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Allozyme variation in Czech populations of the invasive spiny-cheek crayfish *Orconectes limosus* (Cambaridae)

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ABSTRACT

Key-words:
Orconectes limosus, *allozymes, genetic variation, Czech Republic*

The North American spiny-cheek crayfish, *Orconectes limosus*, was most probably introduced into Europe only once, in 1890. The size of the founding population was just 90 individuals. Low genetic variability resulting from a bottleneck effect during introduction might therefore be supposed in European spiny-cheek crayfish populations. On the other hand, the fast spread of *O. limosus* in Europe and colonisation of various habitats suggest that this species does not suffer from inbreeding depression due to an introduction bottleneck. We analysed 14 *O. limosus* populations from the Czech Republic using allozyme electrophoresis to evaluate the level of intra- and among-population genetic variation. Out of eight well-scoring allozyme loci chosen for detailed analysis, six were variable in studied populations, suggesting that sufficient variability was maintained during the introduction. Genetic differentiation of Czech populations of the spiny-cheek crayfish was relatively low and did not show any clear geographic pattern, probably due to long-range translocations by humans.

RÉSUMÉ

Variation des allozymes dans les populations de l'écrevisse américaine, *Orconectes limosus* (Cambaridae), en République tchèque

Mots-clés :
Orconectes limosus, *allozymes, variation génétique, République tchèque*

L'écrevisse américaine, *Orconectes limosus*, a été très probablement introduite en Europe en une seule fois, en 1890. La taille de la population initiale était seulement de 90 individus. Suite à un goulet d'étranglement (bottleneck effect) pendant l'introduction, on pourrait s'attendre à une variabilité génétique réduite. Cependant, la dispersion rapide d'*O. limosus* en Europe et la colonisation d'habitats très variés indiquent que cette espèce ne souffre pas d'inbreeding causé par le goulet d'étranglement. Par électrophorèse des allozymes, nous avons analysé 14 populations d'*O. limosus* en République tchèque pour évaluer le taux de variation génétique intra- et inter-populationnel. Des huit loci choisis pour cette étude, six étaient variables dans les populations étudiées, suggérant qu'une variabilité suffisante a été maintenue lors de la première introduction. La différenciation génétique des populations tchèques de l'écrevisse américaine était assez basse et sans aucun pattern géographique, conséquence probable des translocations à grande distance effectuées par l'homme.

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INTRODUCTION

With the increasing number of species introduced into new territories, the need arises to understand the process of colonisation and factors influencing the distribution potential of those species. Apart from other factors (*e.g.*, adaptations for dispersal and the competitive ability of the species, the character of invaded habitats and communities, the presence or absence of predators and pathogens), the success of invasive taxa may depend on the genetic variability of introduced populations (Sakai *et al.*, 2001). High genetic variability is supposed to be advantageous in invading new areas, because in sexual species it allows more rapid evolution and adaptation to changing environmental conditions (Lambrinos, 2001; Sakai *et al.*, 2001). An example of a highly genetically diverse successful invader is the North American crayfish, *Procambarus clarkii*, in European waters (Barbaresi *et al.*, 2007). However, even organisms with very low genetic variability in their invasive populations can be excellent colonisers. In extreme cases, a single clone of a species may become a widespread invader, as is the case for the tropical alga *Caulerpa taxifolia*, invading a very large area of the North-western Mediterranean Sea (Jousson *et al.*, 1998), or the obligately parthenogenetic clone of the water flea *Daphnia* (interspecific hybrid of two North American lineages of the *D. pulicaria* complex) spreading in Africa (Mergeay *et al.*, 2006). In these cases, low genetic diversity does not obstruct the invader's spread and competition with genetically diverse indigenous species.

As mentioned above, North American crayfish are prominent among successful invasive animal groups in European continental waters. Several species have been introduced into the continent since the end of the 19th century, in attempts to replace lost native crayfish populations decimated by crayfish plague. Three of these invasive crayfishes – the signal crayfish (*Pacifastacus leniusculus*), the red swamp crayfish (*Procambarus clarkii*), and the spiny-cheek crayfish (*Orconectes limosus*) – are extremely widespread in Europe (Souty-Grosset *et al.*, 2006). They inhabit various types of habitats and successfully compete with native crayfishes; moreover, they serve as a vector of the oomycete *Aphanomyces astaci*, a pathogen of the crayfish plague (Vey *et al.*, 1983; Alderman *et al.*, 1990; Diéguez-Uribeondo and Söderhäll, 1993; Kozubíková *et al.*, 2009). Contact of infected American crayfish with native species often results in mass mortalities of the latter (Holdich, 1999; Söderhäll and Cerenius, 1999).

In our study, we focused on the genetic variation of the spiny-cheek crayfish, *Orconectes limosus* (Rafinesque, 1817). It was first introduced into Europe in 1890, when 90 individuals were released into a pond in Pomerania (currently western Poland) (McDonald, 1893; Kossakowski, 1966); this event has most probably been the only successful introduction of this species into Europe (Kossakowski, 1966). *O. limosus* has spread from the point of its original introduction to at least 20 European countries (listed in Holdich *et al.*, 2009), both naturally and through human-mediated translocations. Among those who spread non-native crayfish to both standing and running waters in Central Europe are especially anglers, owners of water bodies, and recreational scuba divers, usually being unaware of the negative impact of such activities on local ecosystems (Petrusek *et al.*, 2006).

Orconectes limosus is the most widespread non-indigenous crayfish in the Czech Republic. Its presence in the country was first confirmed in 1988 close to the border with neighbouring Germany (Hajer, 1989). However, the species was probably already present in the country in the 1960s; it most likely invaded the territory by migrating up the river Elbe from Germany (Kozák *et al.*, 2004; Petrusek *et al.*, 2006). Since the late 1980s, these crayfish have quickly spread over a large area of the Czech Republic, especially in the western part (Petrusek *et al.*, 2006). *O. limosus* can currently be found mostly in large watercourses, lower reaches of their tributaries, and in isolated standing waters such as flooded quarries, sandpits or ponds. The detailed distribution of the spiny-cheek crayfish in the Czech Republic has been reviewed by Petrusek *et al.* (2006) and Filipová *et al.* (2006). However, new scattered localities with the presence of this species are still being discovered. For example, *O. limosus* was found in 2006 in the south-west of the country in a large reservoir in the Bohemian Forest mountains (Beran and Petrusek, 2006), as well as in the north-east (Silesia), in the Prudník brook close to the border with Poland (Ďuriš and Horká, 2007; Kozubíková *et al.*, 2008).

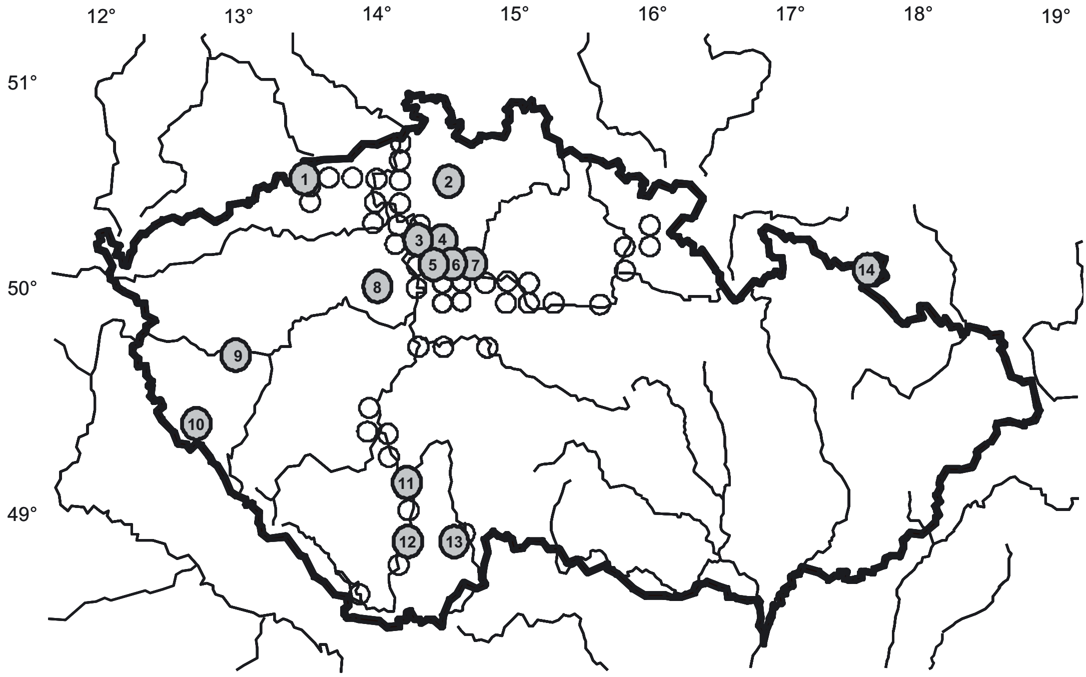

Figure 1
Distribution of O. limosus *in the Czech Republic (empty circles) and localities where individuals were sampled for the present study (grey circles with numbers, corresponding to the codes of localities in Table I).*

Figure 1
Répartition d'*O. limosus* en République tchèque (cercles vides) et sites de prélèvement des individus inclus dans cette étude (cercles gris avec les chiffres, ces chiffres correspondant aux codes des localités dans le Tableau I).

The aim of this study was to assess the level of genetic variability of selected populations of this species in the Czech Republic, using allozyme electrophoresis. We tested the hypothesis that sufficient genetic variation was maintained during the introduction of this species into Europe, so that allozyme markers can be used to analyse the genetic structure of these populations. Although the distribution of the spiny-cheek crayfish clearly suggests a large influence of long-range translocations within the Czech Republic, we wanted to test this by comparing the genetic and geographic distances of the studied populations.

MATERIALS AND METHODS

> SAMPLING

Crayfish were sampled between 2004 and 2007 from various types of localities – brooks, sandpits, lakes, reservoirs and flooded quarries (Figure 1, Table I), in parallel with a study focusing on the distribution of the crayfish plague pathogen in populations of American crayfish in the Czech Republic (Kozubíková *et al.*, 2009). Nearly all samples came from the western part of the country where this crayfish is most abundant; all these populations are assumed to have originated from the initial invasion through the Elbe (Petrusek *et al.*, 2006). An exception in our dataset was the population from the Prudník brook in Silesia, which had been colonised by individuals by upstream or downstream migration from Poland (Ďuriš and Horká, 2007).

Crayfish were mostly captured by hand or while scuba diving. After transporting in cooling boxes, individuals were stored in a deep freezer (at –80 °C). The tissue for analyses was then

Table I

List of sampled localities (codes correspond to numbers on the map in Figure 1), date of sampling and summary of population characteristics: numbers of examined individuals (n), heterozygosity expected non-biased (Hexp. n.b.) [%] and heterozygosity observed (Hobs.) [%], mean number of alleles per locus and Hardy-Weinberg exact probability.

Tableau I

Liste des localités échantillonnées (les codes correspondent aux chiffres notés dans la carte de la Figure 1), leur caractère, date de prélèvement et caractéristiques des populations : nombre d'individus examinés (*n*), hétérozygotie attendue corrigée pour le biais d'échantillonnage (Hexp. n.b.) [%] et hétérozygotie observée (Hobs.) [%], nombre moyen d'allèles par locus et probabilité de conformité à l'équilibre de Hardy-Weinberg.

Code	Name of locality	Locality character	Latitude (N)	Longitude (E)	Date of sampling	*n*	Hexp. n.b.	Hobs.	Alleles/locus	H-W exact probability
1	Záluží	retention pond	50° 33′	13° 36′	Jun. 2007	22	2.50	2.27	1.875	0.007
2	Stará pískovna (Provodín)	sandpit	50° 37′	14° 36′	13 Sep. 2004	10	2.10	1.38	1.75	0.257
3	Cítov (Horní Počaply)	sandpit	50° 21′	14° 26′	12 Oct. 2005	8	2.99	2.59	2	0.216
4	Pšovka (Lhotka)	brook	50° 23′	14° 33′	4 Jun. 2005	10	2.30	1.50	1.75	0.437
5	Kojetice	quarry	50° 14′	14° 31′	20 Jul. 2005	20	2.74	2.38	2	0.172
6	Proboštská jezera (St. Boleslav)	sandpit	50° 12′	14° 39′	3 Sep. 2005	17	2.00	2.28	1.75	0.420
7	Lhota	sandpit	50° 14′	14° 40′	Jul. 2005	35	1.90	1.41	2.25	0.162
8	Smečno	pond	50° 11′	14° 2′	9 Apr. 2006	21	2.55	2.62	1.75	0.128
9	Hracholusky (Přovany)	reservoir	49° 47′	13° 6′	25 Jun. 2006	11	2.17	1.71	1.875	0.293
10	Starý Klíčov (Mrákov)	quarry	49° 23′	12° 57′	23 Oct. 2005	20	3.19	2.88	2	0.131
11	Kořensko (Neznašov)	reservoir	49° 14′	14° 22′	26 Apr. 2004	11	2.36	2.50	1.75	0.249
12	Malše (České Budějovice)	river	48° 58′	14° 29′	12 Sep. 2005	12	1.30	1.04	1.375	0.133
13	Zlatá Stoka (Třeboň)	channel	49° 0′	14° 46′	19 Jul. 2006	14	1.33	1.25	1.625	0.007
14	Prudník (Slezské Pavlovice)	brook	50° 17′	17° 43′	27 Oct. 2006	11	2.79	1.82	1.875	0.181

dissected from legs or claws of the captured crayfish. Altogether, 222 individuals of *O. limosus* from 14 populations were analysed.

> *ALLOZYME ELECTROPHORESIS*

Horizontal cellulose acetate electrophoresis was used for the genetic analyses as described in Hebert and Beaton (1993). This was carried out in a Tris-Glycine buffer system (pH = 8.5) on 76 × 76 mm cellulose acetate plates (Titan III, Helena Laboratories). A small amount of tissue was dissected from crayfish legs or claws and homogenised with a plastic rod in about 10 μL of distilled water. The tissue of one crayfish individual was used as a standard in all analyses to assure comparable scoring. In each run, eleven animals and one standard, loaded in one row, were analysed.

Overall, seventeen enzymes were tested. Some of them did not show sufficient activity for routine screening and were therefore excluded from the analyses: α-amylase (AMY, EC 3.2.1.1), fumarate hydratase (FUM, EC 4.2.1.2), hexokinase (HEX, EC 2.7.1.1), xanthine dehydrogenase (XDH, EC 1.1.1.204), alcohol dehydrogenase (ADH, EC 1.1.1.1), α,α-trehalase (TRE, EC 3.2.1.28), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase NADP$^+$ (ME, EC 1.1.1.40) and adenylate kinase (AK, EC 2.7.4.3). Furthermore, we did not include aspartate amino transferase (AAT, EC 2.6.1.1), although it scored well, as it showed very low migration speed under the conditions used for other enzymes.

Eight enzyme loci were finally selected for further analyses: glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 5.4.2.2), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), malate dehydrogenase (two loci, MDH 1 and MDH 2, EC 1.1.1.40), arginine kinase (ARK, EC 2.7.3.3), lactate dehydrogenase (LDH, EC 1.1.1.27) and aldehyde oxidase (AO, EC 1.2.3.1). The most common allele for each locus was designated M (medium). Other alleles were labelled corresponding to their relative mobility to the M-allele: F (fast), S (slow), or S^- (very slow).

Allelic frequencies, observed and expected heterozygosities, F statistics (Weir and Cockerham, 1984), and genetic distances were calculated in Genetix 4.03 (Belkhir *et al.*, 1996). GenAlEx 6.1 (Peakall and Smouse, 2006) was used to test whether genotypic frequencies at the studied loci are consistent with Hardy-Weinberg expectations. Nei's genetic distance (Nei, 1978) was calculated to estimate levels of genetic distance between tested populations. Based on these results, a UPGMA dendrogram was created in Statistica 6.1 (StatSoft, Tulsa, USA) to depict similarity among studied populations. The relationship between genetic (Nei's) and geographic (log-transformed) distances between populations was tested by the Mantel test, comparing the respective pairwise distance matrices in the software zt (Bonnet and Van de Peer, 2002).

RESULTS

Out of eight loci used in our analyses, two enzymes (LDH and AO) showed no variability; the remaining six (GPI, PGM, MPI, MDH 1, MDH 2 and ARK) were polymorphic, *i.e.*, with more than one detected allele. However, in MDH 1 one of the two detected alleles was very rare (less than 1%). The most variable locus was PGM, with four different alleles detected; the slowest of them, S^-, was relatively rare (5.5%). In four enzymes (GPI, MDH 2, MPI and ARK), three different alleles could be distinguished (Table II).

A summary of population characteristics is shown in Table I. All 14 populations analysed in our study were polymorphic at two or more loci. The highest average number of alleles per locus was in the population from Lhota (2.25 alleles/locus), followed by populations from Cítov, Kojetice and Starý Klíčov (2 alleles/locus). The lowest average number of alleles per locus (1.38) was in the population from the Malše River.

Table II
Allele frequencies [%], number of detected alleles and mean number of alleles per population observed in eight studied loci in 222 individuals of the spiny-cheek crayfish from the Czech Republic.

Tableau II
Fréquences alléliques [%], nombre d'allèles détectés et nombre moyen d'allèles par population, observés dans huit loci chez 222 individus de l'écrevisse américaine en République tchèque.

	Allele frequency [%]				Number of	Mean number of
Locus	F	M	S	S^-	detected alleles	alleles/population
GPI	38.9	19.7	41.4		3	2.71
PGM	18.8	11.4	64.4	5.5	4	2.92
MPI	0.1	83.6	16.3		3	1.64
MDH 1		99.4	0.6		2	1.07
MDH 2	48.0	18.1	33.8		3	2.92
ARK	1.4	95.7	2.9		3	1.35
LDH		100			1	1
AO		100			1	1

The observed heterozygosity of populations was consistent with expected values in most cases (Table I), and the loci studied were in good agreement with Hardy-Weinberg expectations in most populations. However, two of the studied populations, Zlatá stoka and Záluží, exhibited significant deviations from the Hardy-Weinberg equilibrium, both showing heterozygote deficiencies.

These studied Czech spiny-cheek crayfish populations were significantly genetically structured, with a mean F_{ST} value for all loci of 0.160. Nei's genetic distance between populations varied from 0.003 (between populations from Lhota and Malše) to 0.20 (between populations from Zlatá Stoka and Pšovka). The geographically distant population from Prudník (Silesia) did not markedly differ from other populations; it was genetically closest to the population from Záluží, located more than 290 km away.

The UPGMA dendrogram (Figure 2) did not show any apparent clustering. In some cases, even very distant localities were similar, such as the pairs Kořensko and Záluží (160 km) or Lhota and Malše (147 km). The relationship between geographic and genetic distances among populations was not significant (Mantel test, $p = 0.126$); this pattern did not change if the Prudník population (Silesian region), with different colonisation history, was excluded.

Some population pairs could have been expected to be more similar to each other than the rest of the studied Czech *O. limosus* populations, as one was the source of crayfish for the other: the population in Klíčov was founded by individuals from the Hracholusky Reservoir, and the sandpit Lhota was supplied with crayfish from the sandpit Proboštská jezera. However, we did not observe any substantially higher similarity between these populations in comparison with the others.

DISCUSSION

As Dlugosch and Parker (2008) showed, for invasions where a single introduction occurred, allelic richness is generally lower in introduced populations than in native ones. Moreover, reductions in genetic diversity tend to be inversely correlated with the size of the founder population (Merilä *et al.*, 1996). Although the European populations of *O. limosus* were apparently founded just once and by a relatively small number of individuals (at least in comparison with other widespread invasive crayfish in Europe, *P. leniusculus* and *P. clarkii*), the presence of several variable enzyme loci in the studied *O. limosus* populations suggests that the bottleneck effect was not very dramatic, and sufficient variation was retained during the introduction of this species into Europe. This is also supported by its rapid spread and presence in various habitats.

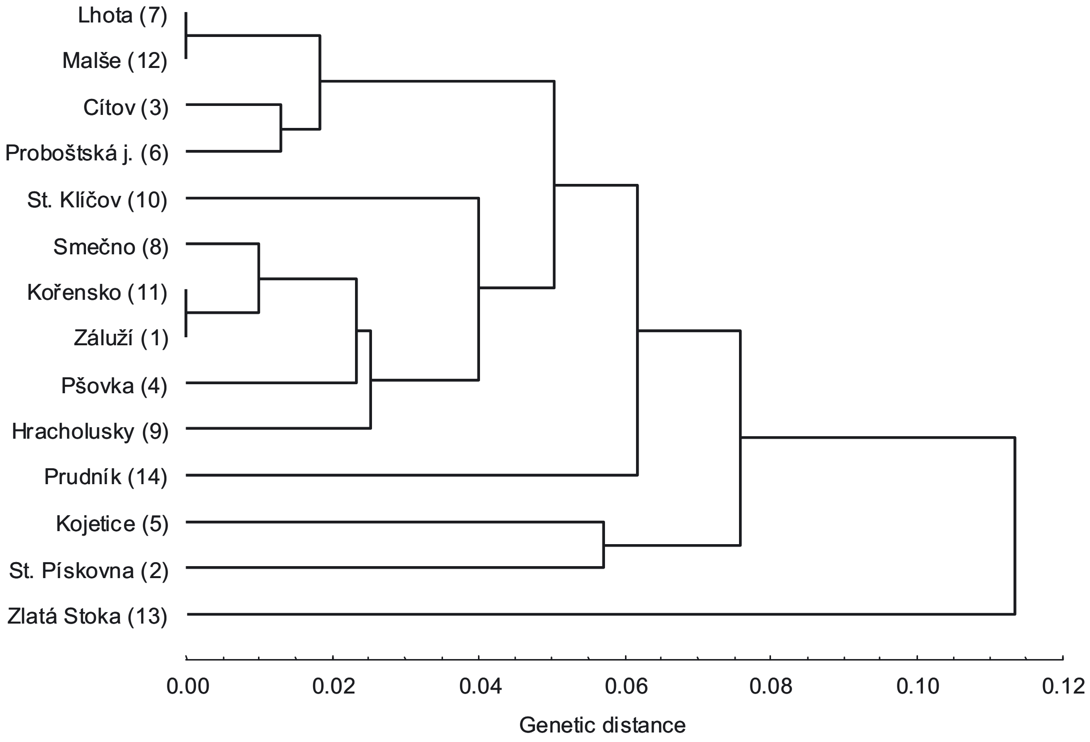

Figure 2
UPGMA dendrogram, using Nei's genetic distance (Nei, 1978), showing genetic similarity of O. limosus *populations included in our study (numbers correspond to the codes of localities in Table I).*

Figure 2
Dendrogramme UPGMA basé sur la distance génétique de Nei (Nei, 1978), montrant la similarité génétique des populations d'*O. limosus* impliquées dans notre étude (les chiffres correspondent aux codes des localités dans le Tableau I).

Nevertheless, the introduction may have resulted in some reduction in genetic variation, as the Czech spiny-cheek crayfish populations showed relatively low allozyme variation in comparison with other previously studied crayfishes such as *Astacus astacus* (Fevolden and Hessen, 1989), *Parastacoides tasmanicus* (Hansen *et al.*, 2001) and *Austropotamobius pallipes* (Lörtscher *et al.*, 1998; Largiadèr *et al.*, 2000). However, the variation we observed was still higher than in several other crayfishes: *A. leptodactylus* (Agerberg, 1990), and ten species of *Procambarus* and two species of *Cambarus* (Brown, 1981; Busack, 1989), in which an almost complete absence of variation was recorded, supposedly due to introduction bottlenecks.

Despite repeated bottlenecks during the colonisation of Czech waters by *O. limosus*, genetic variability has also been maintained in populations at the edge of the species' distribution in the country. Interestingly, the observed allozyme variation was usually higher in isolated populations (which must have been founded by men) than in those from rivers or brooks where crayfish may have dispersed naturally (Table I).

Our analyses did not show any obvious correlation between geographic distance and genetic similarity of the studied populations. This can be explained by human-mediated translocations of the spiny-cheek crayfish into the Czech Republic that influenced the genetic structure of the species. We originally supposed that the geographically distant population in the Prudník brook in Silesia might differ from the remaining Czech populations, as the individuals originated from a different region and stochastic events could have led to substantially different allele frequencies or private alleles. However, the allozyme variation in this population was similar to the others, suggesting that most alleles brought to Europe were transferred into newly established populations of the species. Nevertheless, an analysis of the mitochondrial gene for cytochrome *c* oxidase subunit I (COI) of European *O. limosus* (Filipová, 2008) showed that the Prudník population differed from the rest of the analysed Czech populations in the presence of a rare haplotype found uniquely in several individuals from this population. Our results did not prove that pairs of populations in which one was founded by individuals from the other were genetically closer to each other. This might be due to either insufficient sampling or processes that influenced allele richness and frequency at the newly colonised localities, such as founder effects during introduction or subsequent genetic drift.

Our results on allozyme variation were useful in the selection of individuals and populations for analyses of other genetic markers. While sequencing of the mitochondrial COI gene, even from crayfish carrying unusual alleles, did not reveal any variation in Czech populations (with the exception of Prudník mentioned above), selection of individuals differing at allozyme loci simplified the screening for variable microsatellite markers in this species by cross-species amplification (Hulák *et al.*, 2010). Initial testing of ten microsatellite loci in one Hungarian and two Czech populations (including the one from Starý Klíčov analysed in this study) has subsequently confirmed that populations of the spiny-cheek crayfish are indeed genetically diverse (Hulák *et al.*, 2010). In the future, these variable nuclear markers may allow more detailed population-level studies of this invasive crayfish.

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