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Are different allozyme genotypes of the butterfly *Polyommatus coridon* adapted to resist cold and heat shocks?

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Polymorphisms are widely known in allozymes and are often assumed neutral in the context of phylogenetic and population genetic analyses. However, several studies revealed that, in some loci and species, selection is strongly acting on allozymes and that temperature seems to be an important selective agent. Therefore, we individually determined the recovery time from chill-coma and heat knock-down time of 542 individuals of *P. coridon* sampled at four sites. Afterwards, we analyzed patterns of allozyme variations in these butterflies by allozyme electrophoresis of eleven loci to test the enzymes' performance under thermal stress conditions and to investigate the possibility of adaptive genotypic variation in relation to temperature stress resistance traits. We obtained significant differences of reactions to cold and heat shocks between the sexes and between reared and wild-captured individuals, but no significant differences among genotypes were found for most of the investigated allozyme loci. However, the *Aat1* locus showed significant differences between different genotypes in chill-coma recovery times ($p < 0.001$), and marginal differences in heat knock-down times ($p = 0.073$). Thus, *Aat1* might be a target for thermal selection. Consequently, our results revealed no clear influence of temperature on most allozyme loci and the otherwise observed differentiation in *P. coridon* might be explained by stochasticity, and thus geographic structuring should reflect biogeographic processes.

Introduction

Species adapt to environmental conditions to increase their chances of survival. Genetic diversity is the raw material upon which natural selection acts to produce adaptive evolutionary change (Frankham 2012). Any gene frequency tends to remain constant in the absence of disturbing factors (Wright 1932), but natural populations are frequently exposed to a dynamic balance between local selective forces and the homogenizing effect of gene flow (Coscia *et al*. 2012). Consequently, genetic polymorphisms are of great importance in phylogenetic reconstructions, differentiation among species and studies on the evolution of characters. However, the analysis of allozyme

polymorphisms is also popular in ecophysiological experiments because different genotypes and alleles show different patterns in their kinetics and stability, rendering them susceptible to selection and hence evolutionary processes (Eanes 1999). Allozyme's functional and fitness-related diversity offers an extraordinary tool for gene-based experimental studies of evolution (Watt 1994) as well as analyses of the genetic structure of populations with a focus on conservation and biogeography (Kühne & Schmitt 2010). The opportunity to study differential reactions of genetic variants at different loci to selective pressures, like temperature shocks, may yield powerful insight into microevolutionary processes (Watt *et al*. 1983). The use of allozyme and morphological variation allows these pieces of information to be cross-checked against each other (Goulson 1993). Many studies already dealt with these issues and correlated allozyme polymorphism with a large variety of fitness-related traits, including morphological and physiological ones (e.g. Watt 1977, Watt *et al*. 1983, 1985, Goulson 1993, Karl *et al*. 2008).

Studies on *Drosophila melanogaster*, *Fudulus heteroclitus* and *Colias* butterflies have repeatedly shown that allozyme polymorphisms are associated with functional variation (Eanes 1999). Most of these analyses studied the *Pgi* (e.g. Watt 1977, Watt *et al*. 1983, 1985, 1996, Karl *et al*. 2008) or *Pgm* locus (e.g. Goulson 1993, Watt *et al*. 1985). Thus, Watt (1977) already analyzed the evolutionary potential of different thermal adaptations in the *Pgi* locus in *Colias*. He found a strong relationship between electrophoretic mobility and heat resistance of genotypes. The faster moving *Pgi* alleles in *Colias* are far more heat resistant, and the partial dominance of heterozygotes might be due to heat resistance reasons. Thus, functional differences and differences in kinetic parameters definitively exist among genotypes resulting in advantages e.g. in relation to thermostability (Watt 1977, Watt *et al*. 1983) or male mating success (Watt *et al*. 1985).

Karl *et al*. (2008) discovered that *Pgi* genotypes in the butterfly *Lycaena tityrus* are linked to variation in life-history and stress resistance traits. The *Pgi* genotypes differ in their resistance to cold stress, but not to heat stress. Follow-up analyses revealed that the homozygote genotype 2-2 dominates Alpine populations. In contrast, this genotype is just one of several others in lowland populations. Thus, the 2-2 genotype might be fixed in Alpine populations as a consequence of thermal selection (Karl *et al*. 2009).

Other studies dealt with the association between clinal variation of gene frequencies and environmental factors like temperature and, as a consequence, desiccation and *Hsp70* expression. Analyses on the fruit fly *Drosophila buzzati* detected genetic differences in thermal tolerance among populations. Clearly adaptive patterns were found to heat knock-down resistance and *Hsp70* expression in *Drosophila* (Sørensen *et al*. 1999, 2001, 2005). Sørensen *et al*. (2005) found a negative relationship between heat knock-down resistance and altitude, hence supporting the assumption that this trait is ecologically relevant for heat resistance (Hoffman *et al*. 2002, Sørensen *et al*. 2005). *Drosophila melanogaster* shows temperature adaptation of different alleles in metabolic genes, in particular *Adh* (e.g. Berry & Kreitmann 1993, Sezgin *et al*. 2004). The majority of these studies underline that temperature or temperature-related factors represent rather important selective agents (Goulson 1993, Loeschke *et al*. 2000). Despite these studies, our general knowledge on the consequences of variation at these loci on life-history and stress resistance traits is still limited (Karl *et al*. 2008).

Furthermore, many biogeographic studies also used allozyme analyses, examining the origin and evolution of a given clade and general patterns in the evolution of biota (Härlin 1996). In this context, it is of great importance to select methods, which clearly discriminate between long-term stochastic population processes and selective influences (Besold *et al*. 2008) because natural selection is often strongly influencing the distribution of alleles in a given species. However, if no selection is acting on genotypes, molecular modifications should be due to stochastic processes and, in this particular case, can be interpreted as an unbiased measure for the degree of differentiation among populations.

One species intensively analysed biogeographically based on allozyme polymorphism assuming their evolutionary neutrality is the lycaenid butterfly *Polyommatus coridon* (Schmitt

& Seitz 2001, 2002a, Schmitt *et al*. 2002, 2005, Schmitt & Zimmermann 2012). We here question the possibility of adaptive genotypic variation towards temperature stress resistance traits of allozyme loci in this well-studied butterfly. In the case of no fitness differences, the different alleles at each locus might be evolutionary neutral. On the other hand, correlation with temperature might indicate natural selection acting on these loci. We therefore studied eight polymorphic enzyme systems (representing eleven loci) of *P. coridon* and tested the performance of the enzymes' alleles under thermal stress conditions. In particular, we tested whether: (1) some genotypes are characteristic for higher cold or heat stress resistance, (2) differences in cold or heat stress resistance exist between the sexes (here of reared individuals), and (3) between reared and wild-captured individuals.

Material and methods

Study species

The univoltine butterfly *Polyommatus coridon* is one of the most characteristic lycaenid butterfly species of semi-natural calcareous grasslands (Ebert & Rennwald 1991, van Swaay 2002). Furthermore, it is a model organism for mobile invertebrates with strong habitat dependency (Schmitt *et al*. 2006). This butterfly is widely distributed from northern Spain across most of south and central Europe, north-westwards to southern England, and eastwards through southern Russia to western Kazakhstan (Kudrna *et al*. 2011). The flight period starts in mid-July and extends to the end of August (Ebert & Rennwald 1991). Due to its high genetic diversity at many allozyme loci (Schmitt & Seitz 2001, 2002a, 2002b, Schmitt *et al*. 2002, 2005, Schmitt & Zimmermann 2012), *P. coridon* is an appropriate species for genetic studies.

Sampling and sampling areas

A total of 418 male individuals were hand-netted between the end of July and the beginning of August in 2010 and 2011. Additionally, we collected larvae between 17 and 27 June 2010. These larvae were kept in translucent plastic boxes and were fed with *Hippocrepis comosa*. 123 butterflies (65 ♂♂, 59 ♀♀) hatched between 8 July and 1 August 2010. As sampling sites, we selected two habitats in a keuper hill region of the Eifel mountains (Keuperscharren) in south-western Germany with well-preserved semi-natural calcareous grasslands, surrounded by an agricultural matrix and some forests (Schmitt *et al*. 2006). Additionally, we selected the nature reserves Perfeist bei Wasserliesch and Rechberg bei Olk in a nearby limestone area. The larvae were collected at the latter sample station.

To homogenize fitness of individuals, all butterflies were nurtured after eclosion or capture in the wild for two days with a highly concentrated sucrose solution prior to the stress experiments.

Experimental procedures

A number of authors used cold or heat stress resistance assays to determine the recovery time from chill-coma or heat knock-down time. These assays are utilized as estimators for cold or heat tolerance (e.g. Chen & Walker 1994, Hoffmann *et al*. 2002, Ayrinhac *et al*. 2004, Castañeda *et al*. 2005). To detect the tolerance to extreme temperatures, animals are often exposed to high temperatures until they cannot remain upright or move effectively (Cowles & Bogert 1944, Folk *et al*. 2006). Chill-coma recovery time is characterized as "the time it takes an individual to recover its mobility following chill coma once it has been returned to room temperature" (Castañeda *et al*. 2005).

For chill-coma, we individually transferred the butterflies into small translucent plastic cups of 125 ml. These cups were placed in a refrigerator (–20 °C) for 5 min. After removal, they were exposed to ambient temperature (20 °C) and the individual recovery times were recorded. Individuals not recovering within 45 min were excluded.

For heat knock-down, individuals were also placed in small translucent plastic cups, which were arranged in a tray in groups of 15. This tray was placed in a heat cabinet at a temperature of 50 ± 2 °C (mean ± SD). We observed the but-

terflies through a transparent door and recorded the individual heat knock-down times. After the knock-down point, individuals were removed from the cabinet.

Following these assays, individuals were frozen in a refrigerator at –20 °C. Later, body weights of butterflies were recorded and individuals were then stored at –80 °C until allozyme electrophoresis.

Genotypes were analyzed for 542 individuals. Half of the abdomen of each butterfly was homogenized in Pgm-buffer (Harris & Hopkinson 1978) by ultrasound to release the proteins into solution and centrifuged at 17 000 *g* for 5 min to separate the enzyme homogenate from other compounds. Cellulose-acetate plates were used for allozyme electrophoresis, applying standard protocols (Hebert & Beaton 1993). A total of eight enzyme systems representing eleven polymorphic loci were studied (Table 1).

Statistical analyses

We tested our data for normality with a Kolmogorov-Smirnov test. As most of the data deviated significantly from the normal distribution, we used non-parametric tests, i.e. Kruskal-Wallis (KW) one-way ANOVA and Spearman's rank correlation (r_S). As ANOVA does not show pairwise differences, we used a Mann-Whitney *U*-test as *a posteriori* test between pairs. All tests were calculated with IBM SPSS. We used sequential Bonferroni corrections to account for multiple comparisons.

Results

Heat stress resistance assay

Two hundred and seventy butterflies were subjected to a heat-stress resistance assay. The mean time until heat collapse was 1033 ± 327 s (mean ± SD). Heat knock-down time was not normally distributed. Data were divided into wild-captured males, reared males and reared females. We found a significant difference between these three groups ($\chi^2 = 6.442$, $p = 0.040$; Table 2). In general, female butterflies were more resistant to heat than males. Heat knock-down time of reared male butterflies significantly correlated with their body weight ($r_S = 0.35$, $p < 0.05$; also significant after sequential Bonferroni correction). Such dependence was not found for reared females and wild-captured males.

Chill-coma recovery assay

The chill-coma recovery assay was performed for 272 individuals. The mean recovery time was 514 s ± 473 SD. A Kruskal-Wallis ANOVA indicated significant differences among reared males, reared females and wild-captured males ($\chi^2 = 15.666$, $p < 0.001$; Table 2). Wild-captured males were more cold-shock resistant than reared males (Mann-Whitney *U*-test: $U = 2531.5$, $p < 0.001$) and reared females ($U = 2338.5$, $p = 0.004$). However, means did not differ significantly between sexes if individuals were reared ($U = 664$ $p > 0.05$). No correlation existed

Table 1. Electrophoresis conditions for the different enzymes analyzed for *Polyommatus coridon*. TC: Tris-citrate pH = 8.2, TG: Tris-glycine pH = 8.5 (Hebert & Beaton 1993), TM: Tris-maleic acid pH = 7.0. All buffers were run at 200 V.

Enzyme system	EC No.	Number of loci	Buffer	Homogenate application	Running time (min)
6Pgdh	1.1.1.44	1	TM	3	50
Aat	2.6.11	2	TG	3	45
Hbdh*	1.1.1.30	1	TG	3	30
Idh	1.1.1.42	2	TM	2	50
Mdh	1.1.1.37	2	TC	3	40
$Pep_{Phe\text{-}Pro}$	3.4.11/13	1	TM	4	30
Pgi	5.3.1.9	1	TG	2	40
Pgm	5.4.2.2	1	TG	2	40

* moves towards cathodes.

between body weight and chill-coma recovery time for the reared individuals (r_S = –0.1; p > 0.05). For the wild-captured males, we found a significant negative correlation (r_S = –0.33; p < 0.01; also significant after sequential Bonferroni correction). Thus, individuals with higher body weight appeared to have a shorter chill-coma recovery time.

Effects of genotype variation on stress resistance

All studied allozyme loci (*6Pgdh*, *Aat1*, *Aat2*, *Hbdh*, *Idh1*, *Idh2*, *Mdh1*, *Mdh2*, *Pep*, *Pgi*, *Pgm*) were polymorphic. For all further analyses, we included only genotypes represented by at least ten individuals. Eight loci showed sufficient genotypic variation (*Aat1*, *Aat2*, *Hbdh*, *Idh1*, *Mdh2*, *Pep*, *Pgi*, *Pgm*). Only wild-captured males were evaluated due to insufficient sample size of reared individuals. No significant differences among genotypes were found for most of the allozyme loci for heat knock-down and chill-coma times.

This also applies to *Idh1*. This locus, which is particularly interesting due to its strong geographic structuring (cf. Schmitt & Seitz 2001, Schmitt *et al*. 2005), is represented by three different genotypes (BB, BC, CC) in the wild-captured butterflies. However, the two most common genotypes BB and BC did not show a significant difference in both treatments (Kruskal-Wallis ANOVA for both $p > 0.05$).

Differences in response times between genotypes were found only for the *Aat1* locus represented by five different genotypes. The two most common genotypes, CC (n = 160) and CD (n = 11) in the chill-coma recovery assay varied significantly: CC butterflies showed a significantly shorter recovery time (352 ± 304 s SD) as compared with CD butterflies (935 ± 706 s SD) (Kruskal-Wallis ANOVA: $p < 0.001$; also significant after sequential Bonferroni correction). Regarding the heat knock-down assay, the two most common genotypes AC (n = 12) and CC (n = 186) did not differ significantly (Kruskal-Wallis ANOVA: p = 0.073): the knock-down time for wild-captured butterflies with the AC genotype was 1179 ± 363 s (mean ± SD) and for the *Aat1* CC butterflies 970 ± 331 s (mean ± SD). The results for all loci are given in Table 3.

Discussion

Chill-coma recovery time and heat knock-down time are plastic traits, which are affected by several factors: e.g., temperature conditions in the habitat, annual mean maximum and minimum temperatures, body weight and the genetic makeup of individuals. In this study, we analyzed the differences in knock-down and chill-coma recovery times relating to sex, rearing condition and genotype in *P. coridon*.

Sex and rearing influence

Our results showed differences between sexes in mean chill-coma recovery and heat knock-down times. The heat knock-down time was shorter in males as compared with that in females. A similar pattern was also found in *Lycaena tityrus* in which heat knock-down time was nearly 40% shorter in males than in females (Karl *et al*. 2008). Chill-coma recovery time was longest in reared females; wild-captured males were most

Table 2. Heat knock-down and chill-coma recovery times in seconds (mean ± SD; number of analysed individuals (*n*) in parentheses) of wild-captured males, reared males and reared females of *Polyommatus coridon*. Significance of the differences among the three groups were tested with Kruskal-Wallis (KW) ANOVA. Mann-Whitney *U*-test was used for pairwise tests; significant differences are given by different letters.

	Heat knock-down time (s)	Chill-coma recovery time (s)
Wild-captured males	1021 ± 308[a] (n = 218)	450 ± 440[a] (n = 200)
Reared males	974 ± 410[a,b] (n = 26)	675 ± 510[b] (n = 38)
Reared females	1184 ± 36[b] (n = 26)	712 ± 534[b] (n = 34)
p (KW)	0.040	< 0.001

chill-resistant, followed by the reared males. Similar was also found for *D. melanogaster* whose recovery times were slightly shorter in males than in females (David *et al*. 1998). The relationships between body size and chill-coma recovery time and heat knock-down time were as expected. Individuals with higher body weight had a shorter chill-coma recovery time and a longer heat knock-down time.

In general, heat and cold tolerance are related to temperature conditions in habitats (Goto *et al*. 1998). Hoffmann *et al*. (2002) indicated that populations of *D. melanogaster* living in a cooler environment recovered more quickly from cold stress. Consequently, heat resistance was positively affected by summer conditions, and chill-coma resistance by winter conditions (e.g. Hoffmann *et al*. 2005, Karl *et al*. 2008). In our experiment, reared and wild-captured butterflies developed under different thermal regimes: the former under more constant temperatures with a higher mean. The shorter chill-coma recovery time of the wild-captured individuals might therefore be explained by their exposition to fluctuating temperatures with lower means and considerably lower minima.

Table 3. Mean times ± SD (in seconds) for heat knock-down and chill-come recovery times of *Polyommatus coridon* individuals representing different genotypic constitutions at eight allozyme loci.

Locus	Genotype	Heat knock-down time (s)	Chill-coma recovery time (s)
6Pgdh	BB	981 ± 337	427 ± 410
Aat1	AC	1179 ± 363*	
	CC	966 ± 331*	352 ± 304***
	CD		935 ± 706***
Aat2	DD	1024 ± 345	420 ± 416
	DH	958 ± 298	
Hbdh	FF	980 ± 312	471 ± 481
	FH	980 ± 382	434 ± 443
	HH	1030 ± 255	409 ± 352
Idh1	BB	993 ± 332	453 ± 445
	BC	993 ± 332	476 ± 447
Mdh1	AA	980 ± 344	447 ± 443
Mdh2	AA	978 ± 319	434 ± 441
	AB	1019 ± 339	420 ± 414
	BB	947 ± 335	475 ± 454
Pep	DC	1171 ± 221	312 ± 184
	DD	980 ± 349	491 ± 484
	DF	937 ± 309	386 ± 340
Pgi	CB	942 ± 283	324 ± 180
	CD	947 ± 383	556 ± 568
	CC	1033 ± 318	439 ± 430
	DD	903 ± 443	299 ± 182
Pgm	BB	993 ± 334	488 ± 480
	DB	1016 ± 330	346 ± 233
	DD	961 ± 360	464 ± 477
	DF	1056 ± 346	466 ± 352

Kruskal-Wallis ANOVA: * $0.1 < p < 0.05$; *** $p < 0.001$.

In contrast to the expectation that higher mean temperature should lead to longer heat knock-down times, reared male butterflies had the shortest heat knock-down time. Therefore, it might not be mean temperatures but their extremes, since maximum temperatures were higher for the wild-captured individuals than for the reared ones. Furthermore, time of development in ectothermic animals strongly depends on temperature, and increased temperatures decrease this time and increase growth rates (Fischer & Fiedler 2000, Karl *et al*. 2008). However, at high temperatures, *L. tityrus* males responded with a trade-off by favouring earlier emergence over larger size, which resulted in a loss of weight (Fischer & Fiedler 2000). This feature, at least partly, might explain the lower heat knock-down times of reared *P. coridon* males.

Influence of genotypes

Numerous studies have addressed the question whether allozyme polymorphisms are caused by selection or are neutral. Geographic variations in allele frequencies are often taken as evidence for natural selection acting on the affected allozyme alleles (e.g. McKechnie *et al*. 1975, Berry & Kreitman 1993, Goulson 1993, Rank & Dahlhoff 2002, Van Oosterhout *et al*. 2004, Huestis & Marshall 2006, Rank *et al*. 2007), but often also for the neutral behaviour of allozyme alleles (e.g. Schmitt *et al*. 2005, Besold *et al*. 2008). However, the number of reported cases of positive selection has also increased remarkably over

the last years (Ford 2002). Several allozyme loci in *Drosophila* (*Adh*, *G6pd*, *Sod*, *Pgm*, *Tpi*, *Pgd*, *Amy*) show patterns of variation, which are inconsistent with a simple neutral model, but should be the result of exposure to regionally-varying selection pressures or balanced, evolutionarily young polymorphisms (e.g. Kreitman & Hudson 1991). Furthermore, Van Oosterhout *et al*. (2004) monitored changes in allozyme variation in the butterfly *Bicyclus anynana* and demonstrated that neutrality of molecular markers cannot always be assumed.

Previous studies on the butterfly *P. coridon* showed a geographic pattern in allozyme variation in this species over major parts of its range, and detected two major lineages with a decline in the number of alleles from southern to northern populations (Schmitt & Seitz 2001). These lineages show a remarkable division into eastern and western population groups most clearly seen in the allele distribution at the *Idh1* locus (Schmitt & Seitz 2001, Schmitt *et al*. 2005). This division was argued to be due to two ice-age refugia. Therefore, changes in allele frequencies of *Idh1* and also other loci might have taken place mainly in these two refugia because of the stochastic processes of genetic drift, possibly enhanced by genetic bottlenecks (Schmitt & Seitz 2002a, Schmitt *et al*. 2002). Also, a positive association between genetic diversity and population size, as predicted by neutral theory, was found for allozymes in *P. coridon* (Schmitt & Seitz 2002b).

The main goal of this study was to examine whether the analyzed enzyme loci in *P. coridon* are really neutral or under thermal selection. *Idh1* showed the clearest phylogeographic signal in this species (Schmitt & Seitz 2001). A lack of significant difference in thermal resistance of the individuals with different *Idh1* genotypes indicated that this locus is apparently neutral to thermal selection, and differentiation between the two lineages in *Idh1* might be interpreted as the result of stochastic processes. Therefore, genetic variation at the *Idh1* locus supported the assumption that the division between these two *P. coridon* lineages is most likely best explained by a long period of disjunction in two different ice-age refugia (Schmitt & Seitz 2001), and not by environmental selection.

The *Pgi* and *Pgm* loci are known to be under thermal selection in several species (e.g. Watt *et al*. 1983, 1985, 1996, 2003, Goulson 1993, Karl *et al*. 2008, 2009). In *P. coridon*, we could not find significant differences between *Pgi* and *Pgm* genotypes with respect to heat knock-down and chill-coma recovery times. Thus, both loci seemed to act neutrally in this butterfly so that they might also represent useful markers for biogeographic and ecological analyses requiring selection neutrality. However, this absence of significant differences among allozyme genotypes should not be taken as clear evidence for neutrality of variation at these loci. Due to limited sample size and methods used here, moderate effects might not have been detected. Small effects certainly would not have been detected, but these might cause substantial deviations from neutrality patterns. The fact that some loci show stronger population structure than others (Schmitt & Seitz 2001, Schmitt *et al*. 2005) by itself suggests non-neutrality, but deleterious alleles might already have been selected out. The thermal stress experiments presented here only represent a small subset of all temperature-related natural impacts, and even if no temperature-related variation exists, variation may still be non-neutral with respect to other important traits. It is even possible that non-neutral variation in the protein sequences exist with respect to the measured phenotypes, but that this variation is not detectable by electrophoresis. There is no reason to believe that all important polymorphisms at these enzyme loci involve charge-changing amino-acid replacements. Of course, in this case, allozyme variation (i.e. the one detected by electrophoresis) may be neutral, but this may not apply to all variation at the amino-acid sequence level (i.e. also the one not detected by electrophoresis).

In contrast to all other loci studied here, *Aat1* genotypes seemed to affect stress resistance. The CC genotype exhibited significantly shorter chill-coma recovery times in wild-captured males than the CD genotype. Individuals with the AC genotype showed remarkably longer heat knock-down times than the CC genotype individuals. All other genotypes at this locus had to be excluded from further analysis due to insufficient sample sizes. However, these gen-

otypes also showed strong variation in mean heat knock-down and mean chill-coma recovery times. Despite these data, it is still not possible to evaluate whether the observed genotype variations represent some thermal adaptation or not. It therefore would be of special interest for the understanding of climatic adaptation processes of genotypes to continue this study with a specific focus on geographic gradients. In this context, it would be of particular interest to study the altitudinal distribution of different *Aat1* genotypes of *P. coridon* and to test their climatic adaptation (cf. Karl *et al*. 2008, 2009). However, the allele frequencies of *Aat1* over major parts of the distribution of *P. coridon* showed no major genetic differentiation for this locus (Schmitt & Seitz 2001). Consequently, even if being under selection, this locus is not blurring the general phylogeographic signal in this species.

Overall, these results supported the assumption that the observed geographic pattern of allozyme diversity and differentiation of *P. coridon*, at least mostly, might have been produced by stochastic processes. Our data indicate that the assumption of neutrality is applicable to most of the loci studied (but *see* above), including all the loci with important biogeographic signals. Thus, allozyme electrophoresis should still be a powerful tool to analyze biogeographic and phylogeographic questions in this species.

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