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New methods for analyzing the hybrid zone of our fire-bellied toads (genus *Bombina*)

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Annotation:

In this elaborate the methods that are used for studying the hybrid zone of *Bombina bombina* and *B. variegata* were summarized. The principals, advantages and disadvantages of these methods are mentioned.

Prohlašuji, že svoji bakalářskou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Obsah

1. Introduction

1.1. The *Bombina* toads

The anuran genus *Bombina* (*Anura: Bombinatoridae*) contains 3 European species and 3 Asian species, typically with contrastly coloured belly. The European species are *Bombina bombina* (Linnaeus, 1761), *B. variegata* (Linnaeus, 1758) and *B. pachypus* (Bonaparte, 1838). While *B. bombina* is quite uniform, there were described 3 geographical subspecies within *B. variegata* – *B. v. variegata* (Linnaeus, 1758), *B. v. scabra* (Küster 1843) and *B. v. kolombatovici* (Bedriaga 1890), differentiated in their phenotypes (Vasara et al., 1991), allozymic traits and mitochondrial DNA (Szymura et al., 2000). *B. pachypus* is sometimes considered as a subspecies of *B. variegata*, but beacuse of their molecular differencies they are separate species. However, only the two species from central Europe, *B. bombina* and *B. variegata* are hybridizing there.

Both *Bombina* species differ not only morphologically, but also with their ecological requirements and etological exhibitions as well. Some differences were also found in their DNA content, chromosome length and the position of centromere of the 12th chromosome (Piálek, 1992). The major part of the genome among the two species is differentiated.

B. bombina, which occurs mostly in lowland areas, is much more an aquatic species breeding in rather large permanent waters (Maděj, 1973), with a relatively long breeding season and tadpoles with a longer larval period (Rafinska, 1991) and exhibition of several adaptations against predation (Kruuk, 1997). Smaller egg size in *B. bombina* results in delayed metamorphosis.

B. variegata usually inhabits more hilly or mountainous regions, where the supply of breeding sites is pretty small. Due to its more terrestrial way of life, adults have thicker skin (Nürnberger et al., 1995) and longer legs (Michalowski, 1961) then those of *B. bombina,* which allows them longer migration between breeding sites. Their breeding has an explosive character. Females of *B. variegata* lay smaller clutches, but their

eggs are bigger and tadpoles reach metamorphosis more rapidly (Nürnberger et al., 1995). Males of *B. variegata* lack the vocal pouches and emit weak calls, but generate water waves to maintain spacing (Seidel, 1999).

Both species inhabit either aquatic and terrestrial sites. The distribution of findings in individual months implies earlier entry of activities in *B. bombina* with maximum quantity in May and with phased passing to the terrestrial way of life and so a declining frequency of occurence. In *B. variegata* it is a bimodal frequency of distribution with most observation in July. They overlap in altitudes from 100 to 800 metres above the sea level.

The area occupied by *Bombina bombina* runs through the lowlands of eastern and northern Europe, while *Bombina variegata* inhabits rather higher altitudes in western and southeastern Europe and in the Carpathian mountains. The occurence of *Bombina* in Bohemia and Moravia was decribed in 1898 (Pražák ex Piálek, 1992).

European fire-bellied toads *B. bombina* and *B. variegata*, have a parapatric distribution that is related to their ecological requirements and postglacial dispersal from southern refuges (Arntzen 1978; Szymura 1993). Arntzen (1978) thought both *Bombina* species have arisen in allopatry during glaciation when the Apennine and Balkan mountains served as refuge to *B. variegata*, whereas *B. bombina* found refuge in lowlands surrounding the Black and Caspian Seas. When the climate warmed up the toads expanded to central Europe and formed a hybrid zone. Later studies pushed the time of speciation backwards. Szymura´s (1983) estimates, based on protein electrophoresis analyse, set the age of diversification at 6.8 ±1.8 mil. years ago.

Werner (1897) pointed at the disappearance of species specific characteristics due to hybridization in points with common occurrence. A large number of diagnostic traits differentiating the two species make *Bombina* a powerful system to test hypotheses on the origin and dynamics of hybrid zones (Hewitt, 1988).

1.2. The hybrid zones

Biological species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups. If two populations are to belong to different biological species, reproductive isolation must be complete: no fertile hybrids can be formed (Barton & Hewittt, 1985).

Hybrid zones are narrow regions in which genetically distinct populations meet, mate and produce hybrids. They are often only a few hundred meters wide and yet may be several hundred kilometers long. They are found in a wide variety of organisms. The wide range of genotypes found in a hybrid zone can be used to analyze the genetic differences and selective forces that separate the taxa involved.

Hybridization occurs whenever two or more insufficiently pre- or postzygotic isolated population meet, which results in a gene flow between such populations. In case of producing fertile hybrids the genotypes usually varies from gene to gene. In some loci versatilly beneficial alelles are fixed on either side of the hybrid zone, in others different alleles can be favourable in various environment (Barton & Gale, 1993).

Hybrid zones contain recombinant individuals resulting from many generations of hybridization (Rieseberg & Buerkle, 2002). Hybridization is a common feature in both plants and animals. Hybridization is thought to occur less frequently in animals than in plants; however, quantitative estimates are only available for birds and fish (Arnold, 1997). Between 9% and 10% of bird species hybridize, hybridization frequencies in families of North American freshwater fish range from 3% to 17% (Hubbs, 1955). Application of cline theory requires that hybrid zones be more or less continuous (Szymura & Barton, 1991) and that they be maintained by a balance of dispersal and selection against hybrid (Barton & Hewitt, 1985).

The existence of hybrid zones causes substantial troubles to the biological species concept (Mayr, 1942 ex Harrison, 1993). Although the question of species definition

remains open, hybridization comes up with the possibility to take a look inside the processes of species genesis itself. A much discussed theme is the reinforcement of premating isolation. Since hybrids are often inviable, sterile or exhibit reduced fitness relative to their parental species, selection should push them to evolve and amplify some isolating mechanism (Liou & Price, 1994). On the other hand some authors (Barton, 2001) suppose the hybrid genotype may be in some cases as fitt or even fitter than the parental genotype.

A transect running across the hybrid zone at a certain place, displays a typical pattern of different characters called cline (Barton & Hewitt, 1985). Such characters might be either morphological or can be represented by gene frequencies of alleles typical for parental species (Szymura & Barton, 1986). Most of the phenomena referred to as hybrid zones are in fact clines maintained by balance between dispersal and selection against hybrids. Hybrid zones are usually stable in their characteristics (Barton & Hewitt, 1989). The zone can move under the effect of external intervention. This movement is oriented to the barrier inhibiting the gene flow and change of the allele frequencies can be very fast (Piálek, 1992).

Two basic models of hybrid zones dispersal independent and dispersal dependent (Barton & Hewitt, 1989) are distinguished. The first type (the ecotone model) arises when hybrids occupy some intermediate habitat between the two parental habitats (Moore, 1977), which increases their fitness. Selection leads to a stable equilibrium within each population there. Tension and mosaic hybrid zones diversify in accordance with two different ways of how selection operates there.

A typical tension zone (Key, 1968) is maintained by a balance between dispersal and endogenous selection against hybrids (Barton, 1979). Clines are narrow with sharp transitions in allele frequency in the centre. The hybrids are less fit, due to incompatibility of parental genotypes, and so the barrier against gene flow between parental populations has a genetic basis there. An example of such hybrid dysfunction

provides increased mortality of *Bombina* hybrids during embryonic and larval stage of development or decreased fertility of males (Kruuk et al., 1999). This type of zone is not maintained by a response to local environmental conditions, it can move from place to place to minimize their width. Tension zones are often trapped in areas of low population density, where they reach some local equilibrium, and then they can move by some radical events such as extinction or recolonization. The fact that the clines have similar shape and width across different transects (Szymura & Barton, 1991) document their independence upon the environment.

Great environmental heterogeneity allowed distribution of different genotypes in relation to certain habitat within the zone (Howard, 1986), which means that a barrier to gene flow is here environmentally based. Such zones were named mosaic, because the centre of the zone is created by a mosaic of genetically distant populations instead of a smooth transition from one species to another as it is typical for tension zones. The cline shape might reflect the local environments and its width is quite broad (Štefka, 2003). The zone is maintained by exogenous selection against hybrids, because both parental species are well adapted to their favored habitat. Deviations from Hardy-Weinberg equilibrium refer to assortative mating between adults of each species (Kruuk, 1997), which might increase the probability of reinforcement (Cain et al., 1999).

1.3. Hybridizing in *Bombina*

Both European *Bombina* species are highly suitable for the studies of hybridization and related events because while they are morfologicaly, anatomicaly, ecologicaly and etologicaly sufficiently separated from each other, their genetical closeness still enables their crossbreeding (Havelková, 1999).

The European fire-bellied toads, *Bombina bombina* and *B. variegata* hybridize in narrow, stable zone maintained by selection and dispersal (Szymura, 1993). Strong selection against hybrids is generated by hybrid dysfunction (Szymura & Barton, 1986)

and also by environment-dependant selection against toads in the mistaken habitat. Although the genetic structure of the zone is dependent on environmental complexity related to topography, diagnostic traits differentiating the species, such as morphology, allozymes or mating calls, change in a parallel fashion. Selection against hybrids acts on a large number of loci (approx. 55), which are more or less evenly spread throughout the genome (Szymura & Barton, 1986; 1991).

Szymura (1993) describes three types of hybrid zoners in *Bombina*:

1) tension zone, found in few localities in Poland (Szymura & Barton, 1991), South Bohemia (Horák, 1997; Havelková, 1999) and Croatia (Szymura, 1993; MacCallum, 1994)

2) mosaic zone discovered in Novohradské Hory (Štefka 2000; 2003) and Jugoslavia (both described in the previous part, as is found in another animal species too)

3) residual hybrid zone, which can be derived from the two foregoing. It can be interpreted as the remnant of the ancetstral hybrid zone, where the central populations were destroyed during the habitat destruction (Havelková, 1999). No crossbreeding occurs here, yet still there are signs of formerly ongoing hybridization within the marginal populations. Both populations correspond to the Hardy-Weinberg equlibrium and there was found no F_1 hybrid.

Adaptations to permanent and ephemeral breeding habitats, respectively, have shaped numerous phenotypic differences between the taxa. In larger breeding ponds, the weak mating call of *B. variegata* males (Lörcher, 1969) may not be attractive to *B. bombina* females. The reproductive success of hybrid males may be lowered by their intermediate calls (Gemmell et al., 2004), inferiority to territorial males of *B. bombina* or depressed sperm motility due to depleted energy production.

Today, a narrow hybrid zone runs for 3-4000 km across central Europe and the Carpathian foothills, wherever the areas of both species meet. This separating hybrid zone has a width of 6-20 km. Hybrid zones lies in altitudes of 110-400m

(Szymura, 1988; Gollmann, 1984; Horák, 1997; Štefka, 2003). The gene flow is limited for the area of the hybrid and the gene introgression reaches from the hybridization center to the naximum distance 260km into the territory of *B. bombina* and 280km into the territory of *B. variegata* (Szymura & Barton, 1991).

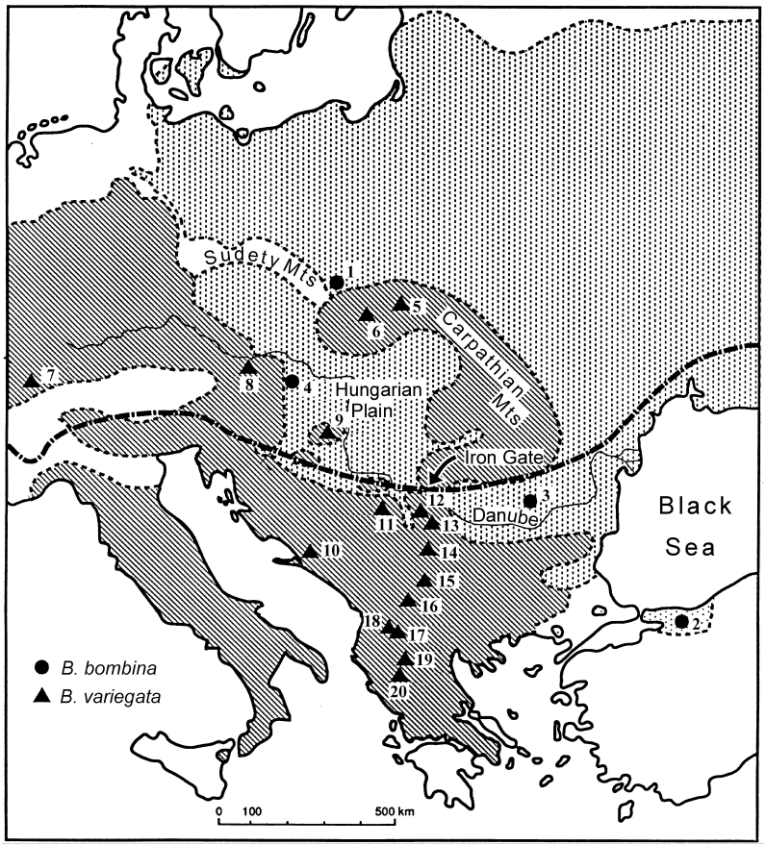

Fig. 1. Distribution of *B. bombina* and *B. variegata* in Europe and location of samples. The thick broken line indicates the southern margin of permafrost during the last glacial maximum. Taken from Szymura et al. (2000).

The first *Bombina* hybrid zone in the Czech Republic was described by Piálek (1992) in Oderské Vrchy. Horák (1997) detected a hybrid zone in southern Bohemia in the Vltava river basin. Havelková (1999 and 2002) made a detailed genetical survey of this zone, which resembles Polish transects within its narrow width (6, 71 km). But since two of the central populations exhibit deviations from Hardy-Weinberg equilibrium and a few F1 hybrids were found, it is possible that some mosaic zone

mechanism could be involved in part. A neighbouring transect running from Malše river to the Novohradské mountains was described using morphological methods (Štefka, 2000; 2003).

1.4. Previously used methods

Increased morphological variability was associated with the interspecific hybridization of the fire-bellied toads. However, morphologicaly distinct individuals were found even in places far away from the hybrid zones (Maděj, 1967). The existence of habitually abnormal specimen has been expalined as a demonstration of ongoing or historically present hybridization (Maděj, 1966), as a result of microevolution including reversed mutation (Sturgen, 1980) or a clinal variability (Sturgen, 1980). From the historical point of view, the examination of both species were paralelly oriented on the morfological studies and later on biochemical ones (Gollmann, 1984; Piálek, 1992; Szymura, 1983; Havelková, 1999).

The quantitative methods of classification of both species and their transient forms were first used in the study of *Bombina* distribution in Poland (Michalowski, 1958). The confirmation of the hybrid ancestry of *Bombina* populations from the points with common occurence were made with the genetic structure analysis using the biochemical methods (Szymura, 1977).

1.4.1. Morphometry

The analysis of the quantitative traits with previous genetical diagnostics has not yet been published. Selection of the morphological marks then included traits that would enable the comparison of published procedures in fire-bellied toads determination, accomplish detailed analysis of quantitative traits in both species and exploit another, originally designed morphological signs in species determination. 16 quantitative traits

can be used (Piálek, 1992). They are as follows: body weight (W), body length (SVL), head width (HW), head length (HL), snout length (SEL), the distance from the edge of the head to the edge of nostrils (SNL), eye diameter (ED), nostrils distance (ND), eye-lid distance (ELW), thigh length (FeL), shin length (CrL), tarsus length (TaL), pedal length (MtL), the amount of ventral and dorsal spots.

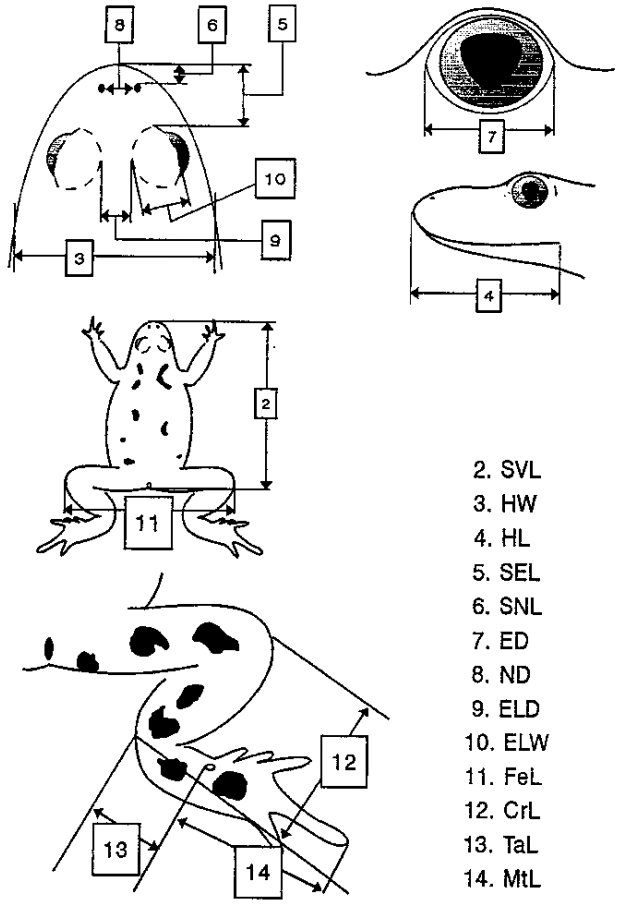

Fig. 2. Measurable quantitative traits of a *Bombina* frog. Taken from Piálek (1992).

1.4.2. Morphology

The traits that are defined in discrete categories can be considered qualitative traits. In the case of *Bombina* toads the body pigmentation is primarily used. „The ventral spot" is a yellow to orange red, sharply bounded site on a grey-black to black background on the ventral side of the body including the limbs. „The dorsal spot" can vary

from a green, olive green, dark-grey to grey-black coloured site on a lighter background of the back, while the borderline between the back and the side is specified with the presence of the papillary nodulation (the sides are smooth).

The connection of the ventral spots has a value of 1 and there are 18 such connections possible. There are 18 further qualitative traits to be distinguished, such as colour of the ventral spots, dorsal body side coloration, the roughness of the pike on the papillary nodulation, white dots on the belly and sides, and so on. The ventral spots can be used for identification during recapturing according to their uniqueness.

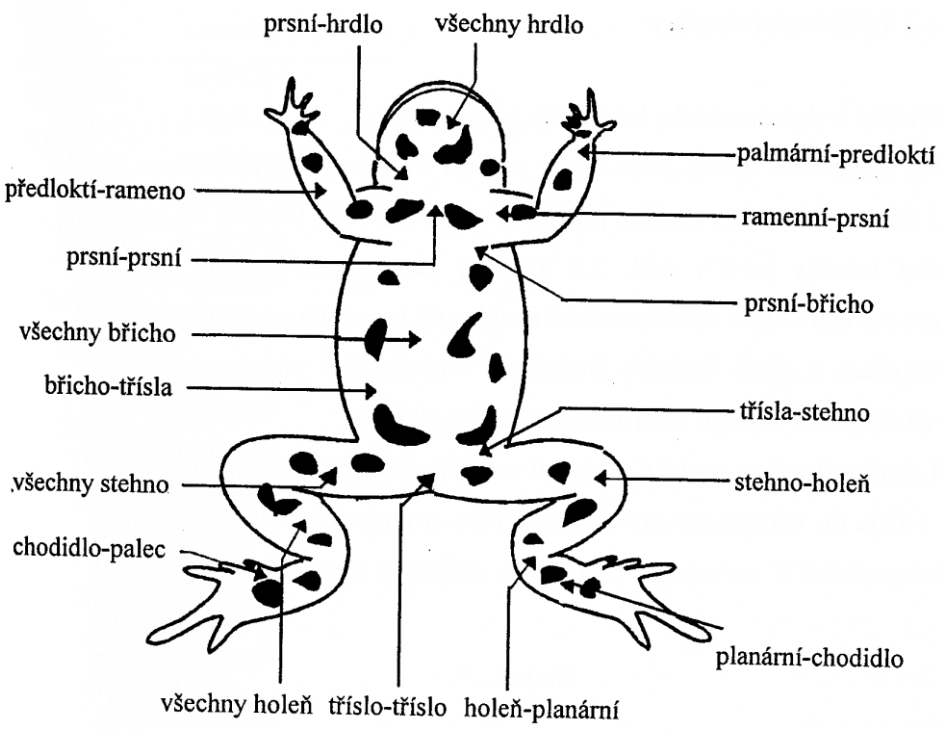

Fig. 3. Ventral spots for contructing the hybrid index, here all the spots with a value 0. Taken from Horák (1997, after Piálek, 1992).

1.4.3. Allozymes

The presumptions for the enzymatic electrophoretic analysis are as follows: 1. the uneven migratory electromorphs of one locus are products of different alleles; 2. they are codominant to each other; and 3. they are not involved in interaction with some other

allele that would modify their effect (Barton & Gale, 1993). The diagnostic locus is a locus with alternatively fixed alleles within *B. bombina* a *B. variegata*. The enzymes used for the electrophoretic assignment of the species classification fulfilled the common characteristics of the allozymes. It is also possible to detect them from the muscle tissue (Gollmann, 1984).

According to Piálek (1992), the electrophoretic analysis of 6 enzymes differing with the species distinct migrality in the direct-current electric field showed the presence of a narrow hybrid zone between the fire-bellied toads with nearly exclusive representation of hybrids. Further study of 29 proteins in two populations of *B.bombina* and *B.variegata* in Poland, coded with 39 loci, another 34 distinct electromorphs within 16 loci were discovered (Szymura, 1983).

1.5. New methods

Hybrid zones are ideally suited for the genetic dissection of phenotypic traits. They involve closely related taxa that have diverged by natural selection, and so can inform us directly about the role of genes of major effect in species divergence. The nuclear genome of the genus *Bombina* (sensu strictu) consists of 12 chromosome pairs, all of which are metacentric or submetacentric (Morescalchi, 1965). *B. variegata* genome is about 12% larger than that of *B. bombina* (Olmo et al., 1982). With an average of about 1010 bp, *Bombina* genome size is among the largest of 228 species of Anura and greatly exceeds those estimated for other members of the sister families *Alytidae* and *Bombinatoridae* (Duellmann & Trueb, 1994). Observations on chiasma frequencies in *B. variegata* suggest a total map length of 22.56 Morgans in males and 30 Morgans in females (Morescalchi, 1965).

The requirements for the QTL (quantitative trait loci) mapping, all that is needed are hybrid individuals that vary with respect to the traits of interest (Rieseberg & Buerkle, 2002).

1.5.1. Mitochondrial DNA

Mitochondrial DNA (mtDNA) is a model molecule in evolutionary, systematic and conservation biology. Identifications of forces behind the dynamics of mtDNA variants in populations are crucial for understanding the long-term evolution of this extranuclear portion of the animal genome. Important features include a high mutation rate, a lowered effective population size due to maternal inheritance and female-mediated dispersal (Hofman & Szymura, 2007). MtDNA, due to its cytoplasmic location, is free of physical constraints and, in many organisms, introgresses more easily than nuclear loci. Maternally inherited mtDNA also provides a measure of female-mediated gene flow.

A unique opportunity for the study of mtDNA dynamics is offered by hybrid zones between genetically differentiated populations, wherein mtDNA haplotypes are found on various nuclear backgrounds across a range of habitats (Hofman & Szymura, 2007). Analysis of the genetic structure of hybrid populations and the spatial distribution patterns of separate genome segments, as well as associations between them, allows for a quantitative assessment of the forces operating within the zones (Barton & Gale, 1993).

Corrected sequence divergence between the mtDNA of *B. bombina* and *B. variegata* amounts to 8.7% (2.3% divergence in amino acids). The control region contains two repeat regions, LV1 and LV2, present in all species except for *B. bombina*, in which LV2 has been secondarily lost.

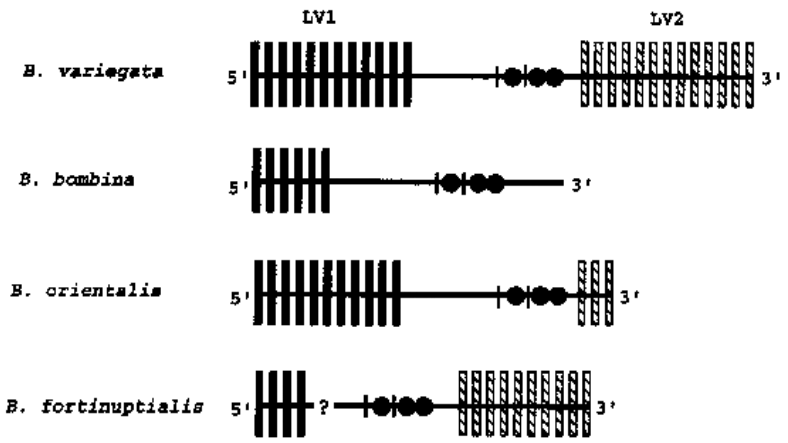

Fig. 4. A comparison of control region structure in European and East Asian Bombina species. Large vertical bars represent tandemly arranged repeat units within LV1 and LV2. Filled circles represent conserved sequence blocks (CSB); small vertical bars among the CSBs are pyrimidine rich regions (PP-1 and PP-2). Length is proportional to the number of nucleotides in each species. The question mark in B. fortinuptialis marks an unsequenced fragment after four repeats. Taken from Pabijan et al. (2008).

The rRNAs and tRNAs are characterized by low nucleotide divergence. The mtDNA phylogeny established the distribution of autapomorphic nonsynonomous substitutions in the mitogenomes of *B. bombina* and *B. variegata*. Nine out of 98 nonsynonomous substitutions led to radical amino acid replacements that may alter mitochondrial protein function. Most radical substitutions were found in ND2, ND4, or ND5, encoding mitochondrial subunits of complex I of the electron transport system (Pabijan et al., 2008). The extensive divergence between the mitogenomes of *B. bombina* and *B. variegata* is discussed in terms of its possible role in impeding gene flow in natural hybrid zones between these two species.

Postzygotic reproductive barriers between *B. bombina* and *B. variegata* have thus been attributed to substantial genetic divergence in the genomes of the toads and the genetic incompatibility (Szymura et al., 1985) that ensues, with differences in mtDNA hypothesized to contribute to the negative epistasis in recombined hybrids (Hofman & Szymura, 2007). The complete mtDNA sequences of the two hybridizing

European species, *B. bombina* and *B. variegata* were described. The gene order of their mitochondrial DNA (mtDNA) was identical to that of canonical vertebrate mtDNA.

Pabijan et al. (2008) quantify divergence between the two genomes and their functional components, including the distinctive organization of their control regions (Spolsky et al., 2006), and study the distribution of substitutions across the mitogenomes. Using a phylogeny based on four species of *Bombina* and five outgroup species, Pabijan et al. (2008) identify regions in the mtDNA of *B. bombina* and *B. variegata* carrying nonsynonymous and radical amino acid substitutions and, conversely, conservative nucleotide and amino acid domains.

aa position	Substitution	Score	Domain
B. variegata			
ATP6 64	L-P	−1	Matrix
ND2 310	P-S	−1	Inter
ND4 20	S-Q	−1	Inter
ND5 23	L-S	−2	Trans
ND5 516	S-P	−1	Inter
ND5 530	N-Y	−2	Inter
B. bombina			
ND4 445	A-M	−1	Trans
ND5 451	S-F	−1	Inter
ND5 512	P-S	−1	Inter

Fig. 5. Autopomorphic and radical amino acid (aa) substitutions in *B. variegata* and *B. bombina* and their location on the peptide chain and topology of the protein. Taken from Pabijan et al. (2008)

Initial interspecific matings within *Bombina* most likely involved *B. bombina* females and *B. variegata* males (Hofman & Szymura, 2007). This asymmetry would have also produced an excess of F_1 individuals with *B. bombina* mtDNA haplotypes onto a *B. variegata* nuclear background due to mating asymmetries and higher fecundity of *B. bombina* maternal lines if they manifested in later generations of backcross hybrids (Hofman & Szymura, 2007).

1.5.2. Microsatellites

Microsatellites, sometimes referred to as a variable number of tandem repeats or VNTRs, are short segments of DNA that have a repeated sequence (such as CACACACA), and they tend to occur in non-coding DNA. A microsatellite consists of a specific sequence of DNA bases or nucleotides which contains mono, di, tri, or tetra tandem repeats (Goldstein & Schlötterer, 1999). Microsatellites owe their variability to an increased rate of mutation compared to other neutral regions of DNA. These high rates of mutation can be explained most frequently by slipped strand mispairing (slippage) during DNA replication on a single DNA strand.

The size of the repeat unit, the number of repeats and the presence of variant repeats are all factors, as well as the frequency of transcription in the area of the DNA repeat. Interruption of microsatellites, perhaps due to mutation, can result in reduced polymorphism. However, this same mechanism can occasionally lead to incorrect amplification of microsatellites; if slippage occurs early on during PCR, microsatellites of incorrect lengths can be amplified (Beebee & Rowe, 2004). Microsatellites are typically neutral and co-dominant. They are used as molecular markers in genetics, population and other studies. It is also the only molecular marker to provide clues about which alleles are more closely related. In diploid organisms, each individual animal will have two copies of any particular microsatellite segment.

1.5.3. SSCP

The single-strand conformation polymorphism (SSCP) is a relatively cheap method, which allows DNA fragments that have been amplified with specific primers and polymerase chain reaction (PCR) to be scanned rapidly for any sequence variation. Under optimal conditions this technique can identify single base differences. The *SSCP*

technique is capable of identifying most sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides in length (Kalvatchev & Draganov, 2005).

Under nondenaturing conditions a single strand of DNA will adopt a conformation (presumably dependent on internal basepairing between short segments by foldback) that is uniquely dependent on its sequence composition. This conformation will usually be different if even a single base is changed. Usually, diluted PCR product is denatured by a brief boiling step, after which the sample is loaded on a nondenaturing "sequencing" acrylamide gel. There are four important variables that must be considered in designing an optimal *SSCP* strategy (Kalvatchev & Draganov, 2005). These are the length of the PCR fragment, the effect of temperature of the gel run, the method of PCR denaturation, and the characterization of the gel. The optimal length of a single strand seems to be 150–200 nucleotides. In this size range, 70–90% of single base substitutions are apparent on *SSCP*s.

2. Study objectives

- Elaboration of the literature search summarizing the knowledge of the modern approach to studies of the hybrid zone of the fire-bellied toads *Bombina bombina* and *Bombina variegata*
- Comparison of these methods
- Selection of a suitable one for further study of the hybrid zone lying south from České Budějovice

3. Materials and methods

3.1. Morphometry

Horák (1997) collected the specimen on previously chosen localities and narcotized them with 2% solution of the MS 222 hypnotic (Sigma-Aldrich). All individuals were released back within an hour. Only specimen bigger than 25 mm (2 years and older) were measured. The immobilized frogs were measured with a slide gauge with 0,1 mm accuracy. Horák (1997, in compliance with Piálek, 1992) used the folowing morphometric traits: body length (L), head width (Ltc), head length (Lc), femur length (F), shin length (T), tarsus length and pedal length (P).

3.2. Morphology

Captured and narcotized frogs were photographed from both ventral and dorsal side in works of Horák (1997), Havelková (1999; 2002) and Štefka (2000). Photographs of the ventral spots were then classified by Gollmann´s (1984) method. The connection of the ventral spots has a value of 1, the absence, on the other hand had a zero value. In more extensive areas (belly) the occurence of one big spot (e.g. the connection of two or more smaller ones) was additionaly evaluated as 1 and the presence of two or more spots as 0 (Horák, 1997). Altogether 18 traits can be evaluated from the ventral pigmentation (Piálek, 1992). The sum of the trait values is called a (morphological) hybrid index and has values from 0 (pure *B. bombina*) to 18 (pure *B. variegata*). Horák (1997) used the hybrid index of only 17 points, due to unusable picture of the palm spot in his photographs.

3.3. Allozymes

The electrophoreticly detectable variability of 16 supposed codominant loci was used by Piálek (1992) to determine the genetic structure of populations of *B. bombina* and *B. variegata*. Different authors use different tissue sample, most usual being the muscle from hind limb toe. Clear extracts are then subjected to starch electrophoresis (Szymura, 1995). The allozymes used by Piálek (1992) are encoded by loci of the following enzymes: lactate dehydrogenase (Ldh-1), malate dehydrogenase (Mdh-1), isocitrate dehydrogenase (Idh-1), creatine kinase (Ck), adenylate kinase (Ak), glucose-6-phosphate isomerase (Gpi) and nucleoside phosphorylase (Np). Individual electromorphs were evaluated according to their relative mobility.

3.4. Mitochondrial DNA

A single anuran hind leg toe-tip was removed by Hofman & Szymura (2007) under anesthesia in 0,2% MS 222 and stored at -75°C until DNA was extracted from tissues. Mitochondrial cytochrome *b* sequences were obtained by amplifying a fragment of 1200 bp by using primers designed on the basis of a cloned *B. variegata* mtDNA sequence.

Each of the 83 mtDNA samples was digested using five restriction enzymes that recognize specific hexanucleotide sequences. Fragment sizes were determined by comparisons with molecular size markers. The proportion of shared fragments was calculated for each pair of mtDNA haplotypes. Fragments were considered homologous or shared if they migrated the same distance in side-byside comparisons (Hofman & Szymura, 2007).

A single individual of *B. variegata* and *B. bombina* were used by Pabijan et al. (2008) as sources of mtDNA. The mtDNAs were purified as by Szymura et al. (1985; 2000), radioactively labeled and used as a probe to screen for clones containing mtDNA fragments. *Bombina* specific primers amplifying overlapping fragments spanning the entire mtDNA molecule on both strands were then designed on the basis of the cloned

B. variegata mtDNA for both PCR and sequencing in *B. bombina*. Pabijan et al. (2008) identified 13 protein coding genes by comparisons to the mtDNA genomes of *B. orientalis* and *B. fortinuptialis*.

3.5. Microsatellites

For the construction of a genomic library, high molecular weight DNA was extracted from muscle tissue of adults following the protocol in Sambrook et al (1989). Fragments between 250 and 500 bp and between 700 and 1200 bp, were used (Nürnberger et. al., 2003). Aliquots of the libraries were plated onto agar plates. Three of the 35 plates were also probed with a mixture of four trinucleotide repeats (GTC, CGA, TCC and CCA). All clones that gave a signal on this first screen were streaked out in replicate onto fresh agar plates and rescreened as before. Based on this secondary screen, 49 putative microsatellites were sequenced in both directions (Nürnberger et. al., 2003).

3.6. SSCP

Random clones over 400 bp in length were sequenced by Nürnberger et. al. (2003), and primers were designed to give PCR fragments between 200 and 340 bp (two exceptions: 168 and 157 bp). Microsatellites were separated after amplification with fluorescently labelled primers. The search for SSCPs was carried out using native 0.5mm thick horizontal polyacrylamide gels. Bands were visualised by silver staining.

3.7. Statistical methods

Qualitative traits were evaluated within individuals that geneticaly belonged to one or the other species. Both Horák (1997) and Štefka (2000) used a regression analysis.

Morphological distances for all population pairs were calculated to determine the phenetic differencies (Havelková, 1999), results being the phenetic trees constructed with the cluster method UPGMA. Various statistical methods are used by different authors and in different studies.

4. Results

4.1. Morphometry

Horák (1997) normalized the measured data by outspreading them to the total body length (L) (in 13 from 14 traits the linear dependence on the body length was proved) and so this parameter was excluded from the evaluation. For the six remaining traits Horák (1997) used the discriminant analysis. The discriminant ratios for the component traits indicates the tightness of their relation to the discriminant function and so illustrate which of the traits is the most important one for species determinig. The femur length (F) and pedal length (P) proved to possess the highest importance in differentiation between the two species. The statistical evaluation of discriminant functions indicate that there is a significant difference in the two specie´s morphometric traits.

4.2. Morphology

The number of ventral and dorsal spots is higher within the populations of *B. bombina* (Piálek, 1992). The analysis of the relationship between genetic and morphological structure is based on constucting the hybrid index reperesenting a smooth transition from *B. bombina* individuals (without the diagnostic allele of *B. variegata*) to the pure individuals of *B. variegata* (maximum of diagnostic alelles) (Havelková, 1999; 2002).

4.3. Allozymes

Piálek (1992) found out about the different migrality of electromorphs within 9 loci, 6 others monomorphic in all populations. The criterion of polymorphism of a locus was crossing the 5% frequency of the less numerous allele in the population. The electromorphs most often migrated to the anodic side of the gel and the ratio of fast electromorphs in *B. bombina* and *B. variegata* was 2:7.

Population was considered hybrid when a polymorphism in diagnostic locus were proven. Piálek (1992) detected hybrid population in three geographicaly distant places. The transition of average frequencies of the diagnostic alleles in *B. variegata* occurs in the direction from lowland to higher and bare forests.

4.4. Mitochondrial DNA

There were eleven polymorphic sites distinguished within *B. bombina* and there were seven of them within *B. variegata* (Hofman & Szymura, 2007). Sequences of the most common haplotypes were identical to those obtained from cloned *B. variegata* mtDNA and amplicons from purified DNA.

All mtDNAs examined by Szymura et al. (2000) had three shared fragments. These shared fragments indicated both that 9.5% of the fragments were conserved and that all 83 mtDNA samples in both species were ultimately derived from a single female ancestor.

Within the genus, the *B. variegata* mtDNA genome was the largest, at 18,551 bp, followed by *B. maxima* (Boulenger, 1905) (17,575 bp), *B. orientalis* (17,173 bp) and *B. bombina* (17,154 bp). The size of the control region varies nearly twofold in *Bombina*, consisting of 3072 bp in *B. variegata*, 2373 bp in *B. orientalis*, 1990 bp in *B.maxima* and 1675 bp in *B. bombina*. Uncorrected nucleotide divergence between the hybridizing *B. bombina* and *B. variegata* was lowest at 8.1% or 8.7%, the two East Asian species *B. orientalis* and *B. maxima* at 14.3%. The rRNA and tRNA genes were least

diverged, implying functional constraint. The most divergent mtDNA of the four species compared by Szymura et al. (2000) was that of *B. maxima* that belongs to the group of large-bodied *Bombina* species of Southeast Asia.

The large-bodied species bear a diploid chromosome complement of 2N = 28, while the small-bodied species contain 2N = 24 (Szymura & Passakas-Szymczak, 1988). *B. orientalis* is phylogenetically closer to the Western Palearctic Bombina species than to its East Asian relatives. The average nucleotide divergence in mtDNA between the two hybridizing species, *B. bombina* and *B. variegata*, was 8.1 (Szymura et al., 2000).

Despite ongoing hybridization in spatially and temporally variable hybrid zones, Hofman et al. (2007) have found no evidence for either past or present mtDNA introgression between European *Bombina*, which is consistent with the idea that later-generation hybrids are less fitt because of cytonuclear incompatibility leading to the disruption of mitochondrial function (Burton et al., 2006). There are ample differences between the mtDNA genomes of *B. bombina* and *B. variegata* that may have functional consequences affecting mitochondrial oxidative phosphorylation in hybrids.

4.5. Microsatellites

Among the 49 putative microsatellite clones, there were 41 true positives that contained either a CA (40 cases) or a CGA (1 case) repeat pattern (Nürnberger et al., 2003). Of the 40 isolated CA microsatellites, 19 were compound and typically had a large total number of repeats. Among the compound loci, 17 featured combinations of CA and TA repeats only and showed perfect dinucleotide periodicity (e.g. $(AT)_n(AC)_m$ rather than $(AT)_n(CA)_m$, Bull et al., 1999). Variant interspersed dinucleotides (e.g. a CT embedded in a run of $(CA)_n$) that are indicative of point mutations were frequently observed. The TA and CA repeats typically formed interleaved patterns.

Nürnberger et al. (2003) screened recombinant plasmids with an average insert size of 300 bp. Our 40 microsatellites thus correspond to a density of one CA microsatellite every 1300 kb.

4.6. SSCP

Primers for SSCPs were designed from 22 clones. A distinct pair of SSCP bands with very similar electrophoretic mobility that produced a Mendelian ratio of segregants in the F2 cross was treated as a single locus. Bands of the same mobility in both taxa on denaturing gels were excised, reamplified and then separated on SSCP gels (Nürnberger et al., 2003). Given diagnostic differences and normal segregation, the fragments were treated as allelic to each other.

In all cases studied by Nürnberger et al. (2003), multiple products of a given primer pair showed clear signs of homology, and most of them could be aligned over their entire length. SSCP polymorphisms differed by as few as two substitutions from each other.

5. Discussion

5.1. Hybridizing in *Bombina*

Piálek (1992) collected data from 2682 findings, in 1789 cases *B. bombina* was determined, in 810 *B. variegata* and in 83 cases the hybrid individuals were observed. Under the influence of hybridization in contact places it is possible to find all combinations of parental species.

The size of inhabited water site is usually said to be differing between *B. bombina* and *B. variegata*, does not agree with the real situation (Piálek, 1992). During the spring season the fire-bellied toads are often observed in small puddles in undeflooding meadows surounding the ponds. The yellow-bellied toads in Croatia, on the other hand, inhabit

large basins typical for the other specie. The results of Piálek´s work (1992) confirm that there is no relation of the phenotypic variability on the geographical origin. In the Czech and Slovak republics the more numerous species is *B. bombina*.

In *B. variegata*, electrophoretic subgroups correspond to subspecific categories. The Balkan, western and Carpathian groups also have characteristic mtDNA haplotypes, but the relationships among these groups inferred from mtDNA and electrophoretic comparisons disagree (Szymura et al., 2000).

Allozyme electrophoresis is a standard and almost exclusive way used to study *Bombina* hybridization (Štefka, 2003). No *Bombina* population monomorphic within a GPI *bombina* allele was found either in the south Bohemia or in the south Moravia (Piálek, 1992). The GPI polymorphism of *B. bombina* populations could be explained by introgression of *B. variegata* alleles into these populations within the south Bohemia (Havelková, 2002), but not within the south Moravia, where the species occur too far from the areas of hybridization. Piálek (1992) found there a few pure *bombina* populations polymorphic within GPI too, that could imply that it is *bombina* allele displaying as a *variegata* one.

The electrophoteric analysis discovered two hybrid zones in Moravia (Piálek, 1992) Both are distinguished by intogressive hybridization and except for one locus they have the same character. Almost no gene flow appears there. Piálek (1992) also established five alternatively fixed diagnostic loci and proved that most of the morphometric traits showed the same variability in both species.

Clear geographical trends in heterozygosity within *Bombina* were discovered in a study of allozyme variation (Szymura, 1988; 1993). Northern populations of both species of *Bombina* are less variable than are southern populations, probably reflecting loss of alleles as the two species expanded following the last glaciation (Szymura et al., 2000).

Generation time in *Bombina* is three years, the absolute age of the contact is 17 000 years. Palaeontological, electrophoretic and immunological evidence suggests that *B. bombina* and *B. variegata* diverged 2.5–6.8 million years ago (Ma) (Szymura, 1983). Taking an average divergence between mtDNAs of *B. bombina* and *B. variegata* as 7.0%, it appears that mtDNA in *Bombina* evolved at a rate of 1–2.8% per million years, a rate comparable to that of mammalian mtDNA (Wilson et al., 1985).

Microsatellites appear to be rare in the *Bombina* genome. The *Bombina* screen produced a large number of small CA repeats, and positive controls were reliably detected. It appears therefore that the density of CA microsatellites in *Bombina* is an order of magnitude lower than in birds (one per 136 kb; Primmer et al., 1997), which in turn have a much lower density than humans (1 per 30 kb; Beckmann & Weber, 1992). $(CA)_n$ repeats in vertebrates (Neff & Gross, 2001) show a decline in microsatellite density with increasing genome size. The flanking regions of a number of isolated microsatellites in *Bombina* appeared to be mildly repetitive, which precluded the design of primers. The predominance of TA/CA compound microsatellites in *Bombina* is striking. In *Bombina* most TA–CA microsatellites have a TA motif at the 5′end (Nürnberger et al., 2003).

SSCPs from randomly cloned DNA fragments should in principle provide a limitless supply of diagnostic single-nucleotide polymorphisms. All but one of these loci also amplified equally well in both taxa. However, most of these sequences exist in multiple copies that are sufficiently recent so that a pair of specific primers (20–25 bp in length) amplifies several to many of them and allele identification becomes impossible (Nürnberger et al., 2003). Duplications made this way are part of the process that caused the unusually large genome size in *Bombina*. In the case of coding DNA, the combination of duplication and either subfunction divergence or alternate gene silencing might be responsible for some degree of genetic incompatibility in the hybrids (Lynch & Force, 2000). Randomly cloned and presumably noncoding DNA is not an efficient source of codominant markers in *Bombina*.

5.2. Hybrid zones

The premise is that studying the hybrid zones can help understanding the mechanisms of speciation. The only source of doubts in species classification with *Bombina* is the fact that they are producing fertile hybrids (Piálek, 1992). According to Mayr (1963) the forms with vicariation but without reproduction barriers should be classified as subspecies Pracht (1987) argues that with regard to the hybridization between both species the speciation wasn´t finished yet and proposes convertion to the semispecies (populations with gene flow but limited). There are some arguments against this opinion, for instance that the value of gene flow calculated in the work of Piálek (1992) excludes the possibility of merging of both *Bombina* species due to a gene flow.

Parapatrically distributed forms remain distinct because they are adapted to different environments, or because hybrids between them are less fit. However, both the direct evidence of the close concordance of different characters leads us to believe that the latter is more likely, and that most hybrid zones are in fact “tension zones”.

The “TR_M” transect detected by Štefka (2003) in South Bohemia fits better the mosaic model than the tension one within all selected features. The environment of a mosaic zone provides both parental habitats close to each other, so that selection against hybrid genotype may perform on the habitats. The genetic character of “TR_M” places it to the mosaic type too, that contrasts with the up to now opinion that the zone in south bohemia is purely tension (Piálek, 1992; Horák, 1997).

5.3. Comparisons of methods

Hofman & Szymura (2007) analysed the variation at (mtDNA) and six allozyme loci in two transects across a hybrid zone between the fire-bellied toads *B. bombina* and *B. variegata* in southern Poland. The mtDNA cline was narrower than allozyme clines in one transect and shifted to the *B. bombina* side in both. Cytonuclear associations were

weak. Narrowed mtDNA clines could be a by- product of female demography and lowered effective population size of mtDNA.

MtDNA can cross species boundaries more easily than nuclear genes both because it is not directly linked to genes that are involved in reproductive isolation and because its cytoplasmic location allows free recombination from its nuclear background (Barton & Jones, 1983).

Alternatively, restricted mtDNA introgression is a likely result of negative epistasis in recombinants argumented by environment-dependent selection because divergence of *Bombina* mitochondrial and nuclear genomes is large. The shift of the mtDNA cline, contrary to expectations from initial mating preferences and fecundity differences between the species, suggests that on the *B. bombina* side of the zone hybrid females with *B. variegata* mtDNA have a higher chance of leaving progeny (Hofman & Szymura, 2007).

Occasionally, sampled migrant males would not contribute their mtDNA to the progeny. Patterns at uniparental sex markers, such as mtDNA or mammalian Y chromosome, may be modified by their lower population size and unequal dispersal of the sexes. The mtDNA cline shift towards the *B. bombina* side implies higher dispersal of females carrying the *B. variegata* haplotypes. Narrowed clines may point to stronger selection on a particular segment of the genome. The information content in compact mitogenome is extremely high. Recently, the neutrality of mtDNA has been repeatedly questioned (Hofman & Szymura, 2007).

Hybrid females with a *B. variegata* haplotype in *B. bombina* – like habitat would thus have higher fitness than hybrid males, displacing the mtDNA cline to the *B. bombina* side as observed in Polish transects by Hofman & Szymura (2007). Also heterozygotes were more likely to carry *B. bombina* haplotypes. Experimental data are needed to separate female dynamics and dispersal from the possibility of selection on mtDNA.

The *B. bombina* haplotype was restricted to the north or north-east of the transect in southern Poland (Hofman & Szymura, 2007), whereas the *B. variegata* haplotype was present in the south or south-west, as expected from toad morphology and allozymes (Szymura & Barton, 1986; 1991).

Significant proportions of mtDNA haplotypes of both species were revealed at the centre of the zone studied by Hofman & Szymura (2007). However, in most of the central samples, frequencies of *B. variegata* haplotypes exceeded frequencies of *B. variegata* allozyme alleles. This asymmetry is also evident on the *B. variegata* side of the zone, where no *B. bombina* mtDNA haplotypes were detected in samples with significant frequencies of *B. bombina* allozymes. By contrast, on the *B. bombina* side, *B. variegata* haplotypes were occasionally found on *B. bombina* nuclear background.

Despite extensive hybridization, no introgression of mtDNA has taken place outside of the narrow zones (Szymura et al., 2000; Hofman & Szymura, 2007). Clines in mtDNA are no wider, and are sometimes narrower, than clines at multiple unlinked allozyme loci and morphological traits (Hofman & Szymura 2007).

The most important within the morphometric traits for the species distinction turned out to be the length of femur and the pedal length (Horák, 1997). This result differs from the previous studies (Piálek, 1992). However, that result confirmed the fact that *B. variegata* vary from *B. bombina* with increased length of the hind limbs, that should have higher adaptive value considering its more terrestrial way of life.

The dependence of the hybrid index and frequncy of the V allele (*B. variegata*) is obvious from the regression line comparing morphologic and allozyme results, which verify once again the correctness of using the ventral pigmentation of *Bombina* toads as a reliable traits in the species detrmination (Havelková, 1999).

Broad interspecific correlation among morphology, allozymes and mtDNA types in European fire-bellied toads argues that, despite continuous hybridization (interrupted

perhaps during Pleistocene glacial maxima), little or no mtDNA introgression between the species has occurred outside the narrow hybrid zones that separate these parapatric species (Szymura et al., 2000).

6. Conclusions

- I worked out the elaboration of literature summarizing the knowledge of approaches how the hybrid zone of the fire-bellied toads *Bombina bombina* and *B. variegata* was studied
- I compared old and new methods, all of which have its advantages and disadvantages
- For further study of the hybrid zone lying south from České Budějovice I would like to use analysis of microsatellites or mtDNA because of their accuracy and frequent using with other authors, the disadvantage are the financial costs
- Because the analysis of mtDNA gives narrower clines, I would also like to calculate a morphological hybrid index to compare and eliminate the by- product of female demography and lowered effective population size of mtDNA

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