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Notes on genetic uniformity in the fairy shrimp *Branchipus schaefferi* Fischer, 1834 (Branchiopoda, Anostraca) from Poland

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Abstract. Although large branchiopods are undoubtedly an important part of the temporary water bodies, they remain the least studied group of macroinvertebrates inhabiting freshwaters. One of these species is the fairy shrimp *Branchipus schaefferi*. Its known occurrences across the central Europe are limited almost exclusively to temporary pools on military training grounds. Thus, the species is particularly threatened by changes in military activities, especially by the decline in the number of temporary pools caused by abandoning of training grounds followed by the secondary plant succession. In the present study, we performed genetic analyses of known populations of *B. schaefferi* in military training grounds of Poland using nuclear 18S ribosomal DNA sequences. No genetic diversity was observed among nine populations from four military grounds. Further, very close phylogeographical relationships among *B. schaefferi* from distant geographic areas (Poland, Italy and Algeria) were found (genetic distance of 0.001-0.002 substitutions per site).

Key words: *Branchipus schaefferi*, freshwater crustacean, temporary water bodies, nuclear 18S rDNA, phylogeographical analysis.

Branchipus schaefferi Fischer, 1834 (Fig. 1) is a member of the freshwater crustacean order Anostraca - one of the oldest evolutionary groups of arthropods (Richter et al. 2007). Anostraca and other large branchiopod crustaceans (Notostraca, Laevicaudata and Spinicaudata) are perfectly adapted to live in temporary water bodies. However, due to the climate change and disappearance and devastation of their natural habitats, they are under threat of extinction (Belk 1998, Pyke 2005, Brendonck et al. 2008).

Recent studies on large branchiopods from the Czech Republic (Merta & Roleček 2005), Germany (Maier 1998) and Western Poland (Gołdyn et al. 2012) have shown that *B. schaefferi* could be classified as threatened (endangered) due to the decline of the number of its populations. Current range of this species is limited to patches of suitable habitats clustered in regions of appropriate land morphology - especially in military training grounds, where habitats of this species depend on the specific form of land use (e.g. Gołdyn et al. 2012), and so that such areas may act as refuges for large branchiopods (Vanschoenwinkel et al. 2013). During the last 30 years, due to changes in European geopolitical situation as well as remodeling of military strategies, many training grounds have been abandoned and their ecosystems have changed dynamically – mainly due to the secondary plant succession (Jentsch et al. 2009, Cizek et al. 2013, Zentelis & Lindenmayer 2015). This leads, among others, to a rapid decrease in the number of temporary pools in central European lowland landscape (Maier et al. 1998). This way, populations of *B. schaefferi*, already highly clustered spatially, become even more endangered.

Gołdyn & Bernard (2008) claimed that the site number of *B. schaefferi* in Poland is higher than in other European regions. In spite of this fact there is lack of information on genetic variation and relationships between populations of endangered *B. schaefferi*. We performed genetic analysis using a molecular marker applied for this species also by other authors to correctly resolve taxonomic questions, because systematics of the genus *Branchipus* has been based to date solely on the morphological taxonomy and still remains questionable (Gandolfi et al. 2015). Till now, only one molecular marker - 18S ribosomal gene has been used just for a few specimens of this species, resulting in obtaining one sequence from Algeria (Weekers et al. 2002; GenBank accession number: AJ238068.1) and two sequences from Italy (GenBank accession numbers: AJ307677.1 and AJ421827.1, deposed in 2001 by Van Damme and others). Thus, using 18S rDNA marker, we aimed at investigating genetic variation within and elucidating genetic relationships among isolated populations of *B. schaefferi* from temporary pools in military training grounds of Poland (representing the central

Figure 1. Female (left) and male (right) of *Branchipus schaefferi* (photo by J. Musiał).

European region) in relation to the known data from geographically distant areas in Algeria and Italy (Mediterranean region).

The field study was conducted between 2013 and 2015 and covered all known localities of the species recorded in Poland during surveys since 2005 (Gołdyn et al 2012; Gołdyn, pers. comm. 2016). These were military training grounds in Poznań-Biedrusko (ca 52°29'N, 16°51'E), Piła (ca 53°08'N, 16°48'E), Wrocław (ca 51°11'N, 17°05'E), and Słupsk (ca 54°26'N, 17°03'E; Fig. 2A). Moreover, samples were collected from Drawsko (ca 53°32'N, 15°48'E; Fig. 2B), military area that has not been surveyed previously. In the latter area two new localities of *B. schaefferi* were recorded.

Occurrence of active specimens of *B. schaefferi* is limited in the region of Poland to a relatively short period of the year, when temporary pools are filled by water after heavy spring-summer rains. Thus, for the purpose of our study we used specimens reared under laboratory conditions from the dormant cysts deposited in the bottom sediments of the pools. When in the field, sediment samples (ca 1 dm^3 each) were collected to plastic containers. After transporting to the laboratory, sediment samples were dried. Then, samples were placed in separate glass aquaria filled with 10 dm^3 of deionized water and stored in a temperature- and light-controlled room at 21-23°C and under 12:12 h (light : dark) photoperiod until the adulthood. Adults of *B. schaefferi* were raised from sediment collected in the active military grounds in Poznań-Biedrusko and old tank roads near this military ground (in total five pools), in the active Drawsko military ground (two pools) as well as in the inactive military grounds in Piła (one pool) and Słupsk (one pool). Neither adult nor juvenile specimens were obtained from sediment taken in the inactive military ground in Wrocław, thus that sample was excluded from this study. In total, 45 specimens from nine temporary pools (5 specimens from each) located in four military training grounds were hatched. In both the Piła military ground and the old tank road near the Poznań-Biedrusko ground, military activity is currently replaced by non-military off-road activities, thus the number of suitable puddles for *B. schaefferi* is still high. On the other hand, in the Słupsk military ground, there is a visible loss of puddles (in which this species might occur) due to environmental change resulting in the plant succession.

When adult, the specimens were anesthetized with carbonated water and dissected under a stereomicroscope. Muscular tissues of *B. schaefferi* were isolated from the thorax and the total DNA was prepared according to the protocol of the Biotrace Genomic Extraction GPB Mini Kit (GenoPlast). To amplify the complete region of the ribosomal 18S gene we used eukaryote-specific primers complementary to the 5′-terminus (5′-TYCCTGGTTGATYYTGCCAG-3′) and the 3′-terminus (5′-TGATCCTTCCGCAGGTTCACCT-3′) (Weekers et al. 2002). PCR reactions were performed in 20 µL volume containing 0.8x JumpStart *Taq* ReadyMix (1 U of JumpStart Taq DNA polymerase, 4 mM Tris-HCl, 20 mM KCl, 0.6 mM $MgCl_2$, 0.08 mM of dNTP; Sigma-Aldrich, Germany), 0.4 µM of both primers and about 100 ng of DNA. 18S rDNA gene fragment was amplified under conditions as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 52°C for 1.5 min and 72°C for 2 min and ending with 72°C for 5 min.

Blast application (Basic Local Alignment Search Tool; Altshul et al. 1990) was used to browse sequences deposited in the NCBI database for identification of sequences which are homologous with the obtained 18S rDNA gene fragment. All sequences were then aligned manually using BioEdit 5.0.9 (Hall 1999) and consensus sequences were created. Alignments were prepared in ClustalX 1.81 (Thompson et al. 1997). The DNA sequences of the complete 18S rDNA gene fragment of all tested individuals were retrieved using DnaSP v.5.10.01 software (Librado & Rozas 2009). Obtained sequence was deposited in GenBank (accession number: KU645889).

Phylogeographical analyses were performed using the MEGA software package version 6.06 (Tamura et al. 2013) – maximum likelihood analysis with the bootstrap method, Hasegawa-Kishino-Yano model parameter using 1000 replications. Genetic distances were calculated as p-distance values of Nei & Kumar (2000). Homologous sequence of the 18S rDNA gene fragment of *Streptocephalus torvicornis* (Waga, 1842) was used as an outgroup (GenBank accession number: AJ238081.1; Weekers et al. 2002).

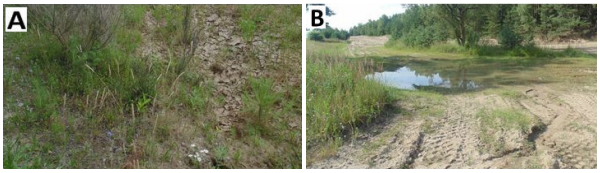

Figure 2. Examples of the sites where sediments were collected for the present study on *Branchipus schaefferi*: a dry pool with patches of terrestrial vegetation in the inactive military training ground in Słupsk (A), and an inundated temporary pool (a new locality of the species) in the active military ground in Drawsko (B).

The length of the 18S rDNA gene was 1688 base pairs (bp). No differences in the DNA sequences between the individuals from the studied populations were observed. A considerable geographic distance between the studied military grounds (from 75 km to 270 km) seems to constitute the main barrier for gene flow between the populations. On the other hand, it is well-known that dormant cysts of large branchiopods may passively disperse, being transported by wind, animals or vehicles among temporary water bodies located in the area of a given military training ground as well as between isolated military grounds (e.g. Beladjal & Mertens 2009, Vanschoenwinkel et al. 2009, 2011). However, Beladjal et al. (2007) showed that vectors of long-distance cyst dispersal do not contribute significantly to the pattern of *B. schaefferi* genetic isolation.

Phylogeographical relationships between *B. schaefferi* sequences of the 18S rDNA gene fragment from different geographic areas of the species range were relatively simple (only two variable nucleotide positions were observed) with low genetic distances (Fig. 3, Table 1). The presented distribution pattern of the studied nuclear marker most probably resulted from the evolutionary history of the species and showed close relationship between *B. schaefferi* from distant geographical regions: Poland, Italy and Algeria. In general, our results are congruent with previous studies on phylogenetic relationships in Anostraca (Weekers et al. 2002). According to these authors, divergence values among anostracan 18S rDNA sequences vary up to 0.060 substitutions per site. The lowest genetic variation was detected within the genus *Streptocephalus* Baird, 1852 – 0.001-0.006 substitutions per site. These genetic distances are similar to our results obtained for *B. schaefferi* (Table 1).

In conclusion, cessation of military operations in training grounds causes successional overgrow of temporary pools with vegetation, which ultimately leads to the habitat loss and extinction of *B. schaefferi* in these areas. This way, geographical distance between the persisting populations of the species elongates, making dispersion and gene transfer more difficult. On the other hand, our research shows that there is no spatial genetic diversity of the *B. schaefferi* populations from the distant Polish military grounds. However, despite the fact that the nuclear 18S ribosomal DNA is a suitable tool for molecular identification of species, it is a too conservative marker for phylogenetic analyses at the species level. Conse-

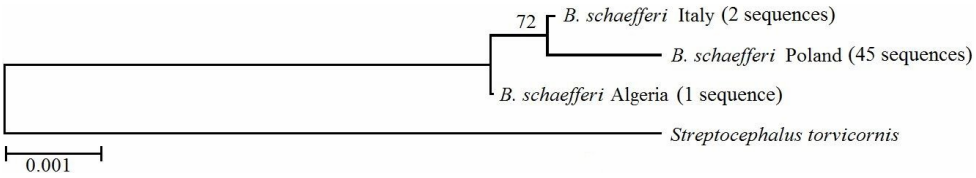

Figure 3. Maximum likelihood consensus tree of 18S rDNA gene sequences of *Branchipus schaefferi* deposed in the GenBank (from Italy and Algeria) and those obtained in our study (from Poland) with homologous sequence of *Streptocephalus torvicornis* as an outgroup. Scale bar indicates an evolutionary distance of 0.001 nucleotides per position in the sequence.

Table 1. Values of p-distance calculated for 18S rDNA gene sequences (below diagonal). Standard error values are given above diagonal.

	B. schaefferi Italy	*B. schaefferi* Poland	*B. schaefferi* Algeria	*Streptocephalus torvicornis*
B. schaefferi Italy		0.001	0.001	0.003
B. schaefferi Poland	0.001		0.001	0.003
B. schaefferi Algeria	0.001	0.002		0.003
Streptocephalus torvicornis	0.012	0.012	0.012	

quently, the further work is needed, with more variable molecular markers, to clarify the genetic variability of *B. schaefferi*, rare species in the territory of Poland. Moreover, intensive long-term surveys are needed to develop effective conservation measures for this species, aiming not only in supporting its known habitats but also sustaining right genetic diversity in central European populations of *B. schaefferi*.

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