

Crossing species' range borders: interspecies gene exchange mediated by hybridogenesis

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The distribution of species is limited by their ability to adapt to local environments. For adaptation by selection, genetic variability is crucial. As founder effects reduce genetic variability, extension of species' range borders is usually slow due to the reduced probability of successful colonization. However, the range limit might be extended by incorporating locally adapted genes. In western Palaearctic waterfrogs, interspecies hybrids show hemiclinal gametogenesis, are fertile and reproductively mimic one parental species. Genetic analysis, using allozyme loci, shows that they mediate gene exchange between the two parental species. Selection analysis provides evidence for local adaptation of single locus genotypes. This suggests that hybridogenesis presents a process which increases the number of neoform parental genotypes, exposing these to selection, and thereby revealing locally adapted genotypes which are essential for species range expansion.

Keywords: adaptability; hybridogenesis; colonization; species' range borders

1. INTRODUCTION

In his fundamental work, Darwin (1859) assumed that interspecific interactions and physical factors limit the geographical range of a species. This idea was later made more specific by arguing that adaptability, mediated by genetic variability, is the major cause of range expansion (Haldane 1956). Low genetic variability would restrict adaptation to environments of expanding species as they reach terrain that is gradually less optimal than the environment at the centre of distribution (Haldane 1956). An equilibrium range limit then occurs where the ability of local adaptation, needed for further range extension, is balanced by the antagonistic effect of gene flow. Species' range borders might be crossed once the migration load can be neglected, and/or if genetic variability is great enough to allow adaptation to environments different to those in the distribution centre (García-Ramos & Kirkpatrick 1997; Kirkpatrick & Barton 1997; Case & Taper 2000).

Another alternative could be the incorporation of locally adapted genes from related species. Several criteria should be met: (i) locally adapted alleles are present, (ii) which are successfully exchanged and incorporated in the parental species' gene pools, (iii) genetic alteration of species' gene pools are frequent enough to compensate for the effects of migration load and (iv) costs of gene incorporation are minimized. Such a process of gene exchange will increase the number of available genotypes substantially, broadening the field of selection much faster than mutation alone could do and with minimal reproductive costs.

Such a system may be provided by hybridogenetic species complexes. These complexes are known from a

range of different taxa (*Bacillus*, Mantovani & Scali 1992; *Poeciliopsis*, Schultz 1966; *Rana*, Berger 1973; *Trophidophoxinellus*, Carmona *et al.* 1997). Each complex is subdivided into several systems, usually consisting of two parental species and a hybridogen. In these hybridogenetic systems the two parental species (PI; PII) hybridize, each submitting one set of chromosomes to the hybridogen (H; figure 1). H excludes the genome of the parental species PI and reproductively mimics parental species PII. The fertility of H is maintained by the exclusion of one whole genome prior to meiosis, thereby avoiding incompatibilities of non-sister chromosomes (Tunner & Hep-pich-Tunner 1991). The H-lineage is maintained in allopatry with PII by backcrosses between the hybridogen and the parental species PI.

In the current study we focused on the hybridogenetic complex of western Palaearctic waterfrogs. The *Rana* complex deviates from other hybridogenetic complexes most obviously by the occurrence of both sexes in the hybridogen. However, the sex ratio in the hybridogen is strongly biased towards females (e.g. Berger *et al.* 1988). *Rana ridibunda* is involved in all of the three currently known systems, and matings with *Rana lessonae* lead to the hybridogen *Rana esculenta* (LE-System), the most widespread hybridogen in Europe. Two systems with a much smaller geographical distribution occur in southern France and northern Spain (PG-system; PI=*Rana perezi*, H=*Rana grafi*) and the Italian peninsula (BH-system; PI=*Rana bergeri*; H=*Rana hispanica*; Berger 1990). Of all waterfrog species, *R. ridibunda* has the largest distribution range, with a distribution centre in eastern Europe (Berger 1990). Its range was further extended by introductions made on gastronomic demand during the early twentieth century (e.g. Zeisset & Beebe 2003).

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ELECTRONIC APPENDIX

This is the Electronic Appendix to the article

**Crossing species' range borders
Interspecies gene exchange mediated by hybridogenesis**

by

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Electronic appendices are refereed with the text; however, no attempt is made to impose a uniform editorial style on the electronic appendices.

1 Appendix to

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Crossing species' range borders

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Interspecies gene exchange mediated by hybridogenesis

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1 Supplementary Table 1: Allozyme systems used to distinguish between the taxa.

2 We used standard procedures of cellulose acetate allozyme electrophoresis (Hebert & Beaton
3 1993) with several buffer systems (TG = Tris-Glycin, TB = Tris-Borat, TC = Tris-Citrat, TM = Tris-
4 Maleat, PP = Phosphat; pH is given as decimal number). N loci = number of scorable loci in the
5 different buffer systems; Locus_{diag} = locus used for identification, EC = Enzyme Commission
6 number

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Enzyme	Tissue	Buffer	N loci	Locus _{diag}	EC
α gdh	Liver	TG 8.5	1		1.1.1.8
6pgdh	Muscle	TB 8.9	1		1.1.1.44
aat	Liver	TC 8.2	2	<i>Aat-1</i>	2.6.1.1
ahh	Liver	TG 8.5	1		3.3.1.1
ck	Muscle	TG 8.5	1		2.7.3.2
ldh	Liver	TG 8.5	2	<i>Ldh-b</i>	1.1.1.27
mpi	Liver	TM 7.0	1		5.3.1.8
mpr	Muscle	TB 8.9	3	<i>Mpr-2</i>	
pgi	Liver	TG 8.5	1		5.3.1.9
pgm	Muscle	TM 7.0	1	<i>Pgm-2</i>	5.4.2.2

1 Supplementary Table 2: Significance of tests for HARDY-WEINBERG-equilibrium at polymorphic
 2 loci.
 3 Bold-faced values remain significant after a population wide Bonferroni correction at the 5%-level.
 4 Pop. = Population number corresponding to Table 1. Values above 0.05 have been left out (empty
 5 fields).

	Pop.	N	<i>ck</i>	<i>αgdh</i>	<i>ldh-b</i>	<i>mpi</i>	<i>mpr-2</i>	<i>pgm</i>	<i>ahh</i>	<i>pgi</i>	<i>6pgdh</i>	<i>aat-2</i>
<i>R. perezii</i>	1	10										
	2	9										
	3	37										0.032
	4	19										
	6	4									0.021	
	7	10										
	8	8										
	9	13										
	11	18										
	13	18	0.002									
	14	5										
	15	2										
	17	3										
	18	7										
19	7											
20	5											
21	6	< 0.001										0.009
23	10								< 0.001			
24	6											
<i>R. ridibunda</i>	6	24										
	10	22					< 0.001					
	15	5										
	17	2									0.046	
	19	4					0.007					
	21	8			0.050		0.002					
	25	27			0.028					< 0.001		
	26	27			0.027					< 0.001		
	27	20								< 0.001	0.019	
	28	28								0.003		
	29	28			0.007					< 0.001	0.038	
	30	28			0.023					< 0.001		
31	29											

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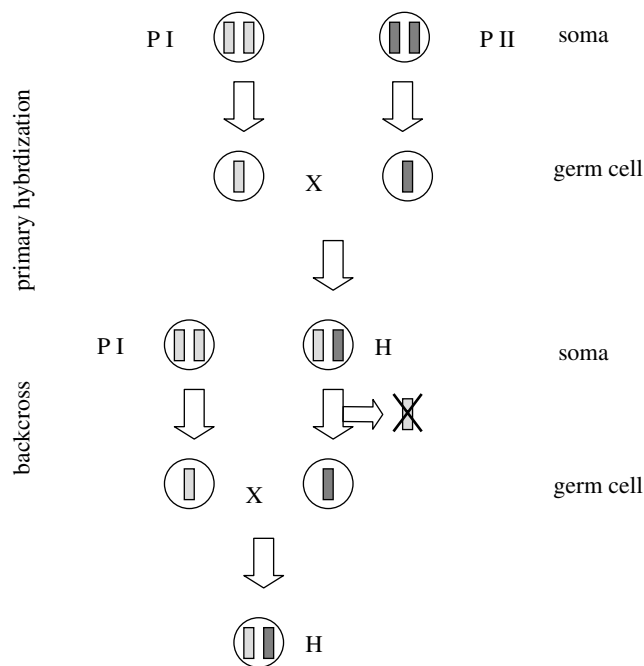


Figure 1. Hybridogenesis. In a hybridogenetic system, two parental species (PI=grey; PII=dark grey) hybridize, each submitting one set of chromosomes to the hybridogen (H). H excludes the genome of PI and reproductively mimics PII, with few exceptions known. The exclusion of one whole genome avoids mispairings of the non-homologous chromosomes of the two parental species, preserving the fertility of H (Tunner & Heppich-Tunner 1991). Hybridogenesis is thought to be a reproductive mode without the opportunity for sexual recombination (Schultz 1969).

R. ridibunda also represents a species group with the largest number of species and cryptic species (Plötner & Ohst 2001). *R. perezi*, with its sister species *Rana saharica*, forms a distant clade to *R. ridibunda*, with a distribution centre in the Iberian Peninsula and northern Africa (Arano *et al.* 1998; Plötner & Ohst 2001). Colonization of France by *R. perezi* occurred after the last ice age, following two pathways over the Pyrenees, closely following the Atlantic and Mediterranean coastlines (Arano *et al.* 1994). In comparison to the central European LE-system, the PG-system therefore has to be considered to be of recent origin.

In the western Palaearctic waterfrogs, several findings contradict non-recombinant hybridogenesis as shown in figure 1. Single males of the hybridogen *R. esculenta* produce sperm containing either the haploid set of *R. ridibunda* or *R. lessonae*, as revealed by flowcytometric analyses, supporting random exclusion of one of the parental genomes during gametogenesis (Vinogradov *et al.* 1991). Further deviations from non-recombinant hybridogenesis have been reported more frequently as the detection of foreign alleles in gene pools of waterfrog species became more common (reviewed in Schmeller 2004). The degree of recombination varies considerably between different studies. In *R. ridibunda*, incorporation of alleles from a sister species was reported to range from 4 to 13.5%, affecting from 7.9 to 85.6% of all sampled individuals. In the two parental species *R. perezi* and *R. lessonae*, the values had been usually lower, ranging from 0.9 to 12.5%, affecting 1.9 to 87.9% of all sampled individuals. There are at least two opportunities for

recombination, (i) the occurrence of aneuploid oögonia (Tunner & Heppich-Tunner 1991) and (ii) nucleus-like bodies (Ogielska 1994), both processes supporting a gradual elimination of a parental genome. The gradual elimination prolongs the time for crossing-over events between the two parental genomes, increasing the probability of successful recombination. However, cytological processes during hybridogenetic gametogenesis are barely understood, leaving room for speculation concerning other opportunities for recombination events.

Studies assessing the effects of gene exchange on the ecology and genetics of *R. ridibunda*, *R. lessonae* or *R. perezi* are not yet available. Gene exchange between two species might have different effects on offspring fitness. It might break up coevolved gene complexes resulting in disturbed epistasis (Mayr 1963) and parental genotypes carrying alleles of the other parental species may therefore be inviable (Mayr 1963; Tregenza & Wedell 2000). The diploid chromosomal system, however, may provide a field of recombination (Carson 1975), where only parts of the whole genome are open to allele exchange between species. Hence, if recombination between species genomes occurs outside balanced gene blocks, newly originating genotypes should be viable (Ortiz-Barrientos *et al.* 2002), and are likely to gain fitness due to increased genetic variability relative to their sympatric conspecifics (Lerner 1954).

Further, mating patterns, following a bigger-is-better strategy of male mate choice, and the strongly female-biased sex ratio in hybridogens, reduce the reproductive costs for both parental species in such a system (for details see Schmeller 2004). Hence, in waterfrogs, the maintenance of a fully fertile hybrid lineage, as opposed to normal interspecies hybridizations, may bear several advantages; (i) it allows for the frequent exchange of locally adapted genes across species with (ii) minimal costs for the species involved. Further, (iii) the hybridogen serves as a container for the hemiclonally transmitted genome (hemigenome). There are, however, constraints with the hemigenomes of the allopatric parental species. Some hybridogenetic lineages appear to be relatively old and have diverged mutationally to the extent that the hemigenome can no longer function in a homospecific background, leading to inviable or fitness-reduced neoform *R. ridibunda* progeny from H×H crosses (Guex *et al.* 2002). There is, however, evidence to suggest that particularly *R. ridibunda* is occasionally propagating from H×H crosses and reaches maturity (Hotz *et al.* 1992; Guex *et al.* 2002). Episodic sexual recombination of the hemiclonally transmitted *R. ridibunda* genome (Vorbürger 2001; Guex *et al.* 2002) may further increase the viability of such neoform *R. ridibunda*, making selection towards viable combinations of two hemiclonal lineages in the current environment possible.

With our study, we aim to contribute to the understanding of hybridogenesis as a process enhancing species range extension by describing (i) the extent of the interspecies gene exchange, (ii) the pattern of this process and (iii) by testing for local adaptation. If local adaptation is the result of directional selection, gene incorporation at different loci should differ in regard to the environment. As waterfrogs are able to migrate over long distances (Tunner 1992; D. S. Schmeller, personal observation), gene flow between populations with different environmental conditions is high, leading to a gradient. Local adaptation might then be detected as a population-wide increase of

recombinant single locus genotypes with increasingly unsuitable environmental conditions, such as high acidity and salinity, and low relative oxygen (e.g. Beattie *et al.* 1993; Viertel 1999; Plénet *et al.* 2000). The number of genotypes with foreign alleles in a population should be higher in populations with less suitable environmental conditions, if they are selectively advantageous, proving local adaptation. By assessing these points hybridogenesis might prove to be a process increasing the number of genotypes, thereby broadening the field of selection, and increasing the probability of local adaptation and range expansion.

2. MATERIAL AND METHODS

We analysed allozyme genotypes of 909 waterfrogs of the genus *Rana* (*Pelophylax*) stemming from 31 populations in southern France (electronic appendix table 2). The frogs were caught by hand at night or by using modified fish traps of different sizes. The frogs were sacrificed in the laboratory by a high dosage of MS222. Samples of muscle and liver were immediately frozen in liquid nitrogen until final storage at -80°C . For reference, we used additional tissue samples of *R. perezi* and *R. grafi* from Spain, *R. ridibunda* from Poland and France, *R. esculenta* and *R. lessonae* from France, Slovenia and northern Italy, *R. saharica* from Tunisia, *R. bergeri* and *R. hispanica* from southern Italy (141 individuals). The different species show diagnostic alleles at several loci (Beerli *et al.* 1996; electronic appendix table 1), which were used to affiliate individuals to species and to determine the degree of allele exchange by the number of foreign alleles found in a species gene pool. The number of foreign alleles was calculated as the proportion of foreign diagnostic alleles at loci *αgdh*, *6pgdh*, *ahh*, *ck*, *ldh-b*, *mpi*, *mpr-2*, *pgm-2*. For allozyme electrophoresis we used homogenates of liver and muscle tissue. Tissues were homogenized in phosphoglucose-mutase-buffer (Harris and Hopkinson 1976). The electrophoretic analyses were conducted on cellulose acetate plates using standard procedures (Hebert & Beaton 1993).

We assigned the individuals to the different waterfrog taxa with the program STRUCTURE v2b (Pritchard *et al.* 2000). Structure uses the genetic mixture analysis and calculates the probability of the number of clusters (K) assumed by the user. The program indicates the probability with which an individual belongs to one of these clusters. As the program does not include a test for the convergence of the Markov chain Monte Carlo simulation, we followed the suggestions of Pritchard *et al.* (2000) and compared the probabilities of several runs. We varied the burn-in rates in the range from 10 000 to 100 000 and report the results revealed for a burn-in rate above 20 000, which produced similar probability calculations. We defined eight clusters involving all our reference samples. Species of other taxa might have been sampled by accident, as *R. esculenta* and *R. lessonae* frequently occur in habitats in the mid-Rhône floodplains (Pagano *et al.* 2001). Further, we know little of the exact distribution range of *R. bergeri*, *R. hispanica* and *R. saharica*. Generally, phenotypes of waterfrogs are difficult to distinguish. Hence, involving all available reference samples ruled out any chance of mis-assignment to the three focal taxa.

We tested for local adaptation with a general regression model. The selection intensity was calculated as univariate linear (β_1) and quadratic (γ_1) selection gradients as well as multivariate linear (β_{multi}) and quadratic (γ_{multi}) selection differentials using standard procedures (Lande & Arnold

1983; Arnold & Wade 1984a,b; Endler 1986). To allow a quantitative comparison of the strength of selection across populations all factors were standardized to a zero mean and unit of variance for each individual sample (e.g. Lande & Arnold 1983). Therefore, we subtracted the population mean from the factor value and divided it by the standard deviation ($z_i = (x_i - \bar{x})/s.d._x$). Further, residuals were tested to ensure no significant deviation from a normal distribution (e.g. Lande & Arnold 1983). We defined two factors for recombination, (i) population-wide frequency of foreign alleles at single loci and (ii) population-wide frequency of recombined single locus genotypes. Selection has been assumed to work through the environmental factors pH, salinity and relative amount of oxygen. These three environmental variables are known to affect the survival of anuran offspring in particular (Beattie *et al.* 1993; Viertel 1999; Plénet *et al.* 2000). To avoid some of the known obstacles with collecting data on environmental variables, we took three measurements per population, one each at midday, during the afternoon and at night. However, it was not possible to monitor these environmental variables over longer periods, of course introducing some uncertainty to our analysis. Further, we only used data from populations with at least five conspecific individuals.

3. RESULTS

Rana perezi, an Iberian species, colonized France after the last ice age by crossing the Pyrenees (Arano *et al.* 1994), but exhibited the highest observed heterozygosity ($H_O > 0.1$) in populations 14, 17 and 18, which are all located in the eastern part of the Camargue, about 400 km north of the Pyrenees (table 1). The mean difference between both regions ($H_{O[\text{Pyrenees}]} = 0.093 \pm 0.003$; $H_{O[\text{Camargue}]} = 0.086 \pm 0.008$) was statistically insignificant ($t_{12} = 0.8$; $p = 0.78$). In *R. ridibunda*, the H_O in populations located in the Camargue ($H_O = 0.184$) is twice as high as in populations along the Rhône valley ($H_O = 0.091$; $t_9 = 12.08$; $p < 0.001$; table 1). In *R. perezi*, most populations were in Hardy–Weinberg equilibrium, whereas in *R. ridibunda* all populations showed deviations at least at one diagnostic locus (Electronic Appendix table 2).

Within taxa, foreign alleles were detected at multiple loci in 16 specimens of *R. grafi* (3.5%), 44 *R. perezi* (22.0%) and 155 *R. ridibunda* (61.0%; figure 2). However, Structure assigned individuals to a species with an assignment probability above 0.6 (figure 2). The frequency of foreign alleles (F_{fa}) was highest in *R. ridibunda* ($6.20\% \pm 0.42$) and was significantly higher than in *R. perezi* ($2.92\% \pm 0.43$; $t_{453} = 5.4$; $p < 0.001$) and *R. grafi* ($0.63\% \pm 0.10$; $t_{705} = 16.6$; $p < 0.001$).

The selection analysis revealed several linear and quadratic trends in both species (table 2). In *R. perezi*, recombined genotypes and foreign alleles at locus *6pgdh* were least frequent under medium oxygen saturation levels. The multivariate test, keeping all other variables constant, shows a significant linear relationship between the foreign alleles at locus *6pgdh* and oxygen saturation. The frequency of recombined *6pgdh* genotypes in *R. ridibunda* has been found to be significantly related to all of the environmental parameters in the multivariate test. While under medium oxygen saturation and salinity levels the frequency of recombined *6pgdh* genotypes was the lowest, we found the reverse for pH (table 2).

Table 1. Location of sites and population composition.

(Population number (no.), the number of different waterfrog taxa, with the number in parenthesis representing taxon percentage in regard to the whole assemblage, the observed heterozygosity (H) of *R. ridibunda* (RR), *R. perezi* (PP) and *R. kl. grafi* (RP) and the coordinates are indicated. Heterozygosities of southern (*R. perezi*), and northern (*R. ridibunda*) populations, relative to Camarguean populations (italic), are bold. Population data used for the selection analysis are indicated with an asterisk (*) for *R. perezi* and a hash (#) for *R. ridibunda*.)

no.	<i>R. ridibunda</i>	<i>R. perezi</i>	<i>R. kl. grafi</i>	H_{RR}	H_{PP}	H_{RP}	coordinates
01*		10 (38)	16 (62)		0.095	0.218	42°38.39' N 4°38.88' E
02*		9 (30)	21 (70)		0.092	0.233	42°39.14' N 2°54.39' E
03*		37 (100)			0.086		43°00.47' N 3°02.56' E
04*		19 (51)	18 (49)		0.099	0.210	43°15.30' N 3°10.90' E
05			17 (100)			0.206	43°48.34' N 3°45.60' E
06#	24 (83)	4 (14)	1 (3)	0.152	0.123		43°33.23' N 3°54.21' E
07		10 (39)	16 (61)		0.046	0.217	43°33.66' N 4°12.61' E
08*		8 (29)	20 (71)		0.072	0.229	43°36.43' N 4°20.34' E
09*		13 (52)	12 (48)		0.057	0.225	43°36.43' N 4°20.34' E
10#	22 (100)			0.200			43°38.86' N 4°35.92' E
11		18 (40)	27 (60)		0.060	0.214	43°33.04' N 4°37.77' E
12			15 (100)			0.231	43°28.38' N 4°38.50' E
13*		18 (53)	16 (47)		0.063	0.229	43°28.71' N 4°38.99' E
14		5 (8)	54 (92)		0.105	0.228	43°29.20' N 4°38.30' E
15	5 (28)	2 (11)	8 (44)	0.192		0.235	43°30.29' N 4°42.26' E
16	1 (3)	2 (6)	29 (91)			0.219	43°30.50' N 4°40.18' E
17	2 (7)	3 (11)	22 (82)		0.110	0.235	43°31.30' N 4°40.44' E
18		7 (19)	29 (78)		0.124	0.222	43°30.31' N 4°38.25' E
19	4 (8)	7 (14)	37 (76)	0.174	0.074	0.240	43°30.37' N 4°41.23' E
20*		5 (21)	19 (79)		0.084	0.214	43°30.32' N 4°40.02' E
21	8 (30)	6 (22)	7 (26)	0.168	0.071	0.241	43°29.26' N 4°43.26' E
22	1 (4)	1 (4)	25 (93)			0.245	43°22.23' N 4°48.33' E
23*		10 (29)	25 (71)		0.071	0.204	43°32.20' N 4°45.31' E
24*		6 (25)	18 (75)		0.079	0.211	43°27.99' N 4°52.86' E
25#	27 (100)			0.088			43°45.16' N 5°13.45' E
26#	27 (100)			0.088			43°47.15' N 4°57.10' E
27#	20 (95)			0.079			43°48.01' N 4°56.10' E
28#	28 (100)			0.097			43°54.10' N 4°40.32' E
29#	28 (100)			0.080			44°02.33' N 4°32.17' E
30#	28 (100)			0.096			44°05.20' N 4°48.33' E
31#	29 (100)			0.109			44°16.30' N 4°38.45' E
∑	254	200	452				

4. DISCUSSION

We have genetically and ecologically analysed 909 specimens of the hybridogenetic waterfrog system of *R. ridibunda*, *R. perezi* and their hybridogenetic associate *R. grafi* to investigate the importance of hybridogenetic gametogenesis for the range expansion of the taxa involved. Our findings suggest frequent incorporation of foreign alleles in both parental species' gene pools, with a significantly higher occurrence in *R. ridibunda*. Further, our selection analysis supports local adaptation due to the incorporated alleles in both *R. perezi* and *R. ridibunda*, even though the signal is weak.

The effectiveness of hybridogenesis as a pathway for the exchange of alleles between two species might be best illustrated by the deviations from Hardy-Weinberg equilibrium. Deviations are particularly frequent in *R. ridibunda*, the species with the highest number of foreign alleles. While, theoretically, panmictic populations should attain equilibrium in one generation, the deviations in *R. perezi* and *R. ridibunda* populations illustrate frequent effects on the gene pool of these species. This might also be one of the reasons why our selection analysis revealed only weak correlations and trends, as selection effects are masked by

the numerous neoform genotypes which have not yet been purged by selection. Additionally, the weak support for local adaptation may be related to sampling and obstacles with measuring environmental conditions. The latter were measured three times in one day, but not over the whole reproductive season, leaving some uncertainty of the exact environmental conditions in the different populations during the active breeding/mating period. Our support for local adaptation of recombined genotypes might best be strengthened by future semi-artificial experiments. However, our study indicates that selection favours recombined genotypes under less optimal conditions. In particular, the haemoglobin of *R. ridibunda* is known to have a low oxygen binding capacity, usually restricting reproductive sites to habitats with frequent freshwater input. Our data, however, suggest that neoform *R. ridibunda*, showing *R. perezi* alleles, perform well in habitats with low oxygen levels. For *R. perezi*, incorporation of foreign alleles counteracts the loss of genetic variability due to founder effects, as can be seen at the high levels of heterozygosity in Camarguean populations. Owing to the high competitiveness of *R. ridibunda* in freshwater habitats (Pagano *et al.* 2001), the ecological niche of *R. perezi* in the Camargue seems to

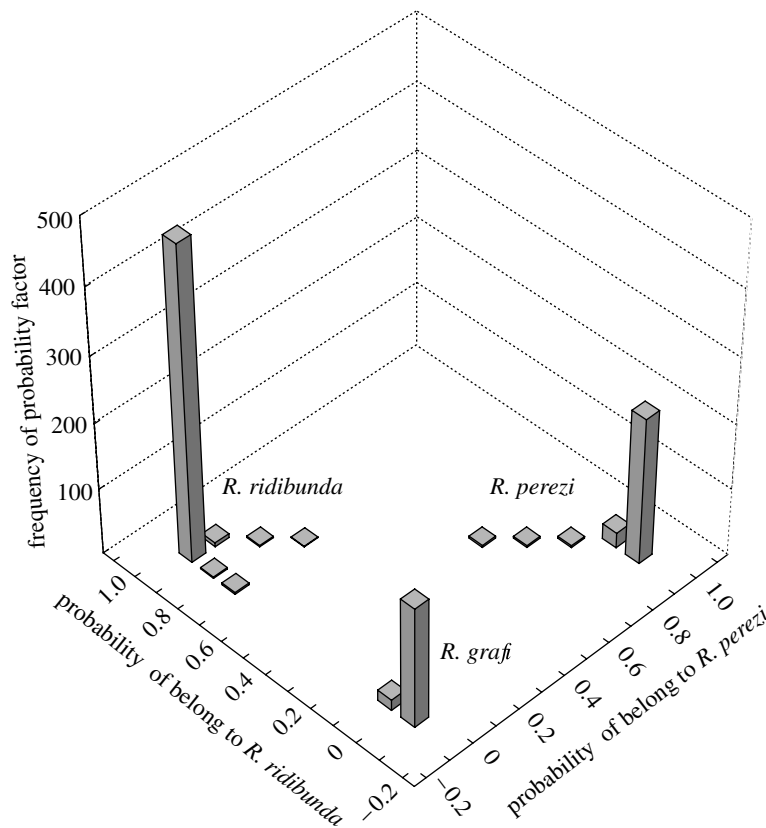


Figure 2. Assignment of individuals to parental species groups. Result of the assignment test from STRUCTURE using the probability values with which one individual was assigned to the parental species groups. All bars with a value different to 1 illustrate individuals that carry alleles from the other parental species, or in case of the hybridogen, show a dominance of alleles of one parental species. However, the majority of individuals incorporated one allele only and have been assigned to the respective taxon perfectly.

be narrowed to low oxygen habitats. Neoform *R. perezi* specimens with modified metabolic pathways therefore may avoid competition with *R. ridibunda* by colonizing habitats yet unsuitable for *R. ridibunda*.

One indication supporting an increased ability to cross species' range borders is the enormous distribution area of *R. ridibunda* in eastern and central Europe (Berger 1988), which might be attributed to the increased ability of local adaptation. Additionally, the range of *R. perezi* has considerably increased since the last ice age. One may, however, argue that the distribution range of these two species should be even larger, if hybridogenesis facilitates local adaptation and colonization success. We can only hypothesize why this has not yet happened. First, competition between other waterfrog species may halt the range expansion. At its current range limits *R. perezi* is in contact with *R. lessonae*, a species with very similar ecological needs (Günther 1990). In such contact zones, competition between these two species probably halts the expansion of *R. perezi* and *R. lessonae*. Second, the duration of the process in the PG-system has been insufficient. The system studied here has originated relatively recently. While the LE-system is thought to have been present in central Europe for several glacial cycles, the *R. perezi* and *R. ridibunda* genomes came into contact only after the last ice-age (Arano *et al.* 1994). This amount of time might not have been long enough to produce a sufficient number of neoform specimens to promote local adaptation to such an extent that colonization of the Iberian peninsula or southern France by *R. ridibunda* became possible.

However, there are concerns that *R. ridibunda* is invading large parts of France (Pagano *et al.* 2003), an indication of its high competitiveness, unusual for a species far from its centre of distribution. Third, there might be a need for adjustments in hybridogenetic gametogenesis to properly detect the one *R. ridibunda* genome. We know little of the detection mechanism that allows for the exclusion of one whole parental genome during hybridogenetic gametogenesis. However, *R. perezi* is less related to *R. ridibunda* than *R. ridibunda* is to *R. lessonae* (Plötner & Ohst 2001), and therefore adjustments might be necessary for the *R. ridibunda* genome to promote the exclusion of the *R. perezi* genome. Such a process would then also have hampered the exchange of locally adapted alleles by the hybridogen, as in early stages hybridogens were perhaps less fertile due to mispairings of non-homologous chromosomes during meiosis, producing few recombined gametes, if any at all.

In conclusion, our results reveal that alleles of the two parental species are successfully exchanged (criterion ii), and that the genetic alterations are frequent enough to compensate for the migration load in the parental species (criterion iii). We further find support for local adaptation, even though it is relatively weak (criterion i). We consider the cost of incorporation low (criterion iv), as the investment of *R. perezi* is limited to 'cheap' sperm due to mating preferences and because *R. ridibunda* regains reproductive potential due to the reproductive mimicry of the hybridogen (Schmeller 2004). Hence, hybridogenesis can be

Table 2. Selection analysis.

(The selection intensity was calculated as univariate linear (β_1) and quadratic (γ_1) selection gradients as well as multivariate linear (β_{multi}) and quadratic (γ_{multi}) selection differentials. Dependent variables were the population-wide frequency of foreign alleles at loci *ahh*, *6pgdh* for *R. perezi*, *α -gdh*, *mpi* and *6pgdh* for *R. ridibunda* and the population-wide frequency of recombined single locus genotypes (F_{rgt}). These variables were related to the three environmental factors salinity, pH and relative amount of oxygen (O_2). The partial correlation coefficients in the multivariate tests are indexed partial. The *F*-test (*F*) and *t*-test (*t*) statistics are shown with indexed degrees of freedom, *p* denotes the level of significance. All trends (below or equal to 10% significance level) are marked italic and bold, while test statistics below the 5% level are marked plain bold. Only the univariate linear test of the population wide frequency of foreign alleles at locus *6pgdh* of *R. perezi* remained significant following Bonferroni correction.)

<i>R. perezi</i>		β_1	β_1^2	$F_{1,8}$	<i>p</i>	y_1	y_1^2	$F_{2,7}$	<i>p</i>
salinity	<i>ahh</i>	0.217	0.047	0.397	0.546	0.401	0.161	0.670	0.542
	<i>6pgdh</i>	0.211	0.045	0.373	0.558	0.360	0.130	0.522	0.615
	F_{rgt}	0.276	0.076	0.657	0.441	0.349	0.122	0.485	0.635
pH	<i>ahh</i>	0.495	0.245	2.601	0.145	0.505	0.255	1.201	0.356
	<i>6pgdh</i>	0.225	0.051	0.426	0.532	0.226	0.051	0.189	0.832
	F_{rgt}	0.400	0.160	1.522	0.252	0.400	0.160	0.668	0.543
O_2	<i>ahh</i>	0.414	0.172	1.658	0.234	0.440	0.194	0.841	0.471
	<i>6pgdh</i>	0.596	0.355	4.405	0.069	0.889	0.790	13.165	0.004
	F_{rgt}	0.416	0.173	1.676	0.232	0.802	0.643	6.310	0.027
		β_{mult}	β_{partial}	t_9	<i>p</i>	y_{multi}	y_{partial}	t_9	<i>p</i>
salinity	<i>ahh</i>	-0.226	-0.276	-0.704	0.508	-1.288	-0.683	-1.622	0.203
	<i>6pgdh</i>	-0.428	-0.543	-1.583	0.164	0.696	0.627	1.395	0.257
	F_{rgt}	-0.485	-0.582	-1.755	0.130	0.423	0.287	0.520	0.639
pH	<i>ahh</i>	-0.505	-0.550	-1.611	0.158	0.486	0.449	0.871	0.448
	<i>6pgdh</i>	-0.351	-0.476	-1.328	0.233	-0.211	-0.328	-0.602	0.590
	F_{rgt}	-0.523	-0.619	-1.932	0.102	-0.171	-0.171	-0.300	0.784
O_2	<i>ahh</i>	0.323	0.384	1.018	0.348	-0.662	-0.584	-1.247	0.301
	<i>6pgdh</i>	-0.720	-0.739	-2.690	0.036	0.806	0.813	2.416	0.094
	F_{rgt}	-0.567	-0.645	-2.069	0.084	0.609	0.542	1.117	0.345
<i>R. ridibunda</i>		β_1	β_1^2	$F_{1,7}$	<i>p</i>	y_1	y_1^2	$F_{2,6}$	<i>p</i>
salinity	<i>α-gdh</i>	0.663	0.439	5.480	0.052	0.836	0.699	6.957	0.027
	<i>mpi</i>	0.008	0.000	0.000	0.983	0.723	0.522	3.277	0.109
	<i>6pgdh</i>	0.063	0.004	0.028	0.872	0.563	0.317	1.394	0.318
	F_{rgt}	0.029	0.001	0.006	0.941	0.694	0.482	2.791	0.139
pH	<i>α-gdh</i>	0.140	0.020	0.140	0.720	0.212	0.045	0.142	0.871
	<i>mpi</i>	0.362	0.131	1.055	0.338	0.407	0.165	0.595	0.581
	<i>6pgdh</i>	0.165	0.027	0.195	0.672	0.304	0.093	0.306	0.747
	F_{rgt}	0.118	0.014	0.099	0.763	0.307	0.094	0.313	0.743
O_2	<i>α-gdh</i>	0.041	0.002	0.012	0.917	0.257	0.066	0.212	0.815
	<i>mpi</i>	0.306	0.094	0.726	0.422	0.314	0.099	0.328	0.733
	<i>6pgdh</i>	0.412	0.169	1.428	0.271	0.543	0.295	1.255	0.350
	F_{rgt}	0.482	0.232	2.118	0.189	0.622	0.387	1.893	0.230
		β_{mult}	β_{partial}	t_8	<i>p</i>	y_{multi}	y_{partial}	t_8	<i>p</i>
salinity	<i>α-gdh</i>	0.655	0.660	1.967	0.106	0.808	0.736	1.539	0.264
	<i>mpi</i>	-0.020	-0.021	-0.047	0.964	-1.054	-0.892	-2.793	0.108
	<i>6pgdh</i>	0.082	0.105	0.237	0.822	-0.305	-0.734	-1.530	0.266
	F_{rgt}	0.045	0.060	0.135	0.898	-0.581	-0.959	-4.765	0.041
pH	<i>α-gdh</i>	0.114	0.126	0.284	0.788	0.757	0.302	0.449	0.698
	<i>mpi</i>	0.279	0.242	0.558	0.601	-0.763	-0.407	-0.629	0.593
	<i>6pgdh</i>	-0.584	-0.530	-1.397	0.221	2.749	0.950	4.297	0.050
	F_{rgt}	-0.570	-0.537	-1.425	0.214	1.928	0.961	4.921	0.039
O_2	<i>α-gdh</i>	0.047	0.053	0.118	0.910	-0.241	-0.101	-0.143	0.899
	<i>mpi</i>	-0.151	-0.134	-0.302	0.775	1.344	0.619	1.114	0.381
	<i>6pgdh</i>	-0.736	-0.620	-1.765	0.138	-2.623	-0.946	-4.123	0.054
	F_{rgt}	-0.800	-0.667	-2.004	0.101	-1.837	-0.958	-4.715	0.042

described as a process facilitating the range expansion of *R. perezi* and *R. ridibunda*. Whether this holds true for the other known hybridogenetic systems of waterfrogs, and moreover, for other hybridogenetic complexes, is a matter of future research.

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