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Development of microsatellite loci for two *Agabus* diving beetle species from the pooled DNA and testing their utility in mountain lake populations

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ABSTRACT

Diving beetles (Dytiscidae) are the most speciose water beetle group, occurring on all continents except Antarctica. They inhabit various lotic and lentic habitats and play an important role in ecosystem functioning. In this study, we developed functional polymorphic microsatellites for two widely distributed species from one of the most diversified dytiscid genus *Agabus*: *Agabus bipustulatus* (Linnaeus, 1767) and *Agabus* cf. *guttatus* from the *Agabus guttatus* group sensu Foster and Bilton (1997). For microsatellites development, pooled DNA and NGS pyrosequencing were used. Microsatellites are still very useful genetic markers for studying recent population changes, but for dytiscids, highly diversified and ecologically important group of freshwater invertebrates, only 8 microsatellite loci are available for one North American species. To test utility of the developed markers, we used several populations of alpine lakes, situated in the Tatra Mountains (Western Carpathians). From the 60 tested markers, 13 loci for *Agabus bipustulatus* and 8 loci for *A.* cf. *guttatus* showed polymorphism. The number of alleles per locus varied from 2 to 10 and no significant linkage disequilibrium was observed. Observed/expected heterozygosity varied from 0/0.077 to 1/0.834 within populations of *A.* cf. *guttatus* and from 0/0.056 to 1/0.837 within populations of *A. bipustulatus*. The significant deviation from HWE was probably caused by presence of null alleles or undetected biological processes. Bayesian cluster analysis revealed differences in the cluster proportions, confirming applicability of the developed markers for future studies of population structure of both *Agabus* species.

1. Introduction

Freshwater ecosystems are among the Earth's most threatened habitats due to anthropogenic impact reflected primarily in global and local species loss (e.g. Sala et al., 2000; Dudgeon, 2010). Moreover, the species diversity is declining faster in freshwaters than in other ecosystems (e.g. Ricciardi and Rasmussen, 1999; Vaughn and Hakenkamp, 2001), but the consequences of this negative trend for the genetic diversity or population structure of aquatic species are still poorly understood.

Widespread freshwater macroinvertebrates are ideal candidates for studying demographic histories, and also for detecting recent habitat disturbances using molecular data (Mamos et al., 2016; Macdonald et al., 2017). While the previous studies used preferably EPT taxa as model organisms (e.g. Theissinger et al., 2012; Elbrecht et al., 2014; Macdonald et al., 2017), the aquatic beetles, representing the most diversified group of insects, were overlooked. This was probably also due to the taxonomic difficulties associated with larger groups like for example dytiscids (Arnott et al., 2006). The diving beetles play an important role in ecosystem functioning. It was suggested, that their distribution is strongly influenced by the environment (Larson, 1985; Ribera and Vogler, 2000), which increase their potential as bioindicators of habitat quality (Fairchild et al., 2003). In this study, we chose two species from the family Dytiscidae representing the most speciose genus *Agabus* (Ribera et al., 2001): *Agabus bipustulatus* (Linnaeus, 1767) and *Agabus* cf. *guttatus* from *Agabus guttatus* group sensu Foster and Bilton (1997). They occur in various freshwater biotopes, including remote and sensitive alpine lakes situated in more or less isolated valleys separated by elevated mountain ridges. Those lakes resemble habitat islands with different level of isolation (Steinbauer et al., 2016), and provide excellent system for studying speciation, gene flow, or colonization processes. Although the studied species are relatively closely related, their different dispersal ability and habitat preferences (Foster and Bilton, 1997) may have led to the development of different population genetic structure within the same area. In European mountains, there are several thousands of alpine lakes (Catalan et al., 2009) and despite their recent origin (mostly Holocene), they harbour specific and valuable communities of oligostenothermic organisms. The

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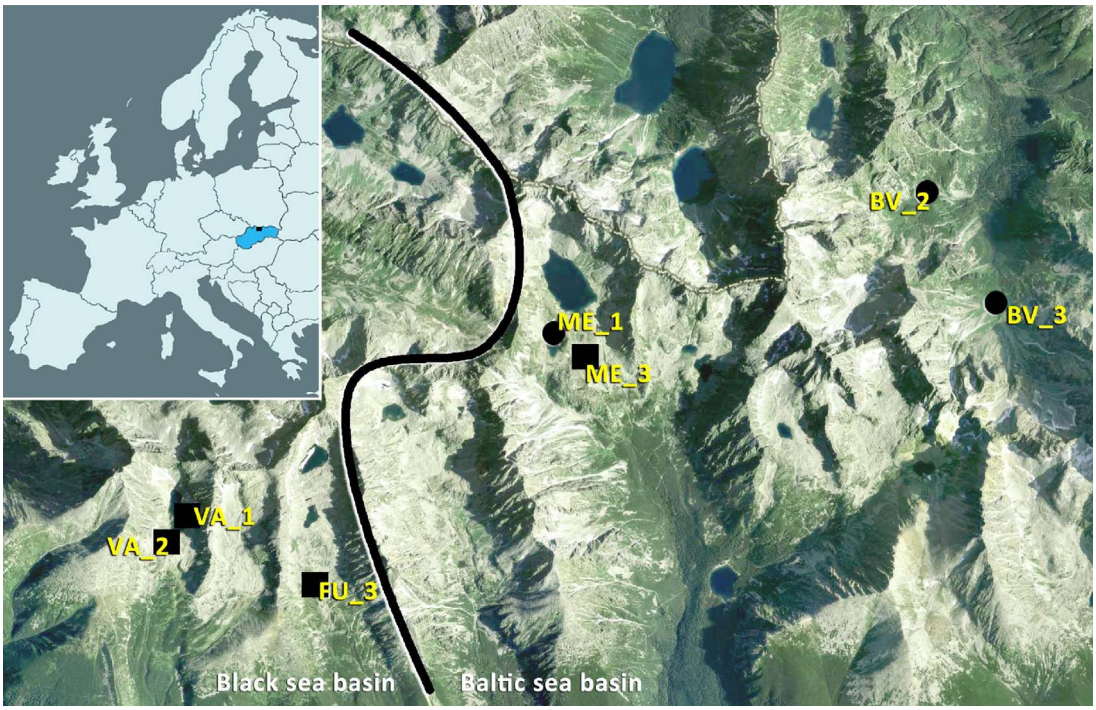

Fig. 1. Sampling sites of the studied *Agabus* species in the Tatra Mts (■ − sites of *Agabus* cf. *guttatus*: VA_2 Malé Krivánske pleso, VA_1 Zelené Krivánske pleso, FU_3 Sedielkové pliesko 3, ME_3 Hincovo oko 1; ● − sites of *Agabus bipustulatus*: ME_1 Malé Hincovo pleso, BV_2 Ťažké pleso, BV_3 Zelené Kačacie pleso).

lakes are very sensitive to atmospheric forcing, and so they could serve as early warning sentinels of global change (Catalan et al., 2009). The past events of the mountain lakes can be detected using molecular tools, but except few works (Petrusek et al., 2007; Hamrová et al., 2012; Ventura et al., 2014) a majority of genetic studies of alpine aquatic biota is focused on mountain streams (e.g. Finn and Adler, 2006; Lehrian et al., 2009; Geismar et al., 2015; Jordan et al., 2016).

To observe the genetic structure of populations and possible gene flow, microsatellites still represent a convenient genetic marker (e.g. Sunnucks, 2000; Selkoe and Toonen, 2006). Although microsatellites are useful and frequently used to estimate recent population changes (Crandall et al., 2000), there is only limited number of these markers available for freshwater invertebrates (e.g. Geismar and Nowak, 2013; Elbrecht et al., 2014; Goffová et al., 2015; Lopes-Lima et al., 2016). Regarding Dytiscidae, a family with more than 4000 known species (Nilsson, 2016), only 8 microsatellites are available for one North American species (Phillipsen et al., 2015). This work is thus among the first proposing development of microsatellites for diving beetles, and the first focused on the palaearctic species.

The development of microsatellite markers is usually tedious process, and the markers are developed for one species due to their high species specificity. Börk et al. (2008) described simultaneous development of microsatellites for two sister species of sturgeons, but they used classical methods employing enzyme digestion of the DNA and cloning. Recently, the high throughput sequencing techniques (HTS) become an efficient approach for microsatellites development (Gardner et al., 2011; Zalapa et al., 2012; Schoebel et al., 2013). To increase effectiveness of the process, we aimed to use pooled DNA of the two species (from one genus, but not sister related) in combination with modern HTS techniques. For subsequent testing their utility for population studies, we used samples from alpine lakes in the Tatra Mountains (Western Carpathians).

2. Material and methods

2.1. Sample collection

During 2009–2016, benthic samples from around 180 lakes and ponds in the Tatra Mts (Western Carpathians, Central Europe) were collected. For this study, 7 localities were selected based on the presence of *Agabus* specimens and the distance (neighbouring and distanced lakes) between sites (Fig. 1; Table 1). Samples were collected using kick-sampling method (Frost et al., 1971), and the collected material was fixed with ethanol in the field. The beetles were subsequently sorted out and stored at −25 °C in 96% ethanol.

2.2. Species delimitation

The specimens were assigned to species using the identification key (Nilsson and Holmen, 1995), and partial mitochondrial cytochrome c oxidase subunit 1 gene (COI) using Kimura-2 parameter distances (Kimura, 1980). Gathering mtDNA data followed Čiamporová-Zaťovičová and Čiampor, (2017). The sequences of *A. guttatus* and *A. bipustulatus* from Tatra Mountains were compared with DNA barcodes in BOLD v4 (Barcode of Life Datasystems http://v4.boldsystems.org/index.php). The sequences were aligned using MUSCLE algorithm (Edgar, 2004) in MEGA 7 (Kumar et al., 2016) and cropped to the equal length of 543 bp. Fabox (Villesen, 2007) was used to collapse sequences into haplotypes. The tree was reconstructed by the maximum-likelihood method in MEGA 7 with 1000 bootstrap replicates. The Tamura-Nei model (TN93 + G; Tamura & Nei, 1993) was used as suggested by MEGA as the best-fitting model.