequency of the HI allele by hitchhiking. Whether this is a general mechanism or not must await further similar studies, but introduces an intriguing fact to Darwin's statement that HIs evolve as by-products of environmental adaptation.

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