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Nuclear markers, mitochondrial DNA and male secondary sexual traits variation in a newt hybrid zone (*Triturus vulgaris* × *T. montandoni*)

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Abstract

The smooth and the Montandon's newts (*Triturus vulgaris* and *T. montandoni*) are genetically similar sister species with highly divergent male secondary sexual traits involved in complex courtship behaviour. Their parapatric ranges overlap at moderate elevations in the Carpathian Mountains where they hybridize readily. Here we present a detailed study of genetic and morphological variation in populations from the area of sympatry. Analysis of variation at seven nuclear markers, mtDNA and male sexual secondary traits was complemented with an ecological survey of breeding sites characteristics. Extensive hybridization was revealed with back-cross individuals similar to either parental species predominating among hybrids. The hybrid zone exhibited a mosaic pattern: the genetic composition of the populations was correlated only weakly with their geographical position. No association with habitat type was found. Departures from Hardy–Weinberg proportions, significant linkage disequilibria and bimodal distribution of genotypes suggest strongly that assortative mating is an important factor shaping the genetic composition of hybrid populations. The pattern of cytonuclear disequilibria did not indicate much asymmetry in interspecific matings. Changes in the frequency of nuclear markers were highly concordant, whereas mtDNA showed much wider bidirectional introgression with 14% excess of *T. montandoni* haplotype. We argue that the mosaic structure of the newt hybrid zone results mainly from stochastic processes related to extinction and recolonization. Microgeographical differences in mtDNA introgression are explained by historical range shifts. Since morphologically intermediate males were underrepresented when compared to hybrid males identified by genetic markers, sexual selection acting against the morphological intermediates is implied. We discuss the implications of these findings in the context of reinforcement of prezygotic isolation in newts.

Keywords: assortative mating, differential introgression, hybrid zones, hybridization, reinforcement, *Triturus*

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Introduction

The evolution of reproductive barriers is, in most cases, a continuous process inevitably resulting in hybridization in areas of contact between species that are not fully reproductively isolated. On one hand the analysis of natural hybridization gives insight into the past processes accompanying speciation, such as adaptive divergence and genome coadaptation (Butlin 1998). Additionally, hybridization itself may be an important factor in speciation. Specifically, it has been argued that hybridization might be the last stage of speciation leading to the evolution of complete reproductive isolation by reinforcement (Dobzhansky 1940; Turelli *et al*. 2001).

Reinforcement is most likely when the correlation between genes responsible for assortative mating and genes causing lowered hybrid fitness persists in spite of hybridization (Butlin 1989; Jiggins & Mallet 2000). Previous detailed studies of natural hybridization involved

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mainly cases where either strong endogenous (epistatic) or habitat-dependent selection predominated with little assortative mating in hybrid populations (Barton & Hewitt 1985; Harrison 1993). In such hybrid zones recombination would prevent the evolution of reinforcement (Butlin 1998). Studies of hybrid zones with a bimodal distribution of genotypes suggesting assortative mating are in the minority (Jiggins & Mallet 2000). Assortative mating is expected to be strong when species divergence was driven mainly by sexual selection. Because in sympatry a species recognition system involves some sexual selection, it should facilitate reinforcement (Liou & Price 1994).

Here we present results of genetic analysis of natural hybridization between the smooth (*Triturus vulgaris* (L.)) and Montandon's newt (*T. montandoni* (Blgr)) (Amphibia, Salamandridae). Hybridization between these two sister species is of special interest for several reasons.

Their overall genetic divergence is low ($D_N = 0.14$; mtDNA divergence 2.5%) (Rafiński & Arntzen 1987; Zajc & Arntzen 1999), so no strong endogenous selection against hybrids might be expected. At the same time, both species exhibit striking morphological sexual dimorphism with pronounced interspecific divergence in male epigamic traits (Halliday 1977; Pecio & Rafiński 1985). The most conspicuous characteristics of the nominal subspecies *T. v. vulgaris* with which *T. montandoni* hybridizes include a large dorsal denticulate skin crest, toe flaps, blue spots combined with a deep orange stripe along the tail and large dark spots on the flanks and belly. While *T. montandoni* males lack the dorsal crest, flaps or dark spots, they do possess dorsolateral ridges, a long dark tail filament and a different combination of colours on the tail.

Mating in newts takes place in water and involves elaborate courtship, which culminates in the transmission of a spermatophore that is deposited first on the substrate and then picked up by a female (Halliday 1977). During courtship, male epigamic traits are displayed to the female together with tactile and chemical cues (Halliday 1977; Pecio & Rafiński 1985). Successful transmission of the spermatophore depends on strict cooperation between the sexes, thus one can conclude that male epigamic traits are under strong sexual selection, as was shown experimentally for the dorsal crest in *T. vulgaris* (Green 1991; Gabor & Halliday 1997). The importance of sexual selection as a driving force during *T. vulgaris*/*T. montandoni* speciation is also suggested by the observation that pronounced morphological variation among *T. vulgaris* subspecies is confined almost exclusively to the male epigamic traits (Raxworthy 1990). Laboratory and garden-pond experiments showed that the behavioural sexual isolation between these two species is strong but not complete. In heterospecific matings *T. montandoni* females appear to be more discriminating than *T. vulgaris* females (Michalak *et al*. 1997; Michalak & Rafiński 1999).

T. vulgaris is predominately a lowland species, distributed broadly in Eurasia (Fig. 1a) whereas *T. montandoni* is confined to the Eastern and Western Carpathian Mountains, as well as the easternmost Sudetes Mountains (Gasc 1997). At lower elevations where both species often cooccur (Juszczyk 1987; Rafiński 1988; unpublished; Babik & Rafiński 2001), individuals with intermediate morphology were found in many localities (Hofmann 1908; Fuhn 1963; Fuhn *et al*. 1975; Juszczyk 1987; Kotlík & Zavadil 1999). Their hybrid origin was confirmed by allozyme electrophoresis (Rafiński 1988; Kotlík & Zavadil 1999) but the extent and dynamics of natural hybridization between these two newt species have never been studied in detail.

Newts have a complex life cycle; adults enter small water bodies in early spring, in which they court, mate, lay eggs and where the larvae develop. After the breeding season the adults leave water and spend the rest of the year on land. *T. vulgaris* and *T. montandoni* breed in small reservoirs of standing water, mainly man-made sites such as deep wheel-ruts and puddles, water-filled clay- and gravel-pits, and occasionally larger oxbows. This indicates that they readily colonize newly available and transient breeding habitats. Rapid colonization of new habitats was shown for *T. vulgaris* in the Netherlands (Stumpel & Voet 1998).

Differential habitat associations for these two species were not found at lower elevations in the Carpathians where their ranges overlap (Babik & Rafiński 2001). This suggests that environment-dependent selection against hybrids and/or habitat preference might be not a major factor influencing the fitness of hybrids. On the other hand, complex mating behaviour in newts provides ample opportunity for mate choice and together with striking interspecific secondary sexual differences point to the importance of sexual selection. To test these predictions we analysed the patterns of variation in the hybrid zone in three sets of traits which are expected to be influenced differently by selection, in relation to habitat and geographical setting. At the same time we expected our analysis to indicate whether the hybrid zone meets the conditions for the evolution of reinforcement.

We compared the variation in molecular nuclear markers, mtDNA and male secondary sexual characters. The hybrid status of individuals was established on the basis of their multilocus nuclear genotypes and mtDNA haplotypes. MtDNA, due to its maternal inheritance, haploid nature, lack of recombination and smaller effective population size than of nuclear genes, could be expected to behave independently and be more sensitive to stochastic processes than nuclear loci. Additionally, the mtDNA variation pattern might indicate the direction of hybridization. To see if genes subjected to different selection regimes show a concordant pattern of variation we also studied male secondary sexual traits that are most likely under direct influence of sexual selection.

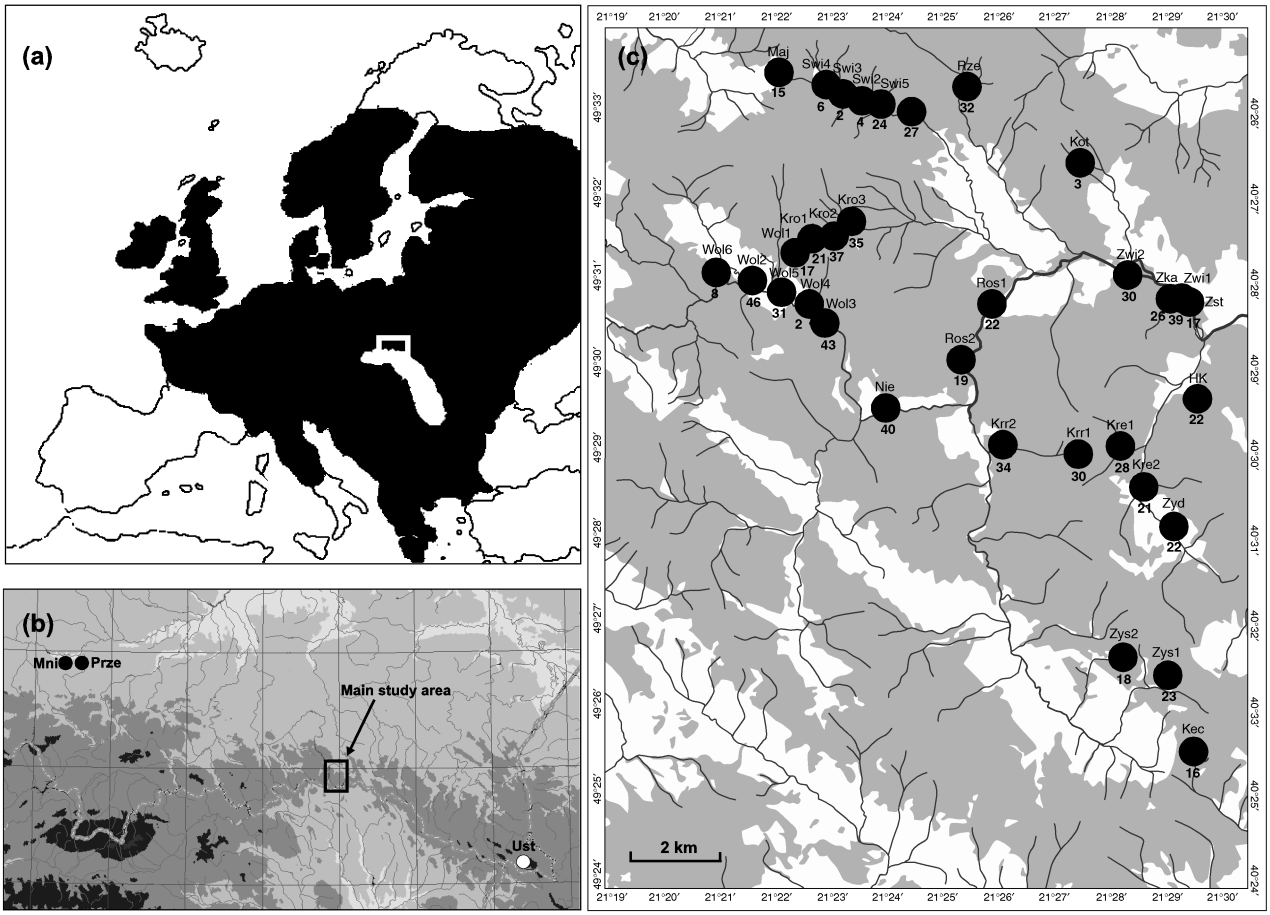

Fig. 1 Maps showing: (a) the distributions of *T. vulgaris* (black) and *T. montandoni* (grey); the range of overlap is poorly known. (b) Southeastern Poland with the reference populations and the main study area indicated; shading from light grey to black indicates elevation (200, 500, 1000 m a.s.l.). (c) Distribution of the populations sampled in the area of sympatry with the respective acronyms and sample sizes; shading indicates forests.

Materials and methods

Sampling

To establish the allele frequencies in the parental species we sampled two allopatric *T. vulgaris* populations from lowland localities in the environs of Kraków (Mni, $n = 32$, Prz, $n = 31$) and one allopatric population of *T. montandoni* from the Bieszczady Mountains (Ust, $n = 43$) (Fig. 1b, Table 1).

Our main study area is located in the Magura National Park in the Beskid Niski Range in southeastern Poland (Fig. 1b). These are low, mainly wooded mountains about 400–800 m a.s.l. The area was searched thoroughly for amphibian breeding sites between 1997 and 2000 (Babik & Rafiński 2001). The present work concentrated on an expanse of about 120 sq km, within which 33 newt breeding sites were sampled from 2000 to 2002, most of them only once. These sites represent the majority of breeding populations present in the study area. The location of the populations, their acronyms and the sample sizes are given in Fig. 1c. For each breeding site nine habitat characteristics were recorded: elevation above sea level, water body surface area, depth, bottom characteristics, soil type, aquatic vegetation, permanence (in arbitrary units), surrounding terrestrial habitat and relief type. The characteristics were later coded as ordinal variables (for details see Babik & Rafiński 2001). A more detailed quantitative description of the habitats would be unwarranted, as most of the breeding sites were small, temporary puddles or water-filled wheel ruts. The environmental characteristics of such sites often change rapidly as they depend on weather conditions and are therefore related to the date of inspection.

Newts were caught during the breeding season in April and May by dip-netting. In small and shallow water bodies netting was a very efficient way of obtaining representative samples. Most of the animals were collected from such

Table 1 Allele frequencies in reference populations of *T. vulgaris* and *T. montandoni*. For microsatellite *Tv4Ca9* alleles are designated by their lengths in base pairs. Only alleles present in the reference populations are shown

		Pep L-T		*Mdh-1*			*Me-1*			*Pgm-1*				*Mpi*		*Gpi*			*Tv4Ca9*						
	N	a	b	a	c	d	a	b	c	a	b	c	d	b	c	b	c	d	134	144	145	146	148	152	154	156
T. vulgaris																										
Mni	32	0.28	0.72	—	0.06	0.94	0.13	0.84	0.03	0.05	0.28	0.62	0.05	—	1.00	—	0.27	0.73	—	0.72	0.11	—	0.14	0.03	—
Prz	31	0.13	0.87	—	0.03	0.97	0.14	0.86	—	0.10	0.15	0.65	0.10	—	1.00	0.02	0.34	0.64	—	0.60	0.11	—	0.16	0.13	—
T. montandoni																										
Ust	43	1.00	—	0.15	0.85	—	1.00	—	—	0.04	0.80	0.16	—	1.00	—	—	1.00	—	0.04	—	—	0.81	—	—	0.01	0.14

sites. For this reason small sample sizes for several populations were also included because they represent small but real breeding populations (Fig. 1c). After transferring the newts to the field station the animals were anaesthetized in 0.2% MS 222 (3-aminobenzoic acid ethyl ester), assigned a number and photographed. Tail-tips were removed and placed in liquid nitrogen. Newts were returned to their original breeding sites within 3 days after capture. The tail-tips were stored at –75 °C until analysis.

Laboratory techniques

Tail-tips were homogenized in 0.05 M Tris-HCl pH 8.0 containing 0.1% v/v of Triton x100 and centrifuged at 13 000 r.p.m. for 2 min. The supernatant was used for allozyme analysis, and the cell debris for DNA extraction.

We initially surveyed the following 30 enzyme systems: AAT, ACOH, ACP, ADA, AK, CK, DIA, EST, FBP, FUMH, G3PDH, G6PDH, GLCDH, GLYOI, GTDH, HK, IDH, LDH, ME, MDH, MPI, PEP, PGDH, PGI, PGM, PNP, PK, SOD, TPI and XDH. The variation in the allozymes was assessed by cellulose acetate electrophoresis using six individuals from the reference populations of each species. Six allozyme loci, *Mdh-1*, *Me-1*, *Mpi*, *Pep Leu-Tyr*, *Pgi* and *Pgm-1* exhibiting substantial differences in allele frequencies between reference populations, good activity in the tail-tip tissues and easily interpretable variation, were chosen for further study. The allozymes were scored by cellulose acetate electrophoresis (Hebert & Beaton 1993) using Titan III cellulose acetate plates (Helena, Beaumont, TX, USA), except for *Pgi*, which was genotyped using starch gel electrophoresis (Murphy *et al*. 1996). Electromorphs (alleles) were coded alphabetically in the order of relative mobility from the origin with the most anodal allele as '*a*'. Among gel comparisons of all alleles were made by rerunning selected individuals on cross-correlation gels.

DNA was extracted from cellular debris using the Wizard® Genomic Purification kit (Promega, Madison, WI, USA) with the Proteinase K digestion step.

To establish nuclear DNA markers, we surveyed between-species genetic variation at 12 microsatellite loci developed by T.W.J. Garner, University of Zurich, Switzerland (Babik *et al*. in prep.). One locus (*Tv4Ca9*), proved to be fully diagnostic for *T. vulgaris* and *T. montandoni*. This microsatellite locus was amplified in 20 µL reaction mixtures containing ~100 ng of genomic DNA, 2 µL of 10 × polymerase chain reaction (PCR) buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of both forward and reverse primer and 0.5 U of *Taq* polymerase (Promega). The cycling scheme was: 94 °C for 3 min, 25 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s with the final extension step at 72 °C for 2 min. The allelic variation at *Tv4Ca9* was scored after electrophoresis on Spreadex gels (Elchrom Scientific, Cham, Switzerland)

and SYBR Green staining (Molecular Probes, Eugene, OR, USA) against the M3 size marker (Elchrom).

Codominance of nuclear markers was confirmed by the analysis of the genotypes of F_1 hybrids from laboratory crosses. Linkage relationships among the loci are unknown, as producing progeny necessary for such an analysis would take a minimum of 3 years. None of the loci appeared to be sex-linked.

To assess variation in mtDNA, a 472-bp mitochondrial DNA fragment was amplified using primers L9858 (5′-CTCCTCCTTAATGATATGCCACA-3′) (Zajc & Arntzen 1999) and TR-A6-H (5′-GATAGTTGGGTAGTTGGGGTT-3′). This fragment, which covers the 3′ end of tRNA-Lys, mtATPase subunit 8 and the 5′ end of ATPase subunit 6, corresponds to positions 7740–8211 in the mitochondrial genome of *Mertensiella luschani* (Salamandridae) (Zardoya & Meyer 2001). Amplification was performed in 20 μL reactions containing ~100 ng of genomic DNA, 2 μL of 10 × PCR buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of both forward and reverse primer and 0.25 U of *Taq* polymerase (Promega). The cycling scheme was: 94 °C for 2 min, 50 °C for 45 s, 72 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min and at final extension step at 72 °C for 3 min.

Both *vulgaris* reference populations, Prz and Mni, were fixed for the mtDNA haplotype which contained two *Hae*III recognition sites (positions 34 and 392). The *montandoni* population was fixed for the haplotype with a single *Hae*III recognition site (in position 34). Detailed data on the mtDNA sequence variation will be presented elsewhere (Babik *et al*. in prep.).

In three populations (Kro3, Tys2 and Zwi2) a unique mtDNA haplotype lacking the *Hae*III recognition site was found at frequencies of 0.03, 0.05 and 0.14, respectively. This haplotype was not found outside the area of sympatry. The sequence of this unique haplotype differed from the common *montandoni* type by only one nucleotide substitution, so we classified it as a variant of the *montandoni* type (Babik *et al*. in prep.).

Variation in mtDNA fragment was scored by RFLP using *Hae*III restriction enzyme (Hybaid, Ashford, UK). The digested PCR products were separated in 1.5% agarose gels stained with ethidium bromide and visualized under UV light.

Morphological data

After inspecting a large sample of both *vulgaris* and *montandoni* males collected from the reference and other allopatric populations at the peak of the breeding season we chose six secondary sexual traits with clear species-specific characteristics. The score 0 was assigned to a *montandoni* character state, and the score 2 to the corresponding *vulgaris* state. The following male traits were recorded from live animals and from photographs: dorsal crest (absent — 0, present — 2), tail filament (absent — 2, present — 0), lateral dorsal ridges (absent — 2, present — 0), dark spots on the ventral side of the thigh (absent — 0, spots on one thigh only — 1, spots on both thighs — 2), colour of the toe tips (light — 0, intermediate — 1, dark — 2) and dark spots on the ventral side of the belly (absent — 0, present — 2). The values recorded for each specimen were summed to obtain the morphological hybrid index (*Morph*HI) with values ranging from 0 (*montandoni*) to 12 (*vulgaris*).

Statistical analyses

Deviations from Hardy–Weinberg equilibrium and linkage disequilibria between polymorphic loci in reference populations were computed using the exact tests implemented in GENEPOP version 3.1d (Raymond & Rousset 1995). The Type I error level for multiple tests was controlled by applying the sequential Bonferroni procedure (Rice 1989). As most of the available nuclear genetic markers were not fixed for alternate alleles in the parental species but showed frequency differences (see Results), we summarized the genetic composition of the hybrid populations by computing the maximum likelihood genetic hybrid index (*Gen*HI) using all seven loci for each individual (Rieseberg *et al*. 1998). *Gen*HI is the sum of probabilities over an individual's alleles that they are derived from one of the parental species (*vulgaris* in our case). The probabilities for particular alleles were set to the frequency of the given allele in each parental species. As we sampled two *vulgaris* reference populations, the weighted mean of the frequency of the individual alleles in both populations was taken as the estimate of the allele frequency in this species. At several loci, the alleles absent in the reference populations were detected in very low frequencies in the area of sympatry. In such cases their frequency was arbitrarily set to 0.001 in both parental species and the frequency of the most common allele was lowered accordingly, so that the frequencies summed to 1. Wherever possible, the multiple alleles diagnostic for one of the parental species were pooled.

Habitat characteristics were summarized using principal component analysis (PCA) of the correlation matrix. We realize that categorical variables used to quantify the breeding sites characteristics do not satisfy the assumptions of multivariate parametric methods, but we use them only for descriptive purpose as a convenient method of summarizing the habitat variation.

We tested for the correlation between the genetic composition of the populations with their geographical position and habitat characteristics using multiple regression with PC1 and PC2 habitat axes and X, Y site coordinates as explanatory variables. The square root-arcsin transformed sample mean *Gen*HI was the dependent variable.

For the populations from the area of sympatry the departures from Hardy–Weinberg equilibrium as measured by F_{IS}, were computed using the maximum likelihood approach (Szymura & Barton 1986, 1991; MacCallum *et al.* 1998) as implemented in ANALYSE version 1.30 (Barton & Baird 1996). If estimates were computed over populations the samples were not pooled, because pooling could introduce a heterozygote deficit due to the Wahlund effect. Instead, the individual log-likelihoods were summed to obtain the overall estimates (Szymura & Barton 1986; Barton & Baird 1996). The significance of linkage disequilibria between pairs of loci was estimated using approximations of the Fisher exact test, as implemented in GENEPOP.

The concordance between nuclear markers was quantified using ANALYSE to fit the cubic polynomial model to the data (Szymura & Barton 1991; MacCallum *et al*. 1998):

$$p_i = \bar{p} + 2\bar{p}\bar{q}[\alpha_i + \beta_i(\bar{p} - \bar{q})]$$

where p_i is the frequency of *vulgaris* alleles at the *i*'th locus and $\bar{p}$ and $\bar{q}$ are the average frequencies across all the loci included. The parameter α represents consistent deviations across the allele frequency range and a positive α value indicates a shift in favour of *vulgaris*. Positive β implies greater difference in allele frequency at an individual locus at either end of the spectrum compared to the average frequency across all loci.

We performed two separate analyses of concordance: one for three diagnostic or almost diagnostic markers (*Mpi*, *Tv4Ca9*, *Mdh-1*, see Results) and the second with mtDNA data added.

Cytonuclear disequilibria were computed using the methods of Asmussen and Basten (Asmussen & Basten 1994; Basten & Asmussen 1997) as implemented in the CND program. In most genetic analyses only the samples with $n \geq 6$ (30 populations) were included.

Concordance among male secondary sexual traits was assessed using the method of Kruuk (1997). The population-mean character value was regressed on the population-mean *Morph*HI value omitting that character (both individual characters and the *Morph*HI were scaled from 0 to 1). Linearity of this relationship would be expected if a given character does not deviate from the overall pattern of change. Departure from linearity, measured by the quadratic component of regression, indicates the relative excess of either species character state. The cubic regression component quantifies differential introgression of an individual character in comparison to the remaining ones combined (Kruuk 1997; Rohwer *et al*. 2001). The same method was used to test the concordance between *Morph*HI and either nuclear markers (*Gen*HI) or the species-specific mtDNA haplotypes.

Results

Reference populations

The allele frequencies at the nuclear loci in reference populations are given in Table 1. We found neither significant deviations from Hardy–Weinberg equilibrium for any locus nor significant linkage disequilibria for any pair of the polymorphic loci after Bonferroni adjustment. The *Gen*HI values in the *montandoni* reference population were 0.000 for all individuals, whereas in the *vulgaris* reference populations *Gen*HI ranged from 0.808 to 1.000 (93.8% of newts in the range 0.950–1.000). Thus, we decided to classify as hybrids or introgressed individuals in the remaining populations those animals for which the *Gen*HI value fell within the range 0.001–0.807.

*Morph*HI values in the *vulgaris* reference populations ranged from 10 to 12 (97% of 33 males scored 12), whereas in the *montandoni* population males ranged from 0 to 2 (87% of 22 males scored 0).

Geographic pattern of variation at nuclear markers in the area of hybridization

In total, 765 individuals from the area of sympatry were scored for genetic markers. The frequency of individuals with introgressed nuclear markers as judged by their *Gen*HI value ranged from 0 to 83%, with a mean of 23% (Fig. 2). However, when evidence from both the nuclear loci and mtDNA was combined, introgressed individuals were detected in all samples (Figs 2 and 3).

Most of the newts classified as hybrids were genetically similar to one or the other parental species (Fig. 4), so they most probably represented back-cross individuals.

As a preliminary step in testing for the association between the genetic composition of the populations and the breeding site characteristics, we summarized the latter using PCA. The ordination of the breeding sites against PC1 (which explained 46% of the variation in the breeding sites characteristics) and PC2 (19.2% of variation) revealed no clear grouping (not shown).

Multiple regression was applied to test for association of the mean *Gen*HI value with the geographical position of the populations and with the breeding site characteristics. Only the Y (North–South) axis explained a significant proportion of the variance in mean *Gen*HI ($P = 0.023$), whereas the X (East-West), PC1 and PC2 (describing the breeding site characteristics) were all nonsignificant.

Populations with a relatively high proportion of *vulgaris* nuclear alleles were present in the northern part of the area, but three populations with a relatively high proportion of *vulgaris* alleles (Kro3 – *Gen*HI = 0.433, Ros1 – 0.589, Wol2 – 0.747) were found in the south, predominated by

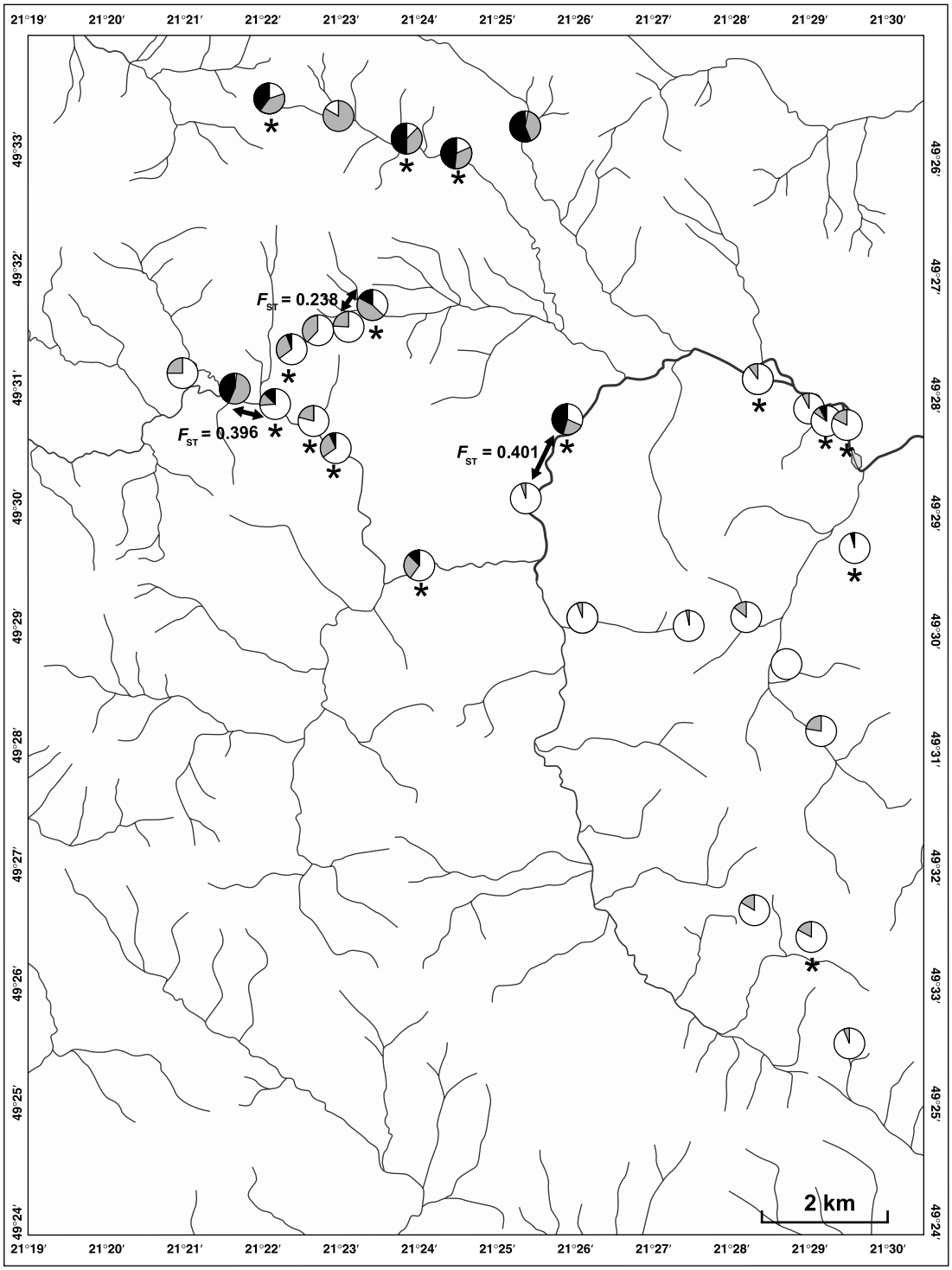

Fig. 2 Pie diagrams showing the frequency of *vulgaris* (*Gen*HI > 0.807, black), *montandoni* (*Gen*HI = 0.000, white) and hybrid individuals (grey) in populations. Significant positive F_{IS} indicated with asterisks. Only populations with $n \geq 6$ are shown.

montandoni-like populations. The genetic composition of these three populations differed dramatically from that of the nearest populations (F_{ST} = 0.238, 0.401 and 0.396, respectively) (Fig. 2).

Genotypic composition of populations

Populations were assigned to groups according to their mean *Gen*HI value. As there were no populations with

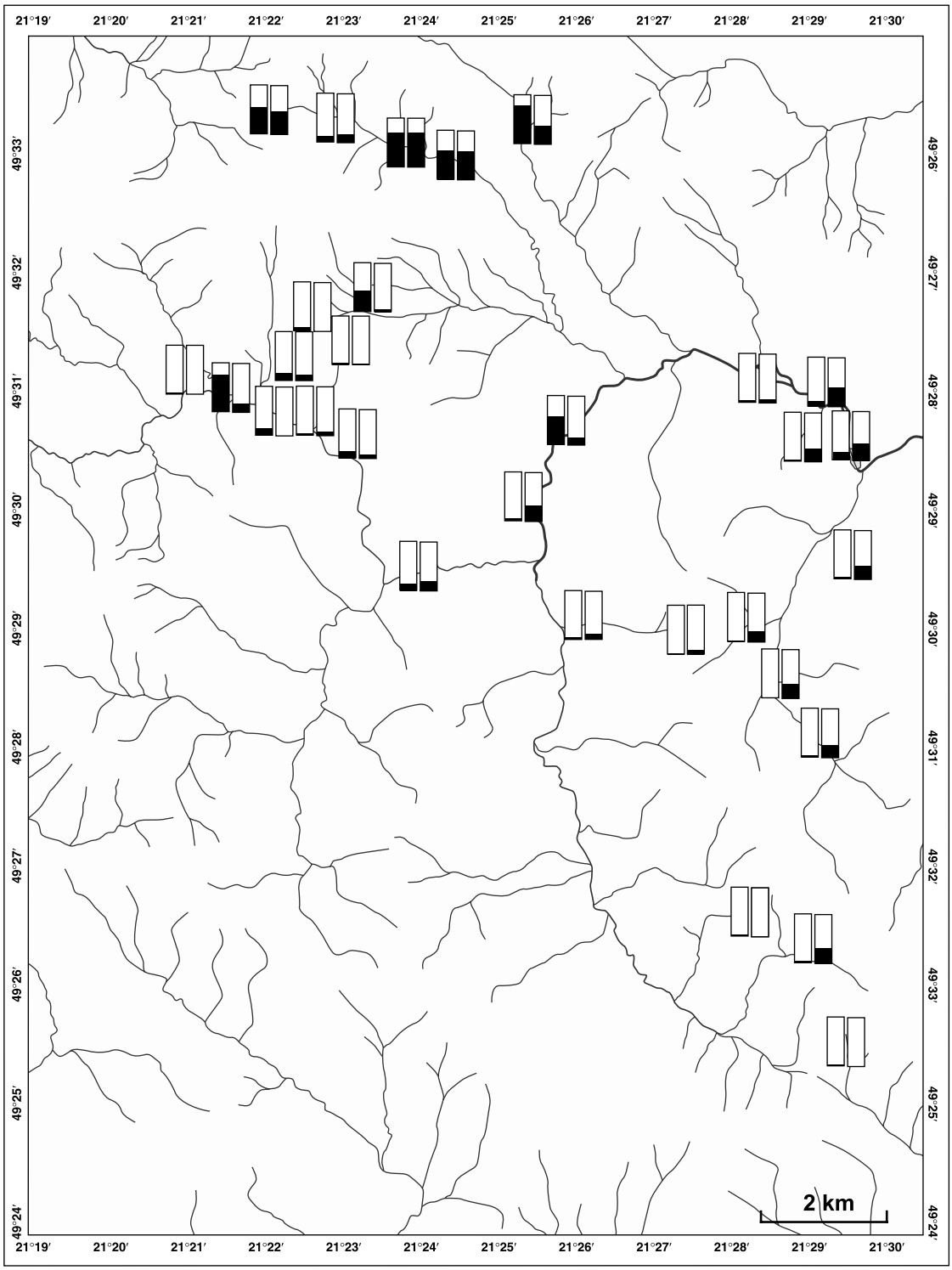

Fig. 3 Mean *Gen*HI (approximate mean frequency of the *vulgaris* alleles, black): left bar, and mean frequency of the *vulgaris* mtDNA haplotype (black) in populations: right bar.

mean *Gen*HI values in the range 0.2–0.4, all the populations were divided into six groups. The individuals in these groups were pooled into seven classes according to their individual *Gen*HI values (Fig. 4). Inspection of the plot shows that (1) 'pure' individuals of both species were present in all groups of populations; (2) the majority of hybrids were either *vulgaris*- or *montandoni*-like recombinants; (3) the frequency of individuals in the intermediate

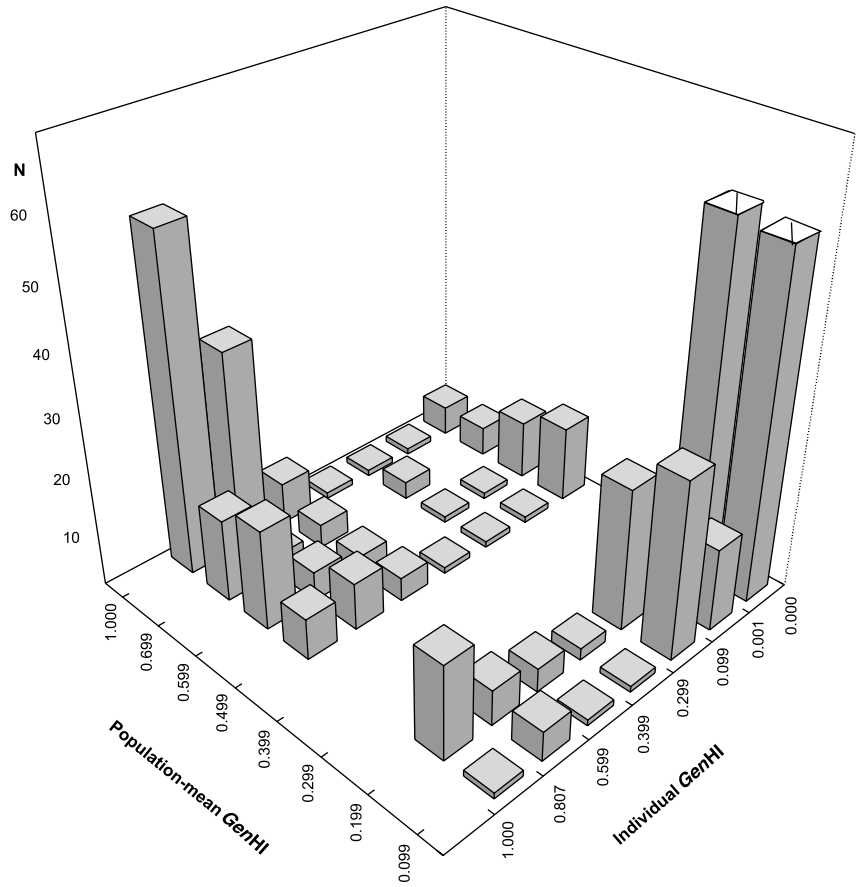

Fig. 4 Histogram showing the frequencies of individuals clustered into seven classes according to individual *Gen*HI values, and six groups based on their population-mean *Gen*HI. Populations within groups were pooled. Bars for 'pure' *montandoni* in groups with mean *Gen*HI 0–0.099 and 0.100–0.199 were truncated.

classes (*Gen*HI from 0.300 to 0.599) was low, but they were present in all population groups and (4) most of the groups displayed a clear bimodal genotypic distribution with parental types more frequent than the hybrids. This was especially evident in the two most intermediate groups (*Gen*HI from 0.400 to 0.599).

Heterozygote deficit, as shown by positive F_{IS}, was found in 15 of 30 populations (only samples ≥ 6 individuals included) (Fig. 2), mainly when both parental species were present syntopically, or in populations with a large proportion of hybrids. Overall F_{IS} value was 0.3187 (2-unit SL 0.283 and 0.354), with significant heterogeneity found both among sites (2Δ Log L = 125.2, d.f. = 29, $P < 0.0001$) and among loci (2Δ Log L = 93.80, d.f. = 7, $P < 0.0001$) (Table 2). Very high F_{IS} for the *Tv4Ca9* locus may indicate the presence of a null allele at this locus.

When populations were divided into four groups on the basis of their population-mean *Gen*HI, the heterozygote deficit was strongest in the two intermediate groups (Table 3). The 2-unit support limits for these groups did not overlap with SL for the *montandoni*-like and *vulgaris*-like groups, which exhibited similar F_{IS} values (Table 3). Heterogeneity in F_{IS} among populations was found in the *vulgaris*-like group only (2Δ Log L = 21.84, d.f. = 2, $P < 0.0001$). In this group F_{IS} was significant in only one (Swi5) of the three populations.

Table 2 F_{IS} values for individual loci across populations. SL: 2 log L support limits

	Lower SL	F_{IS}	Upper SL
Pep L-T	0.3789	0.5464	0.6477
Mdh-1	0.2317	0.3356	0.4623
Me	0.1066	0.2084	0.3131
Pgm	0.0681	0.1356	0.2038
Mpi	0.3196	0.4287	0.5319
Gpi	0.1721	0.2732	0.3748
Tv4Ca9	0.5718	0.6648	0.7819

Significant linkage disequilibria were revealed in all of the populations where both parental species and hybrids were present. In such populations, alleles at the absolute or almost diagnostic marker loci (*Mpi, Tv4Ca9, Mdh-1*) showed significant disequilibria more often than other loci and the strength of the associations between them was highest (detailed data available upon request). No significant disequilibria were detected in populations with a

Table 3 The F_{IS} values over loci in population groups classified on the basis of their population-mean *Gen*HI value

Group	Lower SL	F_{IS}	Upper SL
1 (*Gen*HI < 0.1)	0.1138	0.1793	0.2494
2 (0.1 < *Gen*HI < 0.2)	0.3898	0.4572	0.5344
3 (0.4 < *Gen*HI < 0.7)	0.3695	0.4396	0.5064
4 (*Gen*HI > 0.7)	0.0779	0.1479	0.2201

unimodal distribution of genotypes. These are present mainly in the southeastern part of the area.

Mitochondrial DNA

In 26 populations both mtDNA types were present, the frequency of the *montandoni* haplotype ranged from 0.29 to 0.97 and seven populations were fixed for the *montandoni* haplotype (two of them were represented by only two and four individuals, respectively) (Fig. 3). Nevertheless, in all populations fixed for the *montandoni* mtDNA type, *vulgaris* nuclear alleles were present. On the other hand, there was only one population (Kre2) where no introgression was detected at the nuclear loci (*Gen*HI = 0.000, corresponding to pure *montandoni*), while the *vulgaris* mtDNA haplotype was found at a considerable frequency of 0.29.

A large proportion of newts assigned by their nuclear genotypes to one or the other parental type had a foreign mtDNA type. Sixty-six (59.4%) of 111 individuals with nuclear *vulgaris* genotypes had the *montandoni* mtDNA, and of 488 nuclear *montandoni*, 74 (15.2%) had *vulgaris* mtDNA. This difference is highly significant ($\chi^2 = 99.07$, d.f. = 1, $P < 0.0001$).

Cytonuclear disequilibria between the mtDNA haplotypes and three diagnostic or almost diagnostic loci (*Mpi*, *Tv4Ca9* and *Mdh-1*) were computed for all populations where both nuclear and mitochondrial markers were polymorphic. Significant positive associations between the respective mtDNA types and the species-specific homozygous nuclear marker genotypes were found in several populations, mainly in the northern part of the study area (Maj, Swi1, Swi5). None of the tests remained significant after the sequential Bonferroni correction. When the analysis was performed on the entire pooled data set, significant positive associations between mtDNA types with the homozygous nuclear genotypes were found for all of the markers. Such a result is not surprising, as it simply reflects the preponderance of the species-specific mtDNA in pure or almost pure individuals of both species and pooling increased the sample size, making associations easier to detect.

Tests for associations between the heterozygous genotypes and either mtDNA types gave different results. The marginally significant positive association of heterozygous genotypes at *Mdh-1* with the *vulgaris* mtDNA haplotype was found only in the sample Kre1. No test remained significant after the populations had been pooled.

Concordance among the genetic markers

The concordance among the genetic markers was computed in two ways (Tables 4 and 5, Fig. 5). First, we included in the analysis only three fixed or almost fixed nuclear loci, i.e. *Mpi*, *Tv4Ca9* and *Mdh-1*. A significant $\alpha/2 = 5.5\%$ excess of *vulgaris* alleles was found only at the *Tv4Ca9* locus. This shift in favour of *vulgaris* alleles could reflect the differential action of natural selection on this

Table 4 Concordance between nuclear markers. 2 Log L support limits given in parentheses

	Mdh-1	*Mpi*	*Tv4Ca9*
α	–0.041 (–0.046; 0.018)	–0.063 (< –0.063*; 0.008)	0.114 (> 0.000*; 0.198)
β	–0.084 (–0.210; 0.026)	0.035 (–0.065; 0.136)	0.017 (– 0.096; 0.141)

*Reliable estimation of the lower support limit with the ML algorithm implemented in ANALYSE was not possible.

Table 5 Concordance between nuclear markers and mtDNA. 2 Log L support limits given in parentheses

	Mdh-1	*Mpi*	*Tv4Ca9*	mtDNA
α	0.008 (–0.025; 0.028)	–0.008 (–0.032; 0.022)	0.146 (> 0.000*; 0.209)	–0.276 (–0.352; –0.216)
β	0.104 (0.032; 0.207)	0.196 (0.140; 0.260)	0.181 (0.086; 0.275)	–0.957 (–1.176; –0.733)

*Reliable estimation of lower support limit with the ML algorithm implemented in ANALYSE was not possible.

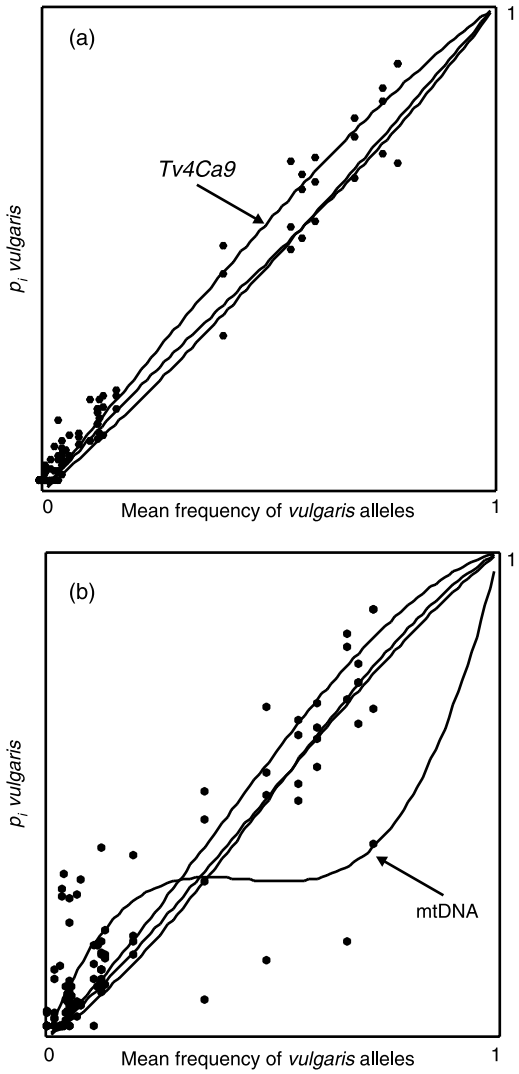

Fig. 5 Concordance between the fixed (*Mpi*, *Tv4Ca9*) or nearly fixed (*Mdh-1*) nuclear markers (a), and between nuclear markers and mtDNA (b).

particular locus or on the chromosomal segment linked to it. An alternative explanation, in our opinion more likely, is that a null allele(s) was present at this locus in *montandoni* (see Discussion).

The support limits for β for all three loci included zero so there was no evidence for differential bidirectional introgression at these three nuclear markers (Fig. 5a).

Strong discordance between the mtDNA and nuclear markers was evident in the second analysis. There was a 14% excess of the *montandoni* mtDNA type over the expected value estimated from the mean frequency of the *montandoni* nuclear markers. The inclusion of the mtDNA data resulted in significant and positive β-values for the nuclear markers. It reflects the inflation of the mean introgression by an overwhelmingly high value of β for mtDNA [β = –0.957, 2 log L SL –1.176 –(–0.733)] (Fig. 5b).

A closer look at the geographical pattern of the mtDNA haplotype distribution revealed fine-scale spatial differentiation (Fig. 3). In the southeastern part of the area we found relative excess of *vulgaris* mtDNA. In contrast, the *montandoni*-like populations in the western part showed comparable levels of nuclear and mitochondrial introgression. Seven populations with a high proportion of *vulgaris* nuclear alleles fell into two categories based on the relative frequency of the foreign mtDNA type. Three samples (Maj, Swi1, Swi5), all located along the same stream valley, showed nearly equal proportions of the *vulgaris* nuclear alleles and mitochondrial types, whereas the four geographically scattered populations (Kro3, Ros1, Rze, Wol2) had a significant deficit of *vulgaris* mtDNA type (tests of difference between proportions, *Ps* < 0.005).

Male secondary sexual characters

The presence of hybrid males with various combinations of species-specific epigamic traits implies the genetic control of these traits by multiple loci, probably inherited independently.

Morphologically intermediate males (*Morph*HI from 3 to 9), if present, were represented by single individuals per population. Two intermediate males were found to co-occur with 'pure' *montandoni* males in only one population ($n_{\text{males}} = 24$). In total, 14 males with intermediate *Morph*HI values (i.e. 3–9) were found, whereas 86 males were classified as hybrids according to their *Gen*HI (values 0.001–0.807) ($\chi^2 = 58.9$, d.f. = 1, $P < 0.0001$). The difference was nonsignificant when only the individuals with more intermediate GenHI values (0.200–0.650) were classified as hybrids ($\chi^2 = 2.21$, d.f. = 1, $P = 0.13$). Nevertheless, the comparison suggests that males with intermediate morphology are underrepresented relative to hybrid individuals assigned to this category by their genotypic composition (14 vs. 23).

The regression of the population-mean *Gen*HI on the population-mean *Morph*HI value showed a very strong linear relationship ($R^2_{adj} = 0.9841$), with quadratic and cubic terms nonsignificant (Fig. 6a). In contrast, the association between the frequency of the *vulgaris* mtDNA haplotype and *Morph*HI was weak ($R^2_{adj} = 0.2161$), and only the linear component was significant ($F_{1,29} = 7.996$, $P = 0.008$) (Fig. 6b).

To check if the changes in particular male epigamic traits were parallel we assessed the significance of the quadratic and cubic regression components when population-mean values of individual traits were regressed on the *Morph*HI. The latter was computed using the remaining traits (Fig. 7). Quadratic and cubic terms were marginally significant for the tail filament ($P = 0.026$ and 0.028, respectively),

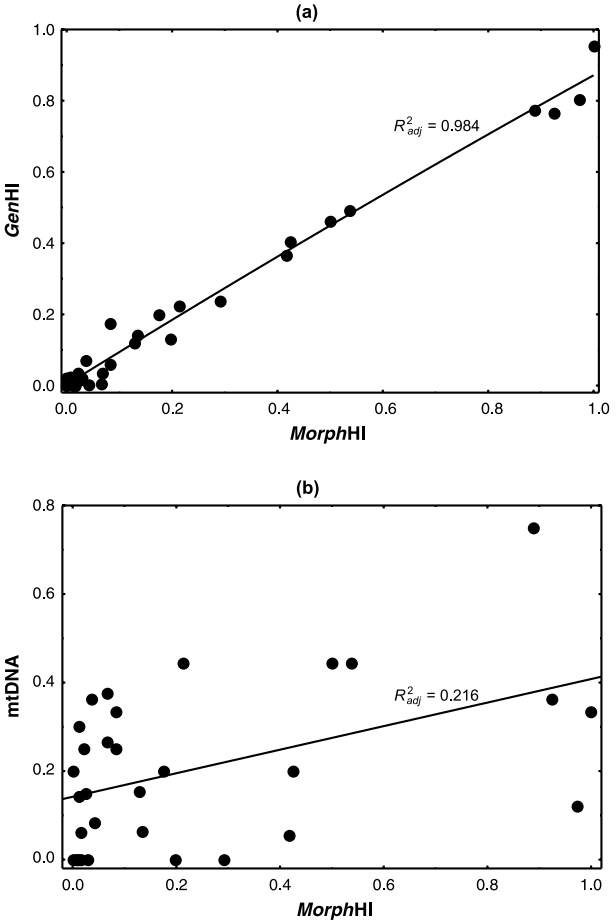

Fig. 6 Relationships between: (a) the nuclear genetic markers and morphological traits (male population-mean *Gen*HI vs. male population-mean *Morph*HI) and (b) mtDNA and morphological markers (male population-mean frequency of the *vulgaris* mtDNA vs. male population-mean *Morph*HI). Adjusted R^2 values and regression lines are shown.

but together they explained only 0.06% more of the variance than the linear model (R^2_{adj} = 0.9947 and 0.9941, respectively). The quadratic term was significant for toe tips colour (P = 0.026), but explained only 0.7% more of the variance than the linear model (R^2_{adj} = 0.9560 and 0.9492). Despite their statistical significance, such small differences have no plausible biological interpretation. It can be concluded, therefore, that changes in the frequencies of species-specific male sexual secondary traits are highly concordant.

Discussion

Extent of hybridization

The first observation of heterospecific matings between *T. vulgaris* and *T. montandoni* in nature and the presence of morphological intermediates were reported as early as the beginning of the last century (Hofmann 1908). Later, the hybrid origin of morphologically intermediate individuals was confirmed electrophoretically (Fuhn *et al*. 1975; Rafiński 1988; Kotlík & Zavadil 1999). Here we present the first quantitative data on the extent of hybridization between *T. vulgaris* and *T. montandoni*.

Our estimates of the frequency of the hybrids at nuclear loci are conservative due to the moderate number of markers used. Nevertheless, the hybrids were relatively common. However, most individuals classified as hybrids did not have intermediate *Gen*HI but possessed genotypes characteristic of backcrosses closer to either parental species. Thus, hybridization leading to the formation of F_1 hybrids appears to be a relatively rare event in most mixed populations. In our study area, *vulgaris* alleles introgressed extensively into *montandoni* populations. We have also found introgressed *vulgaris* alleles in two widely spaced (> 200 km) allopatric *montandoni* populations in the Polish Carpathians (Babik, unpublished). This, together with previous reports from Romania, Poland and the Czech Republic (Fuhn *et al*. 1975; Rafiński 1988; Kotlík & Zavadil 1999), indicates that the two newt species hybridize in the area of contact along the Western and Eastern Carpathians.

The presence of *montandoni* populations not introgressed by *vulgaris* alleles can be expected in the Eastern Carpathians, where allopatric *montandoni* populations are distributed at much larger distances from the *vulgaris* area and where our reference *montandoni* population, Ust, was located (Szyndlar 1980).

Geographical pattern

The geographical pattern of hybridization may provide important information on the form and strength of selection acting on hybrid genotypes. The two most commonly observed patterns depend on the relative strength of endogenous vs. environmental selection. If lowered fitness of hybrids is due primarily to endogenous (epistatic) selection, only a weak association between the habitat and genotypic distribution is expected, and such a tension hybrid zone has a form of a smooth cline (Barton & Hewitt 1985). If the parental species survive best in alternative environments that are distributed in a mosaic fashion and the loss of fitness in hybrids results from exogenous selection, then the distribution of the hybrid genotypes is also mosaic, reflecting the distribution of habitats (Harrison & Rand 1989; MacCallum *et al*. 1998). Both types of selection are expected to act on hybrid genotypes under natural conditions. Several hybrid zones fitting closer to one of the two model were studied in much detail. The hybrid zones of *Bombina* in Poland and *Chorthippus parallelus* in Spain represent examples of tension zones (Szymura & Barton 1986, 1991; Butlin *et al*.

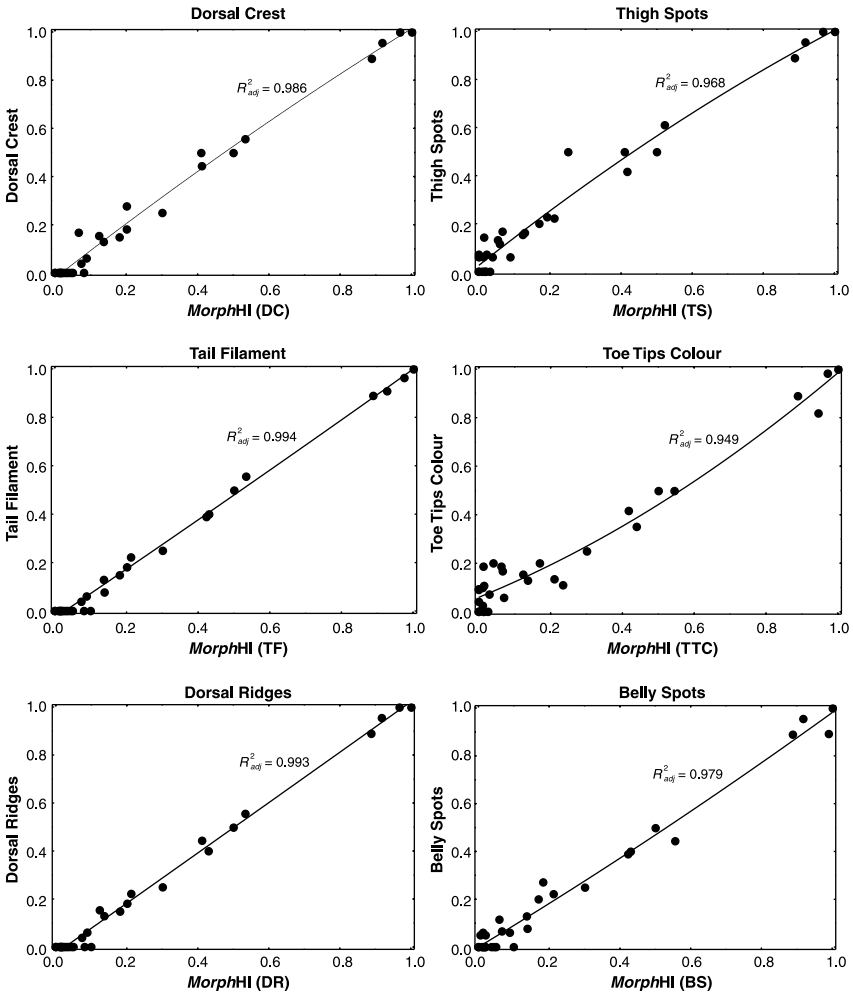

Fig. 7 Relationships between the population-means for individual male epigamic traits and population-mean *Morph*HIs omitting this trait. Adjusted R^2 values of the linear fit and the best-fitting quadratic curves are shown.

1991), whereas *Gryllus* (Harrison & Rand 1989) and *Allonemobius* (Britch *et al*. 2001) in North America are good examples of the habitat-dependent mosaic hybrid zones.

The hybrid zone we studied does not comply clearly with either of these two models. There is a distinct geographical component in the spatial genotypic distribution shown by a weak but significant correlation between the population-mean *Gen*HI and location of the populations along the North–South axis. *Vulgaris* alleles prevail in the northern part of the study area and their frequency declines southward. However, a few populations with a high proportion of the *vulgaris* nuclear alleles (Kro3, Ros1, Wol2) are also found further to the south. The change in the *vulgaris* allele frequency along the North–South axis does not seem to be associated with any obvious environmental gradient. However, as the lowlands north and south from the Carpathian chain are inhabited by *vulgaris* only we can expect a clinal pattern if samples were collected on a larger geographical scale. The clinal or mosaic patterns observed in hybrid zones appear to be scale dependent (Arntzen 1996; Ross & Harrison 2002). We sampled data mainly from the *montandoni* side of the species contact zone due to the scarcity of suitable newt breeding sites north- and southward of the area we studied.

At the geographical scale of our study the hybridization pattern is strongly mosaic. Such distribution of genotypes/species in the hybrid zone may be produced by habitat-dependent selection, active habitat preferences, competitive interactions, and stochastic processes related to the sequence of colonization and extinction. We failed to find any significant associations between breeding site characteristics and genetic composition of the populations therefore diversifying selection seems to us an unlikely explanation of the observed fine-grained mosaic pattern. The lack of association of either newt species with habitat may reflect a narrow range of the available breeding habitats in this area (Babik & Rafiński 2001). Moreover, in a few ponds and oxbows harbouring newts one or the other species predominated, arguing against strong environmental preferences. We cannot exclude however, that the observed mosaic pattern is shaped by natural selection and/or

active preferences related to unrecorded environmental factors, including those acting during the terrestrial phase. Nevertheless, in our opinion this pattern results mainly from stochastic processes as a consequence of extinction/recolonization events and shifts of the species distribution caused by human-mediated landscape changes. Both *T. vulgaris* and *T. montandoni* occupy patchily distributed small water reservoirs that are formed and lost in an unpredictable fashion. Even during a relatively short period of our study some new sites appeared and were colonized by newts, while some others were destroyed or disappeared due to vegetational succession. The initial genetic composition of a new breeding population would be determined by a few founders, whose identity has a large stochastic component related to the proximity and genetic makeup of neighbouring populations. Accordingly, in most cases neighbouring populations have similar genetic composition, but we cannot rule out the possibility that new populations may be founded as a result of episodic long-distance dispersal. Indeed we found a single *vulgaris* male in a *montandoni*-like population (HK) far away from any other *vulgaris* populations. Moreover, in several instances the genetic composition of populations located only several hundred metres apart was dramatically different and with no obvious connection to habitat characteristics (Fig. 2). Because even a small amount of gene flow would homogenize allele frequencies of closely spaced populations, the effective migration between breeding sites must be very low. Newts apparently combine good colonizing ability (Stumpel & Voet 1998) with high breeding site fidelity (Joly & Miaud 1993). Rare episodes of dispersal and colonization might be caused by breeding sites disappearance. Once the population is established, philopatry should be favoured by natural selection. At present this scenario is difficult to evaluate, as very little is known about the newt metapopulation dynamics. A mosaic pattern with no apparent genotype–habitat associations attributed mainly to stochastic processes has been described for fire ants (Shoemaker *et al*. 1996) and grasshoppers (Bridle *et al*. 2001; Bridle & Butlin 2002).

mtDNA introgression

Concordance of nuclear marker frequency changes indicates that natural selection does not act differentially on these markers or closely linked genes. Slight but significant excess of the *vulgaris* alleles at *Tv4Ca9* locus ($\alpha = 0.11$) might be due to the presence of a null allele(s) at this locus in *montandoni*. This interpretation is supported by the absence of a detectable PCR product at this locus in some individuals.

The introgression of mtDNA is much more pronounced compared to nuclear loci (β of the order –1), with an overall 14% excess of the *montandoni* type (Figs 3 and 5). Mitochondrial DNA is regarded generally as being less constrained by natural selection than nuclear genes (Takahata & Slatkin 1984; Rohwer *et al*. 2001; but see Nagao *et al*. 1998; Ballard & Dean 2001; Gerber *et al*. 2001), facilitating wider introgression. This was reported repeatedly, often with unidirectional introgression (e.g. Gyllensten & Wilson 1987; Jaarola *et al*. 1997; Martinsen *et al*. 2001; Rohwer *et al*. 2001). In our case, the mtDNA introgression is bidirectional, a situation not found commonly in hybridizing species (Wirtz 1999).

It is striking that in some populations (Kro3, Ros1, Rze, Wol2) with a large proportion of *vulgaris* nuclear alleles, introgression of the *montandoni* mtDNA was very high (Fig. 3). On the other hand, we did not find a *montandoni*-like population with such high proportion of the *vulgaris* haplotype. This discrepancy may be explained in several, not mutually exclusive, ways. First, assuming that introgressed mtDNA does not influence fitness significantly, the observed excess of the *montandoni* mtDNA type may stem simply from the fact that the *montandoni*-like populations generally predominate in the area. We may expect stochastic processes to lead to the complete elimination of conspecific mtDNA in some *vulgaris*-like populations. Generally, maternally transmitted mtDNA has a smaller effective population size than nuclear loci, making it more susceptible to genetic drift. The considerable effect of genetic drift in the newt hybrid zone is expected due to highly dynamic metapopulation demography. A complete replacement of conspecific mtDNA by a foreign haplotype has been reported several times (e.g. Gyllensten & Wilson 1987; Tegelström 1987; Glémet *et al*. 1998).

However, given much functional interdependence between the nuclear and mitochondrial genomes (Ballard & Dean 2001; Gerber *et al*. 2001), one should expect coevolution between a particular mtDNA type and nuclear genes which may lead to lower fitness in some cytonuclear hybrid combinations. We cannot exclude that the *montandoni* mitochondria perform better on a *vulgaris* nuclear genetic background than in the reverse combination. This hypothesis can be tested experimentally by estimating fitness of the individuals with the foreign mtDNA type (Nagao *et al*. 1998). The relative excess of the *montandoni* mtDNA type might also be related to the larger fecundity of *montandoni* vs. *vulgaris* females (Pecio 1992; Osikowski & Rafiński 2001).

Unexpectedly, the proportion of the *vulgaris* mtDNA was different in western and eastern *montandoni*-like populations (Fig. 3). These two areas may represent microregions, each with a separate colonization history. The study area was depopulated drastically after World War II and according to the old maps the areas formerly cultivated or used for pasture had been more extensive. Our observations from other parts of the Carpathians suggest that

T. vulgaris penetrates mountains along cleared river valleys. Distributional shifts related to changes in forestation were documented in France for another pair of hybridizing newt species, *T. marmoratus–T. cristatus* (Arntzen & Wallis 1991). Similarly, a relatively high proportion of *vulgaris* mtDNA in the southeastern part of the area may be a remnant of a wider distribution of *vulgaris* in the past. The foreign mtDNA as a wake of past hybridization was reported several times (e.g. Jaarola *et al*. 1997; Rohwer *et al*. 2001).

Genotypic composition

The genotypic composition of the hybridizing populations ranges from hybrid swarms, with all individuals showing mixed ancestry and no 'pure' parental species present, to strictly bimodal hybrid populations where only the parentals and F_1 hybrids occur (Harrison & Bogdanowicz 1997; Jiggins & Mallet 2000). The studied hybrid zone is on the bimodal side of the continuum. In many populations, both parental or parental-like individuals predominated and genetical intermediates were generally found in low numbers (Fig. 2). In the remaining populations, mainly in the southeastern part of the area, only *montandoni* and *montandoni*-like individuals were present. Bimodality can result from a recent secondary contact, a constant influx of the parental types into the zone coupled with very strong selection against the hybrids or from the existence of premating reproductive barriers leading to assortative mating or conspecific sperm precedence (Jiggins & Mallet 2000; Marshall *et al*. 2002). The three factors are not mutually exclusive; rather their relative role changes from case to case.

The bimodal genotypic distribution and heterozygote deficit found in newt populations where both *vulgaris*- and *montandoni*-like individuals were present resulted most probably from assortative mating. It is indicated by heterozygote deficit observed in many populations. Random mating would have continuously restored Hardy–Weinberg proportions, even with a substantial influx of pure individuals into the hybrid populations. Assortative mating is also the most probable explanation for the presence of linkage disequilibria in the bimodal hybrid newt populations. Linkage disequilibria can also be generated by continuous immigration of nonrecombined genotypes into hybrid populations. Large differences in genetic composition among neighbouring populations located within the newts' dispersal range indicate that ongoing or recent gene exchange between such populations has not been extensive. For this reason we do not regard constant immigration of nonrecombined genotypes as a plausible explanation of the observed linkage disequilibria. However, we cannot reject the hypothesis that departures from H-W proportions and linkage disequilibria result from differential viability of hybrid genotypes.

Mating asymmetry

We found no genetic evidence for strong asymmetry in the direction of interspecific hybridization as judged by the significance and sign of the cytonuclear disequilibria. The asymmetry in interspecific matings would lead to associations between heterozygous genotypes at nuclear marker loci and the particular mtDNA type. On a broader scale, there was an association of the respective mtDNA types with the corresponding nuclear genotypes. However, a substantial proportion of foreign mtDNA was found on the nuclear background of the other species.

Previous studies on mating behaviour in *T. vulgaris* and *T. montandoni* (Michalak *et al*. 1997) in laboratory and garden pond experiments (Michalak & Rafiński 1999) showed strong behavioural isolation between newts from allopatric populations. Some asymmetry in isolation was also detected, with the *montandoni* females being more discriminating. Assuming neutrality of mtDNA and asymmetry in the heterospecific matings, we would expect much higher introgression of the *vulgaris* mtDNA into the *montandoni* populations than *vice versa*. Rather, the converse was revealed. At least two additional factors may be responsible for this result. First, as argued above, the interactions between nuclear and mitochondrial genomes may modify fitness of the hybrids restricting the introgression of *vulgaris* mtDNA genome into a *montandoni* background. Second, the observed pattern of mtDNA introgression could result from a difference in fecundity between *T. vulgaris* and *T. montandoni* females. Females of the latter species are significantly larger, heavier and lay more eggs (Juszczyk 1987; Pecio 1992; Osikowski & Rafiński 2001).

In newts, mating directionality and the strength of reproductive isolation can be studied directly by analysing mother–offspring data collected from females inseminated in nature.

Male secondary sexual traits

Morphological male secondary sexual traits in newts function as visual signals in complex courtship preceding spermatophore transfer (Halliday 1977). The males of *T. vulgaris* and *T. montandoni* exhibit striking differences in the development of epigamic characters. Together with other male characters (pheromones, behaviour) and with the corresponding female preferences, they constitute a species-specific mate recognition system (Paterson 1986). For this reason, strong disruptive sexual selection on male traits is expected in sympatry. In other words, we predict that hybrid males with intermediate morphology would have lower fitness due to a poor mating success, as is the case in hybridizing flycatchers (Sætre *et al*. 1997) and sticklebacks (Vamosi & Schluter 1999). Our data seem to confirm this prediction. The morphologically intermediate

males (*Morph*HI 3–9) were found in almost half the samples but always in very low frequencies. Their proportion was significantly lower than that of males introgressed at the nuclear markers. This discrepancy may be due to differences in scale between *Morph*HI and *Gen*HI. However, the morphologically intermediate males tended to be underrepresented even when a conservative estimate of the hybrid status based on nuclear markers was taken into account (*Gen*HI 0.200–0.650). Changes in the mean frequencies of particular secondary sexual characters are highly concordant, indicating that natural/sexual selection does not affect them differentially. Moreover, there is a very strong correlation between *Morph*HI and *Gen*HI, because the mean values in our case are not affected by the different level of bimodality of the two distributions. As could be expected from the large discordance between the mtDNA and nuclear markers, the morphological traits are correlated only weakly with the mtDNA type.

Differential action of sexual selection on morphological characters vs. genetic markers was demonstrated for the hybridizing *Manacus* species (Brumfield *et al*. 2001). In *Manacus*, however, the discordance is most probably the result of intrasexual selection controlling the level of male aggression. Discordant patterns of introgression of anonymous nuclear gene markers and alleles at genes responsible for morphology and/or reproductive compatibility was also shown for hybridizing crickets (Harrison & Bogdanowicz 1997).

Lowered fitness of hybrids is a *sine qua non* condition for the reinforcement of prezygotic isolation between the incipient species in the zone of secondary contact (Dobzhansky 1940, 1970; Butlin 1989; Marshall *et al*. 2002). The evolution of reinforcement should be facilitated in bimodal hybrid zones where an initially high level of assortative mating prevents disruption of the mate-choice loci complexes by recombination (Sanderson 1989; Cain *et al*. 1999; Jiggins & Mallet 2000). In hybridizing newts, assortative mating results most probably from sexual selection, as indicated by complex mating behaviour coupled with elaborate species-specific secondary sexual traits. As a result, hybrid males with intermediate expression of epigamic characters are expected to have lower mating success. Another factor enhancing the possibility of reinforcement in the newt hybrid zone is its mosaic structure. Many encounters between two taxa increase the chance that effective reproductive barriers will develop (Littlejohn 1981; Cain *et al*. 1999).

However, there is still much doubt on the relevance of reinforcement in nature (Marshall *et al*. 2002 and references therein), and well-documented examples are few (Noor 1995; Sætre *et al*. 1997; Higgie *et al*. 2000; Jiggins *et al*. 2001). The *T. vulgaris* × *T. montandoni* hybrid zone, exhibiting bimodality, strong assortative mating and mosaic spatial structure, seems to fulfil the requirements for the evolution of reinforcement. If reinforcement is indeed taking place in the area of hybridization between *T. vulgaris* and *T. montandoni*, females from sympatric populations should be more discriminating than females from allopatric sites. This prediction can be tested by comparing the results of the previous studies on the strength of sexual isolation between animals from allopatric (Michalak *et al*. 1997; Michalak & Rafiński 1999) and sympatric populations.

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This study is part of W. Babik's PhD thesis on natural hybridization between the smooth and the Montandon's newts; J.M. Szymura has a long-standing interest in hybrid zones, especially in *Bombina*; The late J. Rafiński had a lifelong interest in newt reproductive biology and phylogeny. This work is dedicated to his memory.
