How large are genetic resources in *Bombina*: Is hybridisation a threat to survival of the fire-bellied toad *Bombina bombina* ?

Jacek M. Szymura

Some history

Over one hundred years ago BOULENGER (1886) showed that there was not one but two species of the fire-bellied toads in Europe. His historical paper, embellished with a fine colour plate portraying *B.bombina* and *B.variegata* side by side, provided an excellent description of these toads. Boulenger pointed out many differences between the two forms, the differences which today we interpret as adaptations to either a lowland or montane environment, or better still to breeding in large and permanent waters (*B.bombina*) or in small, often temporary pools (*B.variegata*).

Darwin's theory of evolution had already appeared on stage, but few of its ramifications were worked out yet. There was no ecology, ethology, genetics or molecular biology. Today, over 100 years later, we have all the benefits that a mature science of biology with a strong theoretical foundations can offer, high-tech equipment for both laboratory and field studies, but sadly we seem to be losing objects of our interest at an astounding rate.

Threats to survival

Decline in numbers

A decline in amphibian populations has been observed worldwide (WAKE, 1991; BEEBEE, 1992) and caused a great concern. The papers presented in this conference provide an alarming report on the status of the fire-bellied toad, *B.bombina* in Germany. Obviously rare species are more likely to go extinct than common ones. Nowadays however, even the common species seem to be threatened because their ranges shrink dramatically and their abundance falls. For instance acid rain or environmental pollution affect large areas, and therefore the whole species' range can be eroded at the same time.

Genetic swamping

Decreasing range and small population size when coupled with hybridisation can lead to extinction of a species by dilution in a more abundant one. The species posing such threat need not even to be the sister species, as hybridisation can also take place between less closely related species.

This threat is particularly acute for endemic species. Their isolation from other congeners is often based on ecological separation rather than on behavioral mating barriers. A dramatic example of such case is the introduction of sheepshead pupfish *Cyprinodon variegatus* into a river inhabited by endemic *C.pecosensis* (ECHELLE and CONNOR, 1989). Only four years after the introduction, the local species have been diluted by the invader, even though initially the introduced form had been less numerous. Other lessons can be learnt from large carnivores: the coyote (*Canis latrans*), the red wolf (*Canis rufus*) and the grey wolf (*C.lupus*) [WAYNE and JENKS, 1991; LEHMAN et al. 1991]. Mitochondrial DNA of coyote was found in retreating populations of the wolf, suggesting that a smaller, more tolerant to presence of man coyote is replacing the bigger and more discriminating species.

Can such situation happen with the fire-bellied toad, *B.bombina* ? This species is indeed retreating in some areas, definitely in the north-west of its distribution, in Germany and Denmark. The extent of population decline in central and eastern Europe is unfortunately unknown.

Comparative aspects

Although we try to focus our attention on one particular species, *B.bombina*, it is illuminating to compare the fire-bellied toad with its closest relative, *B.variegata*. From a biological point of view *B.bombina* and B.variegata are very different organisms, each with unique adaptations to its respective environment (Table 1).

A comparative approach to a study of closely related species is particularly rewarding (HARVEY and PAGLE, 1991). Not only does it help us to appreciate diversity within such a group, but also brings to light uniqueness of each taxon. Moreover, the knowledge of the relationships among the taxa (phylogeny) allows us to learn about how particular traits have evolved. In which respect do the species differ, and why they do so? What are the ecological correlations of the traits? What are the „constant" characteristics by which one group of species differs from other groups etc. The comparisons give also practical guidance to important areas of further research, namely, what information do we need to answer the above questions?

Comparative aspects have no doubt a practical use in conservation. Why should one species disappear faster then other? - is an example of a very basic and seemingly simple question. Nevertheless such question is not an easy one to answer since it requires a comprehensive knowledge of biology of the species. By „the biology of a species" I mean all aspects of the species existence in nature. For the fire-bellied toad, this will include: detailed distribution maps, information on ecology (type of habitat - spawning and wintering sites, abundace in local populations, population dynamics, food), development (fecundity, developmental rate, metamorphosis, age of maturity etc), behaviour (annual and daily patterns of activity, movements, details on mating behaviour). This list is by no means exhaustive. A closer inspection reveals that knowledge of B.bombina biology is far from being satisfactory.

I hope that this report will stimulate the participants to carry out researach in the appropriate areas. Most important at the moment seem mapping projects and identification of causes of population decline. Future studies should also identify life stages subject to greatest mortality. Such research should not be limited to threatened areas only, but also include thriving populations, since the two groups of populations exhibit completely different populations schedules and mortality patterns.

Genetic differentiation in B.bombina and B.variegata

Molecular studies in Bombina

No two fire-bellied toads look alike. The spotting pattern of the dorsal and ventral sides are extremely variable and so unique that it allows easy identification of individuals in the field. Since phenotypes are a product of both genes and environment, it is extremely difficult to assess the extent of genetic variation in natural populations from studies of spotting pattern or other morphological traits. In contrast many molecular techniques such as allozyme electrophoresis, use of restriction enzymes for DNA analysis or DNA sequencing can provide reliable estimates of variation at the genic level.

Studies of allozyme and mitochondrial DNA variation for instance show that in comparison with *B.variegata, B.bombina* is less differentiated geographically and its populations seem to be more similar to each other (Fig. 1, 2). Moreover, southern populations of this species are more variable than northern ones; they possess unique alleles (Fig. 3) and also mtDNA types not present in northern populations. Populations of *B.bombina* can be divided into two broad categories: the southern group - comprising of populations occurring along the Danube and on the Hungarian Plain, and the northern group with populations inhabiting lowlands north of the Carpathian Mts (Fig. 4). Vast expanses of *B.bombina* range in the Ukraine and Russia are too poorly known. Figure 4 shows also that geographically variable populations of *B.variegata* can be divided into four goups.

Molecular studies demonstrate clearly that populations of *B.bombina* in the northern part of its range are impoverished in variation of allozymes (Fig. 3). This pattern also applies to *B.variegata*, and can be explained by post-glacial range expansion from southern refuges located in areas rich in variation. Colonisation of Central Europe by *B.bombina* proceeded along two routes. One group of *B.bombina* moved along the Danube occupying the Hungarian Plain and Bohemia, the other colonised European Plains moving first northwards east of the Carpathins Mts and then to the east and west (ARNTZEN, 1978; SZYMURA, 1988, 1993).

Can we use this information in conservation? Without doubt variation is necessary for a population to respond to a changing environment. One might therefore think then that in order to save northern declining populations of the fire-bellied toad „a new blood" should be added. That is, populations should be supplemented with imports from the south, the Danube Delta populations for example, or reintroduction programmes should use *Bombina*

Tabela 1. Differences between *B.bombina* and *B.variegata*

Measurement	*B.bombina*	*B.variegata*	Reference
Distribution	Lowlands of eastern and central Europe	Mountainous and hilly regions of western and southern Europe and the Carpathian Mts	1,2
Breeding sites	Large permanent waters	Temporary pools, small ponds	2
Habits	Largely aquatic	More terrestrial	2
Skin thickness	134.5 μm	296.6 μm	3
(epidermis/dermis)	(22.8/111.7) μm	(65.2/231.4) μm	
Breeding behaviour	Prolonged breeder, territorial	Explosive breeder, non-territorial	4,5
Rate of calls	22 min^{-1}	95 min^{-1}	6
Call duration	210 ms	160 ms	6
Fundamental frequency	530 Hz	580 Hz	6
Sound pressure at 20 cm, 18°C	10.5 dyn cm^{-2}	1.8 dyn cm^{-2}	6
Vocal sacs	Present	Absent	7
Lung volume in 4.5 cm toad	3.0 - 3.5 cm^3	2.0 - 2.2 cm^3	6
Mean fecundity	363 eggs	116 eggs	8
(larges cluches observed)	(509, 547, 689)	(204, 233, 294)	
No. of eggs per clump	32	17	8
(range)	(9 - 76)	(4 - 58)	
Egg size	1.4 mm	1.9 mm	8
Development time 20°C, egg to toadlet	73 - 75 days	61 - 63 days	8
DNA content per nucleus	18.8 pg	21.1 pg	9
Chromosome no. (identical karyotypes)	24	24	10,11
Nei's D, 29 loci	0.37 - 0.59		12
Albumin distance	2 - 4 IDU		13
mtDNA sequence divergence	5.6 - 7.0%		14
No. of genes under selection in Polish hybrid zones (range)	55 (26 - 88)		15

1. Andrén et al. 1984; 2. Arntzen 1978; 3. Madej 1973; 4. Czopkowa and Czopek 1955; 5. Lörcher, 1968; 7. Boulenger 1886; 8. Rafińska 1991; 9. Olmo et al. 1982; 10. Moreschalchi 1965; 11. Wickbom 1949; 12. Szymura 1983, 1988; 13. Maxson and Szymura 1984; 14. Szymura et al. 1985, unpublished; 15. Szymura and Barton 1991.

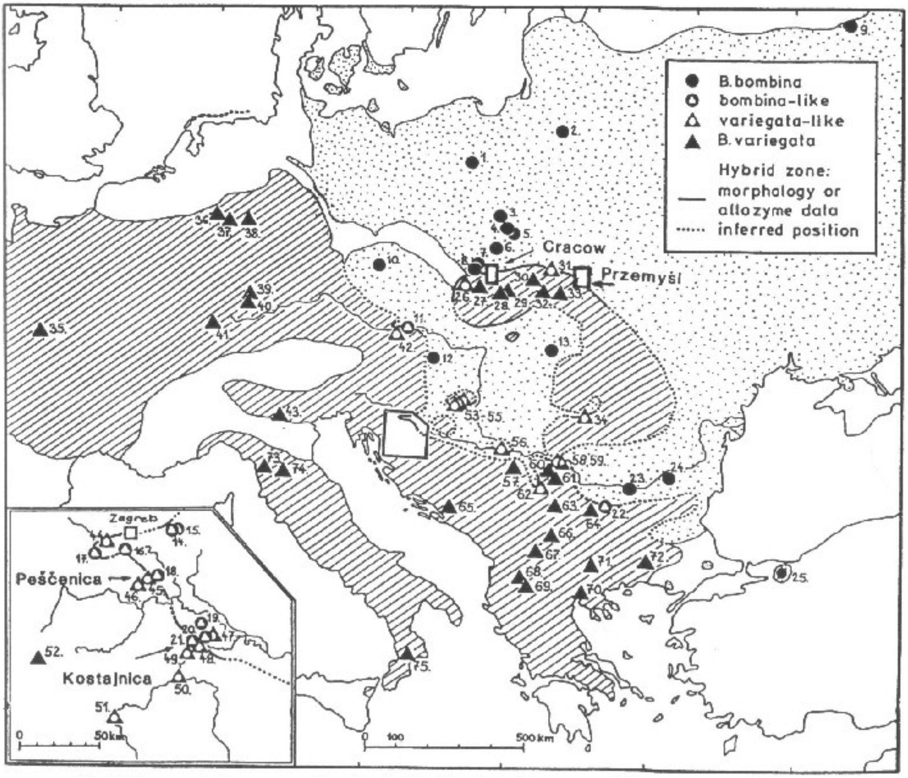


Fig. 1. Geographic ranges of the fire-bellied toads in Europe and location of samples of *Bombina bombina* (circles) and *B. variegata* (triangles). Hybrid populations are indicated by a circle with a triangle (*B.bombina*-like population) or a triangle with a circle (*B.variegata*-like population). A dotted line shows the location of hybrid zones in central Europe. A solid line indicates regions where hybridisation was detected either by earlier morphological studies or by recent electrophoretic analysis. Position of transects across the hybrid zone in Poland and Croatia are indicated by arrows.

of such origin. This is, for several reasons, a completely mistaken idea.
Molecular markers, are but a tiny proportion of the total genome, and may not be representative of polygenic traits and most likely do not influence fitness very much. Regretably, we have no idea how much variation is there in genes responsible for quantitative traits which do affect fitness and thus are of interest to conservation programmes. For instance Polish populations of *B.bombina* are monomorphic at a number of allozyme loci (SZYMURA, 1976, 1988) but are very unlikely to be monomorphic for loci affecting fitness.
Populations possess adaptations to local environmet which have been evolving over long periods of time. For instance *B.bombina* reached the Danish Islands and southern Sweden through a land connection which existed untill 7,000

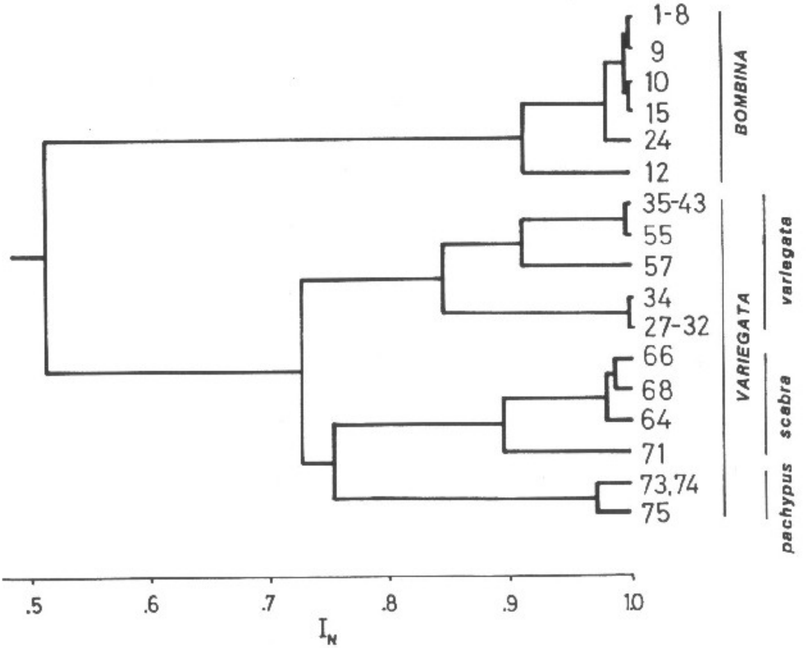


Fig. 2. UPGMA phenogram showing similarities among 38 populations of B.bombina and B.variegata. The phenogram is constructed from Nei`s genetic identities based on nine enzyme loci (Aat-1, Ak, Ck, Gpi, Idh-1, Ldh-1, Ldh-2, Mdh-1, Mdh-2).

years ago, so this species must have colonised Poland and Germany at some earier date. It means that after a fast post glacial range expansion, populations became established in Germany have been adapting to local conditions for thousands of generations. And for this very reason it is wrong to introduce *Bombina* from distant locations. Such practice may even have a negative effect on local populations by introducing genes which disrupt particular local adaptations.

Another danger is an introduction of new pathogenes: parasites or disease with a devastating effect.

Unfortunately we are completely ignorant about geographic variation in the life history of *Bombina* such as growth rate, maturation time, fecundity, or developmental time. All these traits have genetic components most likely with combinations of alleles varying from population to population. For instance individuals of *B.bombina* from southern populations, seem to have smaller size then individuals from northern ones. They may also mature earlier due to a longer growing season, which in turn can affect population dynamics.

Information provided by molecular markes is extreemely useful in tracing origin of populations to broad geographic catergories. More refined techniques, such as DNA sequences or single locus probes, will reveal more geographic structuring, and should help to identify origin of individuals.

Fig. 3a. Allele frequencies at Ldh-1 locus in populations of *B.bombina*. Ldh-1/s - white section of pie diagrams, Ldh-1/f - black sections of pie diagrams.

Fig. 3b. Allele frequencies at Ck locus in populations of *B.bombina*. Ck/s - white section of pie diagrams, Ck/f - black sections of pie diagrams.

Hybridisation between *B.bombina* and *B.variegata*

Earlier work has shown that there does not seem to be any reproductive isolation between *B.bombina* and *B.variegata,* hybrids are viable and fertile (MICHAŁOWSKI and MADEJ, 1969; SZYMURA 1976; GOLLMANN, 1984). Recombination between their divergent genomes takes place easily. Mating seems to be random in the field, at least in some populations (areas) in which ranges of *B.bombina* and *B.variegata* meet (SZYMURA, 1993). Hybrid zones, i.e. areas where genetically distinct groups of individuals meet and mate, resulting in at least some offspring of mixed ancestry (BARTON and HEWITT, 1989; HARRISON, 1990) are common and separate the two parapatric species in Central Europe, so at least a potential threat of genetic swamping which hybridisation can bring about does exist.

Let's examine this danger in detail. When two species meet and begin to hybridise then there are several theoretical possibilities of the outcome (Fig. 5)

1. One species may replace the other if it is superior in a given habitat. This also happens when F1 hybrids are sterile; a more common species will replace the rarer one. (Biological pest control programmes whereby sterilized individuals are released relay on this principle). We would expect that a narrow band of hybrid populations moves in space if this situation applies to *Bombina*. Indeed hybrid zone movements, as biogeographic arguments show, were involved in restricting *B.variegata* range to higher elevations on the Hungarian plains in the past when *B.bombina* invaded this area along the Danube from the east.

2. Hybrid zone at first may widen, if fitness of hybrids is not reduced. The speed of this process is dependent on dispersal capabilities of individuals. The further they disperse, the faster the zone expands. With time differences between species became obliterated and a hybrid swarm forms. So as the zone widens and introgression increases the species should become progressively less distinct and more difficult to tell apart.

3. Hybridization may also lead to a formation of a stable hybrid zone or to evolution of reproductive isolation. Here, none of the hybridising species is lost, though in the latter case hybrid populations will eventually disappear.

The knowledge of the dynamics of a hybrid zone is crucial in order to distinguish between the above possibilities. For simplicity I shall discuss so called „clinal" hybrid zone only, since it is best known. The complications found in other types of hybrid zones can be easily incorporated in a more general model. For a comprehensive anlysis of hybrid zones, the reader should consult BARTON and GALE (1993) and SZYMURA (1993).

A hybrid zone that separates *B.bombina* and *B.variegata* in southern Poland is one of the best known examples of such zones. Since *B.bombina* and *B.variegata* are distinct species (Table 1) they offer a large number of characters whose pattern of change can be analysed in a transect across the zone. The characters anlysed in several detailed studies comprised two groups: molecular traits

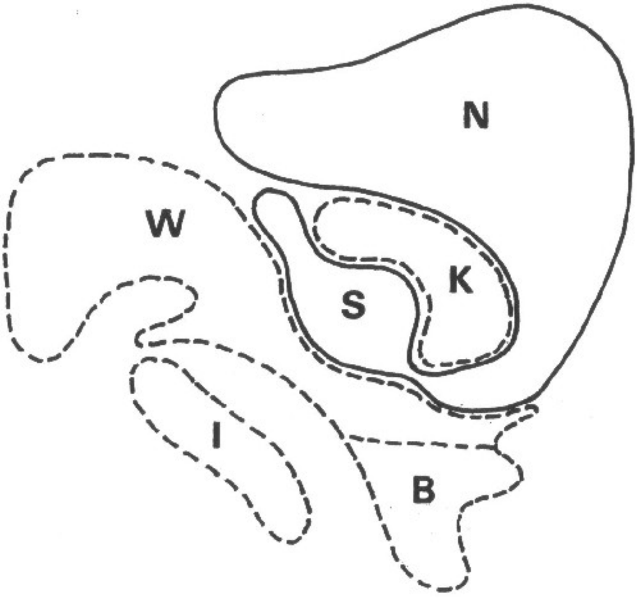


Fig. 4. Subdivision of *Bombina* based on allozyme and mitochondrial DNA variation. N and S = northern and southern groups of *B.bombina*. *B.variegata*: I=italian group (*B.v.pachypus*); B=Balkan group (*B.v.scabra*); K and W = Carpathian and western groups, respectively (*B.v.variegata*).

on one hand, and morphological as well as behavioral traits on the other.

Variation at the molecular level has a simple genetic basis. It can be encoded either in the nucleus (6 independently segregating allozyme loci have been studied in *Bombina*) or in the cytoplasm (sequence variation of mitochondrial DNA). Because the variation at the molecular level is most likely neutral or nearly so, the analysis of genotypic distributions in populations provides insight into processes that take place in populations. Thus type of matings, viability of individuals or extent of selection can be inferred from population structure.

Contrary to the molecules, morphological and behavioral characters have a complex, usually polygenic inheritance and are most likely to have selective values due to either pleiotropic effects of single loci or genetic correlations between loci. For instance differences in the spotting pattern between *B.bombina* and *B.variegata* are associated with other traits such as skin thickness, which have adaptive significance in different habitats occupied by the species. Moreover, spotting pattern itself plays an important role in a distinctive warning response, known as „Unken reflex", triggered by a potential predator. Thus the spotting itself has a great survival value. Mating calls play important role in either mate recognition or territoriality, and therefore are implicated in mating patterns. Therefore comparison of the two types of characters (or their underlying genetic components) can provide information on interaction bet-

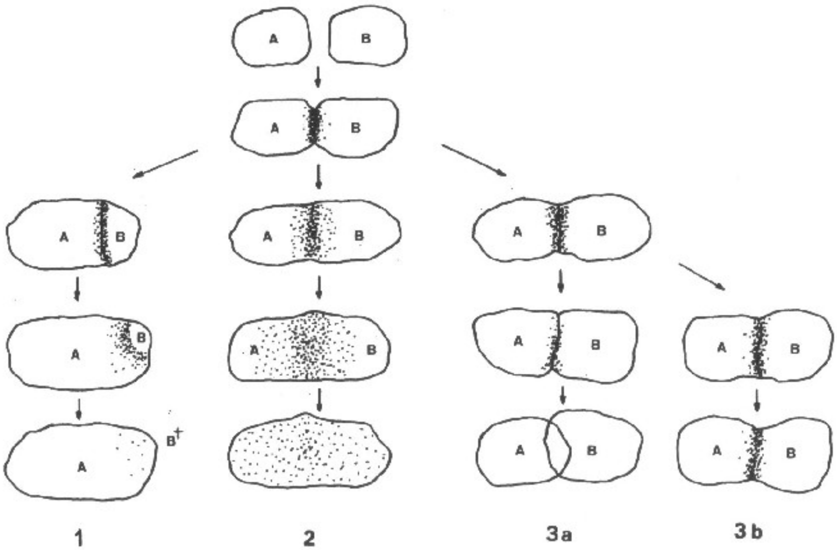


Fig. 5. Outcomes of secondary contact between two genetically differentiated populations A and B. 1. Hybrid zones moves and population A replaces B; 2. Hybrid zone widens and a hybrid population is formed; 3a. Hybrid zone disappears after evolution of isolating mechanisms and the two species can now live in sympatry; 3b. Stable, long lasting hybrid zone forms.

ween two divergent genomes of *B.bombina* and *B.variegata*.
Luckily, historical data also exist on the Polish hybrid zones. Distribution of the fire-bellied toads and their morphology was studied near Przemysl about half a century ago by HORBULEWICZ (1933) and later near Kraków, by MICHAŁOWSKI (1958) in mid-fifties. Therefore contemporary variation pattern can be compared with that of the earlier days.

Patterns of change across a hybrid zone

Allozyme studies show that pure populations are separated by populations composed almost exlusively of hybrids; these are not F1's but further generations hybrids and backcrosses. Mating within hybrid populations seems to be random. There is an excess of parental combination of gametes, most likely due to migration between populations (Fig. 6-8).
Another interesting observation is that independent characters show in fact

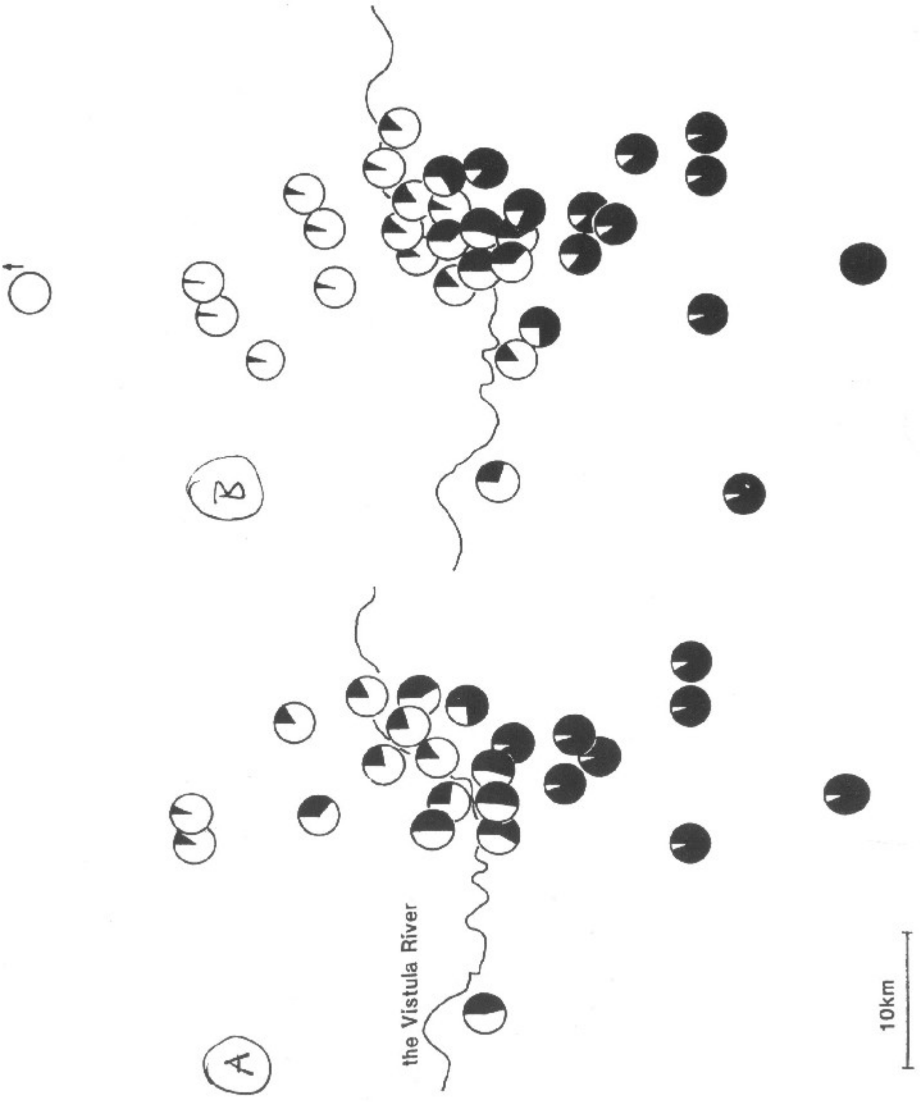


Fig. 6. Morphological and allozyme variation across a hybrid zone near Cracow. A. Average frequency of the *B.variegata* morph averaged over seven diagnostic morphological charakters (black sections of pie diagrams). B. Average frequency of the *B.variegata* alleles (black section of pie diagrams) for five diagnostic enzyme loci (Ak, Ck, Gpi, Ldh-1, Mdh-1).

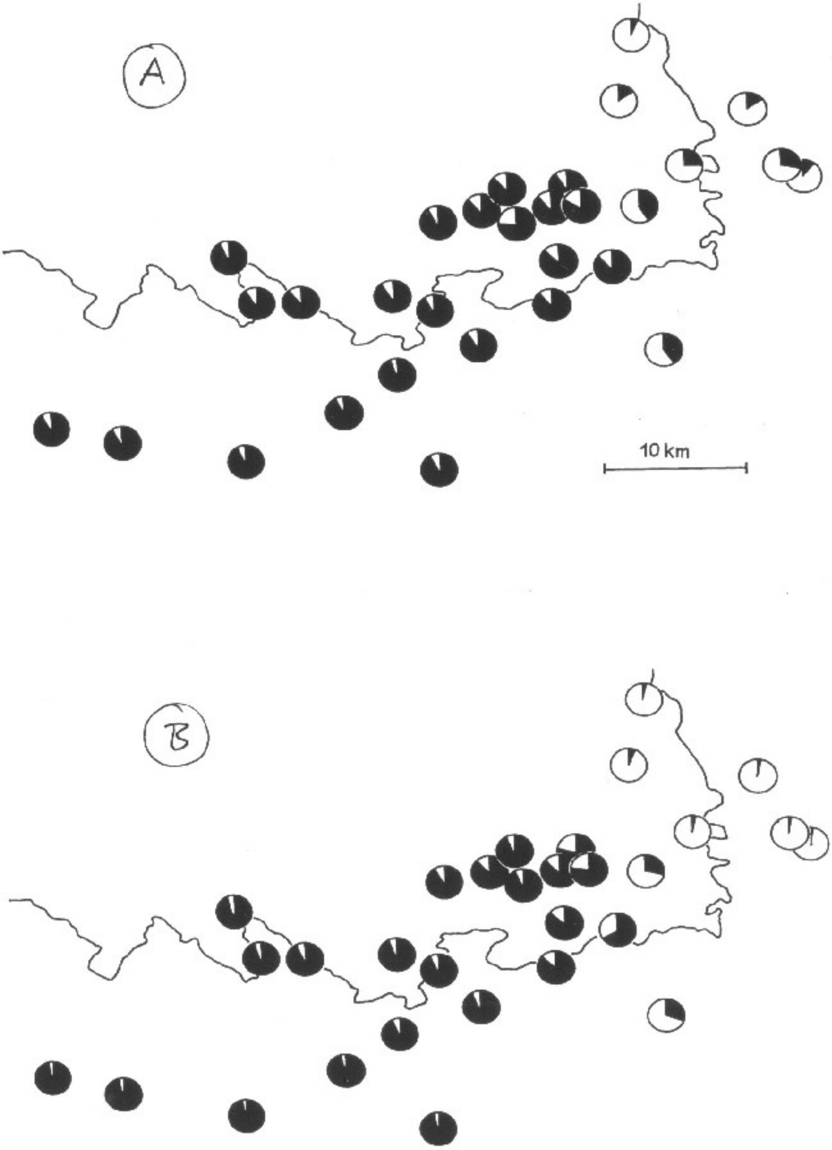


Fig. 7. Morphological and allozyme variation across a hybrid zone near Przemysl.
(See Fig. 5 for explanation.)

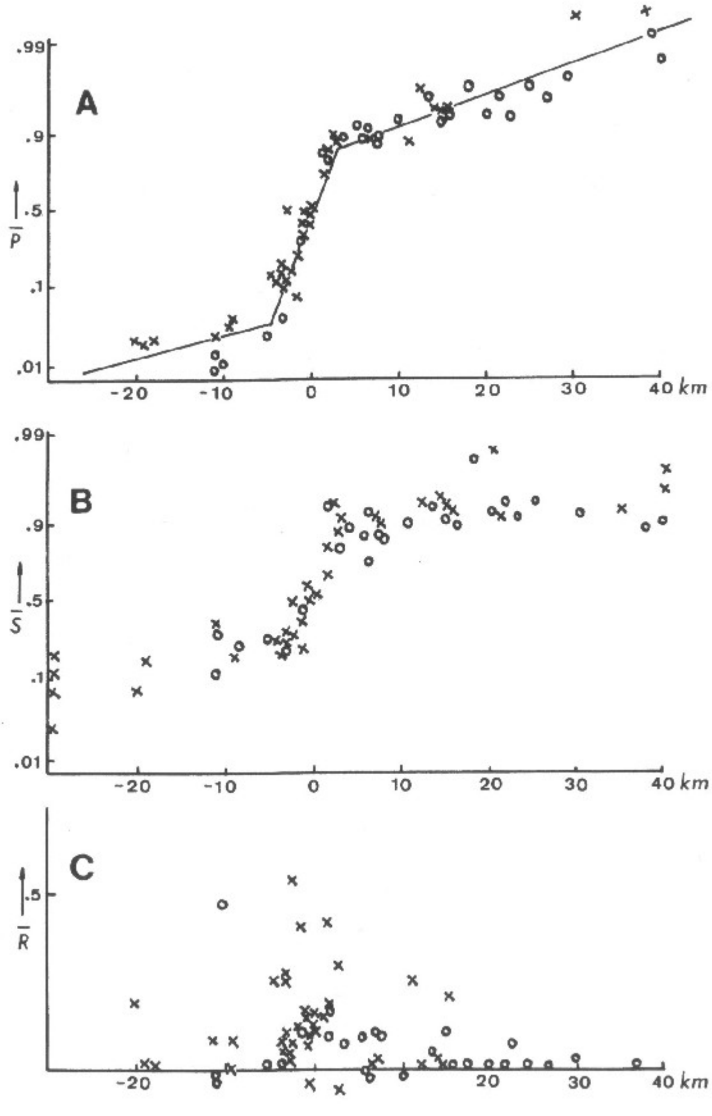


Fig. 8. Comparision of the transects near Cracow and Przemysl.

A. Average frequency of the *B. variegata* alleles (p) for five diagnostic enzyme loci (Ak, Ck, Gpi, Ldh-1, Mdh-1) in relation to the hybrid zone centre (0).

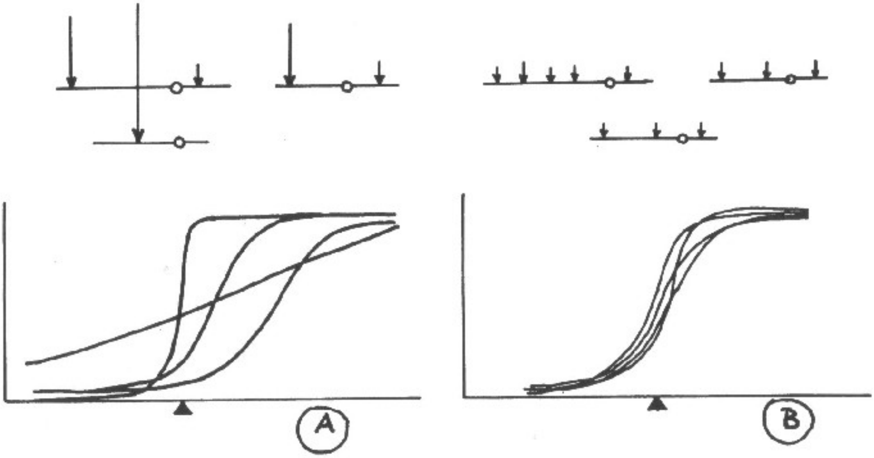
B. Average frequency of the *B. variegata* morph (s) averaged over seven diagnostic morphological characters.

C. Average measure of non-random allelic combinations (R) in hybrid populations. Crosses = Cracow transect; open circles = Przemysl transect.

the same pattern of change. For example six unlinked allozyme markers show parallel and concordant variation in the zone. Moreover, the change observed at the allozyme loci is very similar to patterns seen for mtDNA types, as well as morphological and behavioral traits (mating call) characteristic of *B.bombina* and *B.variegata*. Rather than a complicated patchwork of genotypes, variation across a hybrid zone can be summarized as a smooth clinal change within a narrow central strip only 6 km wide. There is no a priori reason why such unrelated characters should behave in the same way. One would rather expect that separate genes respond to environmental gradient differently and each showing a locus specific pattern (Fig. 9). This is not the case in the Polish transects. Persistence of a narrow hybrid zone (see below) can be explained by a ballance between two opposite forces: selection against hybrids and migration into the zone. Selection against hybrids however does not act on single genes, which would produce staggered clines of different width, but on larger blocks of genes, most likely removing recombinant genotypes.

The assumption in this model is that genes within each species are co-adapted in a separate and unique way, and that upon hybridisation this co-adaptation is disrupted. Genes cannot function well in a foreign genetic environment what results in lowered fitness. This explantion is supported by the fact that there is indeed evidence of increased embryo mortality in Cracow hybrid zone, and individuals also suffer from various morphological abnormalities. Break-down of co-adaptation is expected to be more dramatic with increasing divergence between hybridising species. By biological standards, *B.bombina* and *B.variegata* are very different species (Table 1). The two species differ in any respect, and each can be characterised by a separte suite of features with

Fig. 9. Two models of selection and corresponding cline shapes. Bars represent chromosomes, while selection strength on a particular chromosome segment is indicated by the size of an arrow. A. Varying selection strength produces shifted clines of different with; B. Uniform selection scattered over all chromosomes produces concordant and coincident clines.



adaptive values in lowland or montane environments.

This explanation is further strengthened by the fact that patterns of change are consistent in two Polish transects, near Cracow and near Przemyœl. Both zones are narrow, about 6 km wide, and are bordered by long tails of introgression (Fig. 6-8).

Narrow hybrid zones can be maintained in a variety of ways. In the simplest case, clinal variation results from two opposite forces: selection against hybrids and dispersal into the zone. Application of this model to the *Bombina* hybrid zone allowed quantitative estimates of strength of selection against hybrids, dispersal rates or number of genes under selection (SZYMURA and BARTON, 1991).

Hybrid zones do not exist in a neutral environment, and although they are not located at prominent ecotones, they neverthelss are positioned on a transition from lowlands to mountains. Many physical and ecological parameters change within zone either gradually or form a complicated mosaic. Both on a large geographic scale as well as on a local scale, distribution of the fire-bellied toads, is often related to a type of breeding ponds location of which reflects environmental complexity. Such pattern argues for a significant environmental component determining position, width and genetic structure of the hybrid zone. Juxtaposed lowland and mountain environments may also select for either „*bombina*" or „*variegata*" gene complexes. Enviromental heterogeneity within the transition allows mosaique distribution of the two types to persist due to habitat preferences. However, if the environment complexity within a hybrid zone is low, the mosaique distribution of genotypes colapses into a smooth cline. The position and shape of the cline may eventually reach an equilibrium. This seems to be the case in the Polish trasects.

Position of hybrid zone on a transition between lowlands and the foothills of the Carpthians is expected from ecological requirements of *B.bombina* and *B.variegata*. Comparisons with historic morphological data of HORBULEWICZ (1933) and MICHAŁOWSKI (1958) suggest that the zone, at least in the most recent times, has not changed either position or width (Fig. 10), and it can be argued that it had been established some 8000 years ago (~3000 generations) [SZYMURA and BARTON 1986].

We find no evidence of either large scale replacement of one species by the other, or noticeable widening of the zone in Poland. The zone seem to maintain both its position and width. There is nevertheless introgression of allozyme markers detectable up to 50km from the centre of the zone. Hybrid zones are not absolute barriers to gene flow, they are „semipermeable", some genes can get through them.

How fast is such a process?

This process is slow for neutral alleles, and takes thousands of generations. It can be much faster for advantageous alleles, but it still takes hundreds of

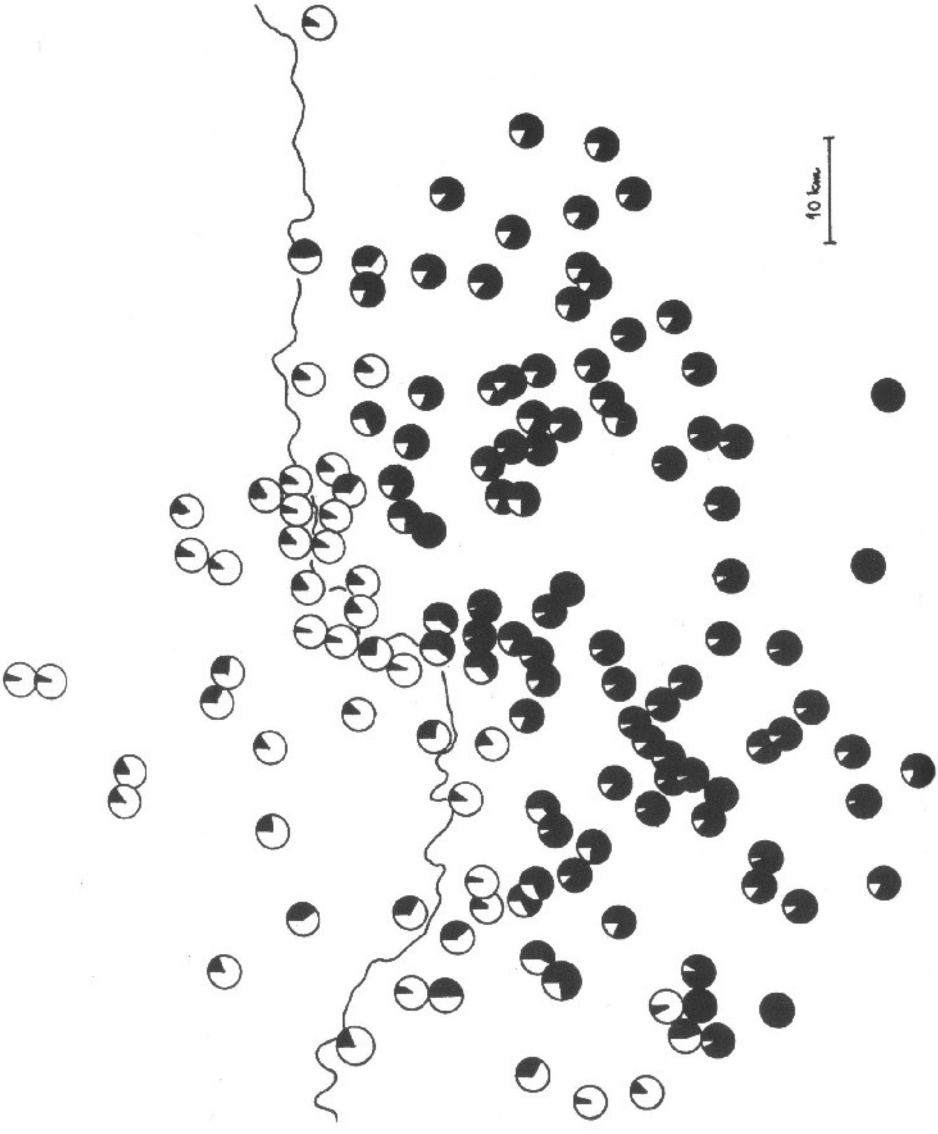


Fig. 10. Morphological variation in a hybrid zone near Cracow based on rescored samples of MICHAŁOWSKI (1958) (SZYMURA, unpubl.). Black sections of pie diagrams show frequency of the *B.variiegata* morph and averaged over seven diagnostic morphological characters. Samples from Fig. 6a are overlayed onto MICHAŁOWSKI samples.

generations (cf. SZYMURA and BARTON, 1986). From the conservation point of view introgression can be ignored unless the two species occur in close proximity, that is within their dispersal distances. One should add that in some areas, where one species forms isolates within the range of the other, or pockets, such populations could be significantly introgressed. For instance populations of *B.variegata*, which occur on small isolated mountain ranges scattered across the Hungarian Plain (Fig. 1), or *B.bombina* near Zagreb show admixture of the other species alleles (SZYMURA, 1988). Studies of Günther Gollmann (this symposium) showed that south of the Carpathians hybrid zone can be more complex than in Poland, more mosaic, with populations introgressed to various extent.

Complexity of environment, and perhaps mating preferences, may also limit extent of introgression. Hybrid zones separating *B.bombina* and *B.variegata* present in complex environments, like the one near Kostajnica on the border between Croatia and Bosnia, depart from the clinal type described above, and seem to be more mosaic with „pure" types of either species occurring side by side (SZYMURA, 1993).

Conclusions

Conclusion is that *B. bombina* is not directly threatened by hybridisation though some local populations close to the *B.variegata* range could be in fact introgressed. Historical, large scale analysis of variation suggest that it was *B.bombina* which displaced *B.variegata* from lowlands, i.e. Danubian Plains and penetrated into *B.variegata* range along river valleys, like for instance near Zagreb, or locally in Poland. Despite widespread and long standing hybridisation the two species do maintain their distinctness through a variety of mechanisms. The analysis clearly shows that *Bombina* do not have problems with their genes, and the retreat of *B.bombina* in Germany is connected with unfavourable environmental changes brought about by human activities.

Conservation projects should focus on preserving local populations, especially those at he present distributional limit. Resettlements programmes, should be the last resort, and *must* use animals from the nearest locality. Adding „new blood has no scientific ustification, and may cause serious damage to local populations.

Fortunately, *B.bombina*, like many other amphibian species, due to their relatively high fecundity (Table 1), can demostrate a high rate of opulation increase. Providing there are suitable habitats, this species need not be doomed to extinction and future generations will enjoy the melacholic calls of the fire-bellied toads.

However, one should not assume a racist attitude to preserve only „pure" populations of either species, but take a more comprehensive view. Hybrid zones wherever they exist should be protected too, as they are naturl heritage, and very interesting natural laboratories (HEWITT, 1988) where one can try to understand interactions of divergent genomes. This of course can only be achieved by protecting habitats or whole regions, a sort of evolutionary national parks, or bio-diversity parks.

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Anschrift des Verfassers:
Jacek M. Szymura
Department of Comparative Anatomy, Jagiellonian University,
Ingardena 6, PL-30-060 Kraków