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Molecular and morphometric analysis of *Veronica* L. section *Beccabunga* (Hill) Dumort.

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ABSTRACT

The Mediterranean is home to rich variety of aquatic plants. Yet, they are less prominent as other groups of plants in this global hotspot of biodiversity. *Veronica* sect. *Beccabunga* is a common member of the semi-aquatic plants in a variety of moist to aquatic habitats in the region. Species numbers vary between two and fifteen with many subspecies and varieties and taxonomic problems. However, most studies involve only regional floras and no global biosystematic analysis is yet available. Here, we present a morphometric and molecular study based on plastid and nuclear ribosomal DNA of the group to provide a phylogenetic framework for the group using 101 specimens of 24 taxa in the morphometric and 65 specimens for 28 taxa. Further, flow cytometry has been used to reveal the ploidy level, especially of the Egyptian endemic taxa. The analyses demonstrate the division in three subsections but fail to resolve further groups within these subsections consistently. Reasons for this lack of resolution are likely a combination of ancient polymorphisms, hybridization and phenotypic plasticity. Especially, the latter two have been shown to be frequent in the group. The study forms the basis for any further study by demonstrating the necessity to analyze the group globally and/or in more in-depth using highly variable molecular markers.

1. Introduction

Despite their importance in many habitats, aquatic plants are highly understudied in modern biosystematic studies. Aquatic plant species are characterized as morphologically highly variable but taxonomically little differentiated (Santamaría, 2002). Indeed, in general, a substantial part of the interspecific variation in aquatic plants is cryptic due to reduction and convergence of certain plant parts (Santamaría, 2002). Additionally, hybridization, clonal reproduction and phenotypic plasticity contribute to the problem (Ito et al., 2010; Santamaría, 2002) and molecular techniques are necessary to differentiate taxa (e.g., Ito et al., 2010; Prančl et al., 2014). Nevertheless, aquatic habitats are important areas for understanding plant evolution and conservation, especially since aquatic plant species are severely threatened by pollution through agricultural intensification (Steffen and Leuschner, 2014).

An important area for aquatic plants is the Mediterranean basin representing one of the 25 global biodiversity hotspots (Myers et al., 2000), with 10 percent of the world's plants found in about 1.6 percent of the Earth's surface. About 60% of the native taxa found in this region are endemics (Greuter, 1991; Thompson, 2005; Thompson et al., 2005). The Mediterranean is especially well known for its oromediterranean flora (Stevanovic, 1996), its richness in annuals (Ackerly, 2009), the diverse insular floras (e.g. Mansion et al., 2009) and its varied coastal plants (e.g., Pfosser and Speta, 2004). However, it is also home to a variety of aquatic plants (Cook, 1983).

One of those aquatic to semi-aquatic taxa in the Mediterranean area is *Veronica* section *Beccabunga* (Hill) Dumort. *Veronica* includes about 450 species (Albach et al., 2008). It is distributed over much of the Northern Hemisphere and beyond and is ecologically highly diverse, with species living in aquatic to dry steppe habitats, from sea level to high alpine regions (Albach et al., 2004a,b). Most of the 10–15 aquatic species (and up to 20 taxa including commonly accepted subspecies and varieties) of *Veronica* occur in *Veronica* sect. *Beccabunga*, which is distributed over most of the Northern Hemisphere and even some parts of the Southern Hemisphere due to human migration. The center of diversity of this section lies in the Mediterranean basin especially in Egypt, Turkey and Iran, which each are home to more than 10 taxa (Abd El-Ghani et al., 2010; Fischer, 1981; Öztürk and Fischer, 1982). Endemism in *V.* section *Beccabunga* is apparently especially well pronounced in the Egyptian flora, which has five endemic and near-endemic taxa (*V. anagallis-aquatica* var. *nilotica; V. catenata* subsp. *pseudocatenata; V. anagalloides* subsp. *taeckholmiorum; V. kaiseri* and *V. scardica* subsp. *africana*) but their relationship has never been rigorously been compared with conspecifics. The high rate of endemicity

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has been attributed to the arid climate in Egypt and mountainous ecosystem at Sinai (Omar, 2014). Four endemic taxa are restricted to the Nile Delta District (*V. anagallis-aquatica* var. *nilotica; V. catenata* subsp. *pseudocatenata; V. anagalloides* subsp. *taeckholmiorum* and *V. scardica* subsp. *africana*), which has been *ascribed* to local restriction of the region surrounded by desert (Zohary, 1973).

However, the section has a long history of taxonomic dispute over species delimitation (Marchant, 1970; Schlenker, 1936). These problems are partly considered due to hybridization, partly due to phenotypic plasticity and partly due to convergence caused by related selection pressures in ephemeral, aquatic habitats (Marchant, 1970; Sellers, 1983). Aquatic plants in general, are characterized by high phenotypic plasticity, the plastic response of a genotype to an ecological factor (Puijalon and Bornette, 2006; Puijalon et al., 2008; Santamaría, 2002; Torres Boeger and Poulson, 2003). Most prominent among aquatic plants in general and *Veronica* in particular is the difference between submerged and terrestrial forms (Arber, 1920; Glück, 1911). Many plant species exposed to environmental stresses display plastic responses in their developmental, morphological, physiological, anatomical, or reproductive traits that can support functional adjustments, possibly compensating for the detrimental effect of stress (Sultan, 2000, 2003). Nevertheless, such plastic responses may augment variation within species and similar changes of related species to the same ecological factor may blur species distinction (Santamaría, 2002). Much research has been devoted to unravel how plasticity may influence and contribute to diversity among individuals, populations and species (Forsman, 2015).

To understand the importance of phenotypic plasticity and hybridization, phylogenetic analyses of markers unrelated to the phenotypic changes are necessary to determine phylogenetic relatedness. In recent years, many authors extensively studied *Veronica* phylogenetically using DNA sequence data from nuclear and plastid DNA regions starting with Albach and Chase (2001) but used only three or four samples from *Veronica* sect. *Beccabunga*. More recently, Meudt et al. (2015) increased the number of samples to ten and demonstrated that plastid and especially nuclear ribosomal DNA can further resolve relationships within the section. Important insights into the relationships of *Veronica* sect. *Beccabunga* have additionally been gained by Abd El-Ghani et al. (2011). In addition, the importance of studying ploidy levels in the section has been highlighted by Öztürk and Fischer (1982) and reviewed in Albach et al. (2008). Here, we provide a comparative analysis of morphometric measurements and phylogenetic analyses based on nuclear ribosomal and plastid DNA data for *Veronica* sect. *Beccabunga* with additional information on ploidy levels of respective taxa.

For that purpose, we generated DNA sequence data for 63 individuals representing all taxa currently recognized at the species and intraspecific level in *Veronica* sect. *Beccabunga*. As molecular markers, the plastid 3'*rps*16-5'*trnK* spacer region was chosen, which is considered to be one of the nine regions in the plastid genome offering most variation in phylogenetic studies (Hollingsworth et al., 2009; Shaw et al., 2007) and considerably more informative than the *trn*L-F-region employed by Meudt et al. (2015). This high variability has previously been confirmed in *Veronica* by Bardy et al. (2010, 2011) and Sonibare et al. (2014). The internal transcribed spacer from nuclear ribosomal DNA (nrITS) is an obvious choice for a supplementary barcode in groups in which direct sequencing is possible (CBOL-Plant-Working-Group, 2009; Hollingsworth et al., 2011). It has frequently been used in *Veronica* at the genus-wide level (e.g., Albach and Chase, 2001) and among closely related species (Albach and Briggs, 2012). Thus, our aim of this study was to provide an improved basis for further systematic and ecological work in the section, which we consider has the potential to form a model system for the evolution of aquatic species and the importance of phenotypic plasticity.

2. Experimental

2.1. Plant material

The present study is based on specimens, both own collections and herbarium specimens from Africa (Egypt, Ethiopia), Europe (Turkey, Germany, Czech, Austria), Asia (Armenia, Azerbaijan, Georgia, Pakistan, Kyrgyzstan, Afghanistan) and North America (USA and Canada). A total of 101 specimens representing 24 taxa of section *Beccabunga* was used in the morphometric analysis (Suppl. Table 1) and 65 specimens representing 28 taxa for the DNA sequence analysis (Suppl. Table 2) including all but one (*Veronica undulata*) of the commonly accepted species and all commonly accepted intraspecific taxa for the other species except for those in *V. peregrina*.

2.2. Flow cytometry

DNA ploidy levels were estimated for seven Egyptian and Kyrgistanian taxa by flow cytometry from three plants each raised from seeds taken from herbarium specimens. Samples were run on a CyFlow ML (Partec GmbH, Munster, Germany) equipped with a green laser (Cobolt Samba 532 nm, Cobolt AB, Solna, Sweden) and using *Zea mays* or *Solanum pseudocapsicum* as internal standard. The 1C value for *Zea mays* is 2.715 pg (Lysák and Doležel, 1998) and for *Solanum pseudocapsicum* 1.2946 pg (Temsch et al., 2010). Propidium iodide staining was used following the protocol of Baranyi and Greilhuber (1996) with either 30 min (Otto buffer) or 15 min staining (woody plant buffer).

2.3. Morphometric analyses

For the morphometric analyses, we scored 27 morphological characters from across all plant organs (Table 1), including both quantitative and qualitative characters. All analyses of the morphological data were computed in R (R-Development-Core-Team, 2011) unless stated otherwise.

The correlation between each pair of characters was calculated using the function cor with Kendall's tau-b coefficient (Yau, 2013). The Euclidean distance matrix was computed, which was the basis of a principal coordinate analysis (PCoA) using the function Biplots of the BiplotGUI package (La Grange et al., 2009).

A cluster analysis was conducted with the R-package pvclust (Suzuki and Shimodaira, 2005, 2006) to assess the uncertainty in hierarchical cluster analysis. Pvclust calculates probability values (*p*-values) for each cluster using bootstrap resampling techniques. We have calculated AU *p*-values with multiscale bootstrap resampling since it is considered superior to BP values calculated by ordinary bootstrap resampling (Suzuki and Shimodaira, 2006). The hierarchical cluster analysis was conducted using Ward's method applying squared Euclidean Distance as distance measure.

2.4. Phylogenetic analyses

Genomic DNA was isolated from silica gel-dried leaves or herbarium material in commercial kits according to the manufacturer's instruction (innuPREP Plant DNA-Kit; Analytik Jena; Jena; Germany).

The quality of the extracted DNA was checked on 1% SB-agarose gels and the concentration was measured spectrophotometrically. The PCR reactions for the nrITS and *rps*16-*trnK* regions were conducted in a total volume of 25 μl with 1 μl of the extracted DNA, 1 μl (pmol/μl) of each of the primers 0.5 μl dNTPs (20 mM epicenter,