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Research Article

Investigation of the molecular signatures of selection on ATP synthase genes in the marine bivalve *Limecola balthica*

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Abstract – We used transcriptomic sequence data to describe patterns of divergence and selection across different populations of a marine bivalve (*Limecola balthica*). Our analyses focused on a nuclear gene (*atp5c1*) that was previously detected in an F_{ST} scan as highly structured among populations separated by the Finistère Peninsula in France. This gene encodes the gamma subunit of the F_O/F_1 ATP synthase, a multi-protein complex that is paramount to cellular respiration and energy production. Analysis of non-synonymous to synonymous mutation ratios revealed that 65% of the gene is highly conserved ($dN/dS \leq 0.1$, min = 0), while 6% of the gene is likely under positive selection ($dN/dS \geq 1$, max = 2.03). All replacement mutations are clustered on a 46 residues portion of the protein, within an inter-peptide interaction zone. Comparative genomics suggests that these mutations are evolutionarily stable, and we hypothesize that they are involved in inter-population genetic incompatibilities with other subunits of the ATP synthase complex. The protein stability of the gamma subunit conferred by southern variants was inferred to be higher under warmer temperatures, suggesting that environmental conditions may contribute to the strength of genetic barriers in *L. balthica*.

Keywords: Molecular evolution / local adaptation / genetic incompatibilities / selection / ATP synthase / *Macoma balthica*

1 Introduction

It is now well-accepted that marine organisms can show local adaptation to environmental conditions despite their high dispersal potential, which was long thought to prevent adaptive differentiation (e.g. review of Sanford and Kelly, 2011). In fact, recent research has shown that adaptations can emerge and be maintained in spite of gene flow (Tigano and Friesen, 2016). For instance, focusing on the high-gene flow sea urchin *Strongylocentrotus purpuratus*, Pespeni and Palumbi (2013) found evidence for local adaptation to temperature in an environmental mosaic, at multiple protein-coding genes that were previously detected using a genome scan.

Limecola balthica is a highly dispersive marine bivalve, broadly distributed in the northwestern Europe (Väinölä and Varvio, 1989). Its pelagic larval phase is estimated to last 2–5 weeks (Caddy, 1967). In the Northern Atlantic Ocean, two lineages of *L. balthica* occur, due to multiple events of trans-arctic dispersal from the northern Pacific Ocean (Väinölä, 2003; Nikula et al., 2007). A Pacific lineage (*L. balthica balthica*) occurs in the White and Baltic Seas, and an Atlantic lineage (*L. balthica rubra*) is found along the Atlantic coasts from Norway to France and around the British Isles (Väinölä, 2003; Luttikhuizen et al., 2003; Nikula et al., 2007; Becquet et al., 2012). The *rubra* lineage extends to the Gironde Estuary, France (with sparse populations down to Arcachon Basin), which corresponds to the present-day range limit for the species (Bachelet, 1980). A hybrid zone between *rubra* and *balthica* was detected at the entrance of the Baltic Sea (Kattegat Strait; Nikula et al., 2008). Within *rubra*, populations north and south of the Finistère Peninsula (France) show significant mitochondrial and nuclear (microsatellite loci) genetic differentiation (Fig. 1b; Becquet et al., 2012).

An F_{ST}-based genome scan was performed on transcriptomic data from pooled individuals, across the Kattegat and Finistère transition zones, in a preliminary effort to look for loci associated with local adaptation (Pante et al., 2012). Three geographically disjunct populations were sampled (Fig. 1a). The sites of Aytré (Bay of Biscay, France) and Somme Bay (English Channel, France) correspond to two discrete populations of the *rubra* lineage (Becquet et al., 2012). Aytré is located near the southern

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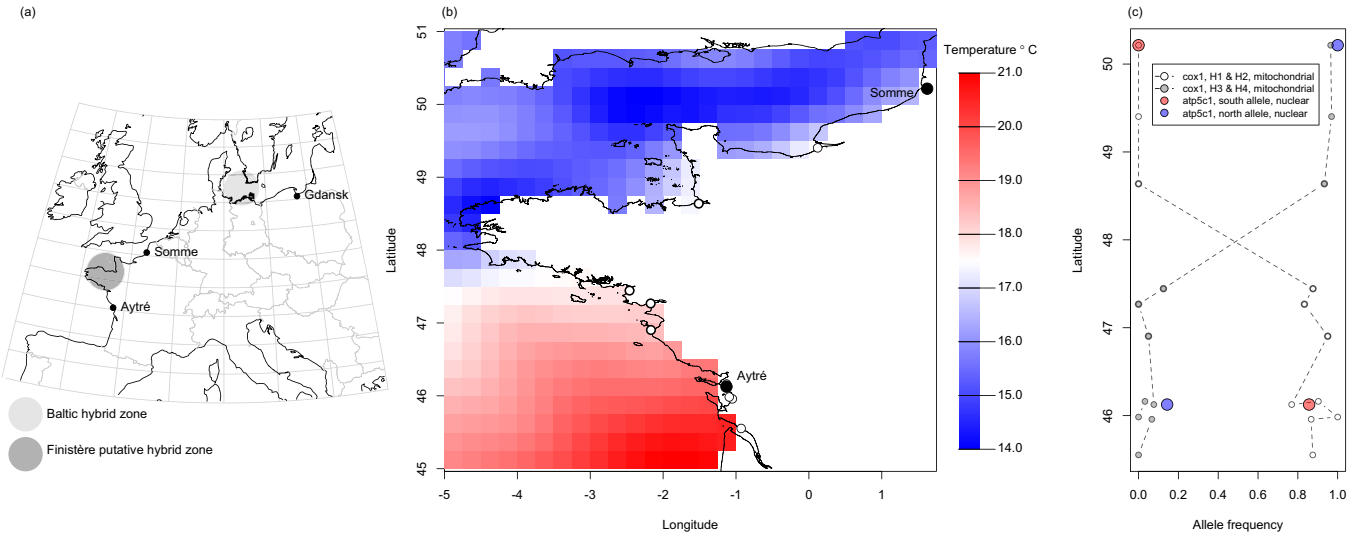

Fig. 1. (a) Sampling map displaying the location of two hybrid zones. (b) Heat map of the Bay of Biscay and English Channel with sampling sites of this study (black dots) and of Becquet et al. (2012) (white dots). SST are monthly averages for July, for 1982, 1992, 2002, and 2012; high resolution SST data provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, USA (Reynolds et al., 2007). (c) Mitochondrial haplotype frequencies at *cox1* (data from Becquet et al., 2012, site locations on panel b) and allele frequencies at the nuclear *atp5c1* (this study). For panels (b) and (c), the sites that are within the putative Finistère hybrid zone (Becquet et al., 2012 and unpublished data) are identified with bolder outer circles.

limit of the species distributional range and is characterized by warmer sea surface temperatures than Somme Bay (Fig. 1b). Furthermore, the Bay of Biscay is subjected to warming surface water temperatures (Goikoetxea et al., 2009). A third population, corresponding to the *balthica* lineage, was sampled in Gdańsk Bay (Baltic Sea, Poland). This F_{ST} scan revealed multiple genes involved in the oxidative phosphorylation (OXPHO) system (including genes coding for subunits of the F_O/F_1 ATP synthase and NADH dehydrogenase complexes, and an ADP/ATP carrier). In particular, the nuclear gene *atp5c1* encoding the gamma subunit of the ATP synthase F_1 rotor, may be under strong selection: 23/41 mapped SNPs were detected as F_{ST} outliers (maximum $F_{ST} = 0.838$). This gene is paramount to the good functioning of the F_O/F_1 complex, as its rotary action promotes ATP synthesis (reviewed in Sielaff and Börsch, 2013).

In this contribution, we examine molecular signatures of selection at *atp5c1* by further analyzing the transcriptomic data of Pante et al. (2012). Our goals were to determine whether SNPs that are highly differentiated among populations cause modifications of the protein structure, and whether these changes may have functional repercussions. Given that the F_O/F_1 complex is generally encoded by 12 nuclear and 2 mitochondrial genes (reviewed in Rand et al., 2004), we asked whether high-F_{ST}, non-synonymous mutations fall within predicted sites of inter-protein interactions, suggesting the implication of genetic incompatibilities in maintaining barriers to gene flow between southern and northern populations of *L. balthica*.

2 Material and methods

2.1 Specimen sampling, and preparation of genetic data

cDNA libraries were prepared from three pools of 10 individuals (one pool per site) and sequenced on a Roche 454 GS-FLX. Details on sequence quality control and assembly, read mapping, polymorphism detection, and gene annotation can be found in Pante et al. (2012). Here, we focus on contig G_c113, detected in this latter study, and identified as coding for *atp5c1* (characterized in Matsuda et al., 1993).

2.2 Sequence evolution and predicted changes in protein function

Differences in allele frequencies between population pairs were tested with Fisher's exact tests, using Popoolation2 (Kofler et al., 2011). The molecular signature of selection was investigated by looking at the ratio between non-synonymous (d*N*) and synonymous (d*S*) substitutions (e.g. Kimura, 1977), calculated using the R package seqinr (Charif and Lobry, 2007). A sliding window (150 nt wide, sliding every 3 nt) was used to detect variation in this ratio along the gene. d*N*/d*S* ratios were calculated based on population sequence consensuses, as in Barreto et al. (2011). The programs SIFT (Ng and Henikoff, 2006; Kumar et al., 2009) and SNAP (Bromberg and Rost, 2007) were used to test whether variations in amino acid (AA) compositions may have an impact on protein function. SIFT predicts whether AA substitutions can be tolerated by aligning and comparing homologous sequences retrieved by PSI-Blast; protein structure is not taken into account. SNAP uses sequence information, but can incorporate functional and structural annotations if such data are available (Bromberg and Rost, 2007). In SIFT, median conservation of sequences was set at 3.00 and sequences ≥90% identical to the query were removed. The hypothesis that local environmental conditions, such as sediment temperature and pH, affect the stability of different alleles of *atp5c1* was tested using I-Mutant 2.0 (Capriotti et al., 2005). Protein stability ($\Delta\Delta G$) was estimated at 5, 15, and 30°C, and at pH 6, 7, and 8

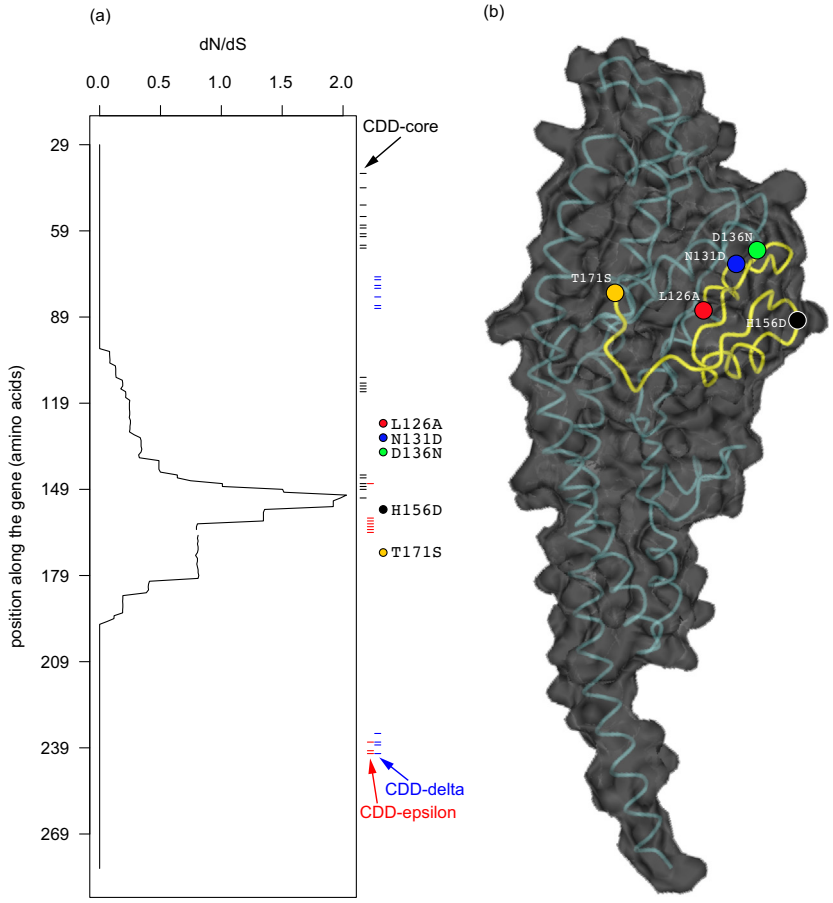

Fig. 2. (a) Sliding window analysis of the d*N*/d*S* ratio along *atp5c1* (window 50 AA wide, sliding every codon). The position of replacement mutations is provided on the right (gray dots), as well as the position of conserved interaction sites detected by CDD (Marchler-Bauer et al., 2013) (black, blue, and red: interactions with core domain, delta, and epsilon subunits, respectively). (b) Three-dimensional model of the ATP synthase gamma subunit of *Limecola balthica* (I-TASSER prediction based on reference contig from Gdańsk). The yellow segment represents the 46 residue-long fragment bearing replacement mutations (gray dots).

(conditions based on observed data; Ekebom, 1999; Lavergne and Barnett, personal communication). In order to further evaluate the impact of AA mutations on protein function, the structure of the gamma subunit was predicted using the I-TASSER server (Zhang, 2008; Roy et al., 2010) and visualized with the UGENE toolkit (Okonechnikov et al., 2012). Finally, we looked for non-synonymous mutations in the other nuclear-encoded F_O/F_1 ATP synthase subunits that could be detected in the transcriptome.

3 Results

3.1 Replacement mutations on *atp5c1*

AA sequences of *atp5c1* from populations sampled some 2800 km apart are, overall, highly conserved, with a global d*N*/d*S* ratio of 0.08. The sliding window analysis (150 nt wide, sliding every codon; Fig. 2a) revealed that 65% of the gene is under negative selection (d*N*/d*S* $\leq$ 0.1, min = 0), while 6% of the gene is under positive selection (d*N*/d*S* $\geq$ 1, max = 2.03). Five AA changes occur between residues 126 and 171, meaning that non-synonymous changes are clustered within a 46-residue fragment spanning 15% of the sequence. This region was predicted by I-TASSER (best model C-score = 0.04; Fig. 2b) to overlap with two known peptide binding sites (Protein Data Bank IDs 2W6I and 2HLD; C-scores of 0.09 and 0.07, respectively). Conserved Domains Database annotation (CDD, Marchler-Bauer et al., 2013) following a BLASTX (Altschul et al., 1990) search for G_c113 suggests that these mutations occur at the interface with the core domain (interaction with the alpha-beta hexamere) and with the epsilon subunit. These non-synonymous changes were predicted to be evolutionarily tolerated based on SIFT and SNAP (expected SNAP accuracy 85%–94%). The first four AA changes (L126A, N131D, D136N, H156D; nomenclature: Aytré considered as mutant) discriminate Aytré from Gdańsk Bay and Somme Bay, while the fifth one (T171S) distinguishes Gdańsk from the two French populations. N131D and H156D have a stabilizing effect on the protein, while all others have a destabilizing effect (I-Mutant tests between 15 and 30 °C at pH 7; Fig. 3). In all cases, a relative increase in $\Delta\Delta G$ was observed with increasing temperature and pH (i.e., in warmer conditions, a destabilizing mutation was less destabilizing). In one case (N131D), $\Delta\Delta G$ changed sign (from negative to

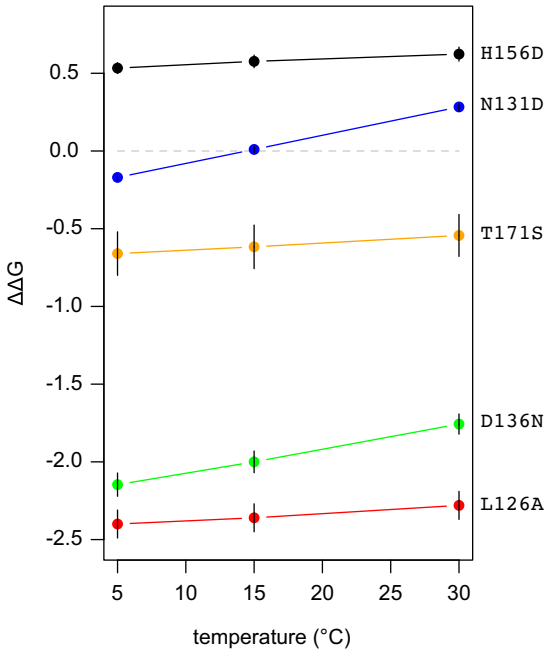

Fig. 3. Effect of temperature and pH on the stability (represented as ΔΔG) of the ATP synthase gamma subunit. Error bars represent one standard deviation of three measures of ΔΔG per temperature, using different pH values (see methods). The name of replacement mutations is provided on the right.

positive) with increased temperature (in all pH conditions), indicating that the expected effect of the mutation shifted from destabilizing to stabilizing. Considering the 4 AA mutations described above, the Aytré population contained a mix of two alleles, the major allele (85.5% of the reads) being unique to Aytré and one corresponding to the type found at Somme and Gdańsk (14.5% of the reads). The median depth of coverage for contig G_c113 was 55, 45, and 18 for Aytré, Gdańsk, and Somme, respectively (Tab. 1).

3.2 Potential for incompatibilities with other ATP synthase subunits

Contig G_c1077 was identified as coding for the alpha subunit and bear one outlier F_{ST} when comparing the samples from Aytré and Somme ($F_{ST} = 0.672$, Pante et al., 2012). This contig could be reliably placed in ORF, which revealed that all nine mutations were synonymous. All mutations segregated Aytré from Somme and Gdańsk. Several contigs were identified as coding for the beta subunit; one (A_c2900) was included in our initial F_{ST} scan but did not stand out as bearing outlier SNPs (in addition, this contig could not be reliably placed in ORF). Unfortunately, the epsilon subunit could not be detected in our transcriptome dataset.

4 Discussion

4.1 Adaptive divergence at OXPHO loci

Mitochondrial function can be significantly influenced by temperature (e.g. Dahlhoff and Somero, 1993). In Dutch populations of *L. balthica*, respiration rate was shown to increase with temperature intra-seasonally (Hummel et al., 2000). In addition, Dutch specimens transplanted in the Spanish estuary of Bidasoa, 200 km south of the known species range limit (i.e. the Gironde Estuary, some 80 km from our sampling site in Aytré, France) showed respiration rates significantly higher than in their native populations (Hummel et al., 2000). Our analyses on the effect of temperature on the stability of subunit gamma suggest a small, positive effect of each replacement mutation in the southern population. While only five replacement mutations were detected, previous studies have shown that even few differences in AA composition can have a significant impact on mitochondrial performance. In the marine copepod *Tigriopus californicus*, three AA changes at the nuclear cytochrome c, separating populations characterized by different thermal regimes, correlated with significant differences in cytochrome c oxidase activity (Rawson and Burton, 2002). In the seed beetle *Callosobruchus maculatus*, metabolic rates were detected between cytotypes that differed by one AA at COI and one AA at Cyt-B (Arnqvist et al., 2010, and see below). In killer whales (*Orcinus orca*), single AA changes at Cyt-B are associated with Antarctic ecotypes, and are therefore possibly implicated in improved mitochondrial performance in polar waters (Foote et al., 2011). Cyt-B was also recently implicated in thermal adaptation in the European anchovy (Silva et al., 2014). Finally, the synergistic impact of temperature and few replacement mutations on protein stability and function was recorded at the nuclear locus encoding cytoplasmic malate dehydrogenase (cMDH) in limpets (Dong and Somero, 2009). As the surface water of the Bay of Biscay is known to be warming at rate of 0.26 °C/decade (1977–2007 time period, Goikoetxea et al., 2009) and the geographical range (Jansen et al., 2007) of southern *L. balthica* is thought to be receding, it becomes increasingly important from a conservation standpoint to understand how genetic diversity at OXPHO genes relates to adaptive potential. Indeed, as gene flow is limited between southern and northern *rubra* populations at *atp5c1*, a narrowing geographical range could result in the loss of adaptive alleles if the southern population does not persist.

4.2 Implications for intrinsic genetic incompatibilities among ATP synthase subunits

Our data suggest that five replacement mutations may interact with other subunits, likely epsilon (encoded by *atp5e* in humans, Tu et al., 2000). This subunit sits on the F_O stator, which is composed of nuclear- and mitochondrial-encoded genes and is embedded in the mitochondrial membrane. While *atp5e* was not detected in our dataset, characterizing genetic variation at *atp5e* and other ATP synthase subunits (including mitochondrial and nuclear ones) across the Finistère transition zone seems central to understanding how intrinsic genetic incompatibilities are involved in maintaining barriers to gene flow in *L. balthica*. The sequencing of mitochondrial genomes from individuals sampled on either sides of the Baltic and Finistère hybrid zones (Saunier et al., 2014) will help us shed some light on possible incompatibilities between the nuclear and mitochondrial genes coding for ATP synthase subunits. In addition, the recent discovery of sex-linked heteroplasmy in

Table 1. Read counts at each position of codons containing non-synonymous mutations, for each population.

		Aytré (A)		Somme (S)		Gdansk (G)		Fisher's exact test			
Locus	Pos	Major	Minor	Major	Minor	Major	Minor	A-S	A-G	S-G	
L126A	376	G (47)	T (14)	T (25)		T (50)		11.3	18.4	0.0	**
	377	C (51)	T (15)	T (26)		T (51)		12.0	19.2	0.0	**
	378	A (49)	G (15)	G (26)		G (52)		11.7	18.8	0.0	**
N131D	391	G (50)	A (13)	A (24)		A (48)		11.7	19.0	0.0	**
	392	A (58)		A (24)		A (47)		0.0	0.0	0.0	
	393	C (55)		C (24)		C (44)		0.0	0.0	0.0	
D136N	406	A (45)	G (13)	G (27)		G (51)		12.0	18.4	0.0	**
	407	A (58)		A (27)		A (53)		0.0	0.0	0.0	
	408	C (58)		C (25)		C (53)		0.0	0.0	0.0	
H156D	466	G (36)	C (14)	C (26)		C (48)		9.8	14.9	0.0	**
	467	A (50)	G (1)	A (25)	G (1)	A (49)		0.0	0.0	0.5	
	468	C (43)	T (5)	C (23)		C (44)		0.8	1.2	0.0	*
T171S	511	A (53)		A (21)		A (42)		0.0	0.0	0.0	
	512	G (51)	C (2)	G (21)		C (35)	G (7)	0.0	15.9	10.4	**
	513	C (56)		C (21)		C (40)		0.0	0.0	0.0	

The significance of differences in allele frequencies are presented as $-\log10$ of p-values from Fisher's exact tests (significance for at least one pair of populations: $^{*}-\log10(p) > 1$; $^{**}-\log10(p) > 10$).

L. balthica suggests that mitochondria in this species are characterised by Doubly Uniparental Inheritance (DUI; Pante et al., 2017, and unpublished male mitogenome draft). In DUI species, the somatic tissues of males are characterized by a "female" mitotype (as in all tissues of females), while the male germ line is characterized by a "male" mitotype that is passed on from fathers to sons (reviewed in Zouros, 2013). Female and male mitotypes sampled from a single individual can be highly divergent, reaching up to 52% in freshwater unionoid mussels (Doucet-Beaupré et al., 2010). In *L. balthica*, genetic incompatibilities could therefore occur in multiple ways, as OXPHO epistatic interactions among nuclear genes, nuclear and female mitochondrial genes, and among nuclear and male mitochondrial genes in the sperm of interpopulational hybrids.

4.3 Interplay between intrinsic genetic incompatibilities and temperature

One fascinating avenue for research is the characterization of interactions between intrinsic genetic incompatibilities and extrinsic environmental forces. Arnqvist et al. (2010) were the first to demonstrate environmental effects on mitonuclear epistatic interactions by crossing mitochondrial and nuclear genomes of *C. maculatus*. In this very elegant experiment, the authors showed that the negative effects of genetic incompatibilities on metabolic rate were only detectable when hybrids were exposed to different temperatures (Arnqvist et al., 2010). In *L. balthica*, the replacement mutations mapped on *atp5c1* (i) are located in an inter-peptide interaction zone (most likely in the area were the gamma subunit interacts with epsilon), and (ii) seem to influence the stability of the gamma subunit depending on temperature and pH. This preliminary study is based on very limited data, as we used few, pooled individuals from three populations to investigate the molecular signatures of selection on *atp5c1*. Future research should therefore focus on determining, with larger population sampling, if subunits of the F_O/F_1 ATP synthase complex are indeed involved in mitonuclear genetic incompatibilities enforcing genetic barriers, and whether these putative incompatibilities are affected by the environment.

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