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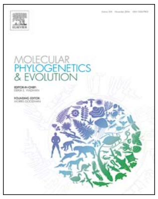

Editor's Choice Article

The challenge of species delimitation in the diploid-polyploid complex *Veronica* subsection *Pentasepalae*

Nélida Padilla-García[a,b,c,*], Blanca M. Rojas-Andrés[a,b,1], Noemí López-González[a,b], Mariana Castro[d], Sílvia Castro[d], João Loureiro[d], Dirk C. Albach[e], Nathalie Machon[c], M. Montserrat Martínez-Ortega[a,b]

[a] Departamento de Botánica, University of Salamanca, E-37007 Salamanca, Spain
[b] Biobanco de ADN Vegetal, University of Salamanca, Edificio Multiusos I+D+i, Calle Espejo s/n, 37007 Salamanca, Spain
[c] Centre d'Ecologie et des Sciences de la Conservation (CESCO, UMR7204), Sorbonne University, MNHN, CNRS, UPMC, CP51, Paris, France
[d] CFE, Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas s/n, 3000-456 Coimbra, Portugal
[e] Institute of Biology and Environmental Sciences (IBU), AG Biodiversity and Evolution of Plants, Carl von Ossietzky Universität Oldenburg, Oldenburg 26111, Germany

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ABSTRACT

A reliable taxonomic framework and the identification of evolutionary lineages are essential for effective decisions in conservation biodiversity programs. However, phylogenetic reconstruction becomes extremely difficult when polyploidy and hybridization are involved. *Veronica* subsection *Pentasepalae* is a diploid-polyploid complex of ca. 20 species with ploidy levels ranging from 2*x* to 10*x*. Here, DNA-ploidy level estimations and AFLP fingerprinting were used to determine the evolutionary history, and species boundaries were reviewed in an integrated approach including also previous data (mainly morphology and sequence-based phylogenetic reconstructions). Molecular analyses were performed for 243 individuals from 95 populations, including for the first time all taxa currently recognized within the subsection. Phylogenetic reconstruction identified four main groups corresponding almost completely to the four clusters identified by genetic structure analyses. Multiple autopolyploidization events have occurred in the tetraploid *V. satureiifolia* giving rise to octoploid entities in central Europe and north of Spain, whereas hybridization is demonstrated to have occurred in several populations from the Balkan Peninsula. Furthermore, our study has established the taxonomic status of taxa, for the most part recovered as monophyletic. Cryptic taxa within the group have been identified, and a new species, *Veronica dalmatica*, is fully described. This study highlights the implications of polyploidy in species delimitation, and illustrates the importance to conserve polyploid populations as potential sources of diversification due to evolutionary significance of genome duplications in plant evolution.

1. Introduction

The delimitation of species boundaries is a classic problem for biologists. Until about seventy years ago, taxonomists have focused on morphological differences between species for their circumscription, rather than on coherence with their evolutionary history. However, since the 1940s, a wider interest in the evolutionary history of organisms arose (Huxley, 1940). In 1950, Hennig published his theory of phylogenetic systematics giving rise to the origin of cladistics, which revolutionized the field of taxonomy (Hennig, 1950). Despite originally considered for the analysis of morphological characters, it is equally suitable for other types of characters that have been used with taxonomic purposes during the last decades. Currently, molecular phylogenies, complementing morphological characters, are the key instruments for biologists and biosystematists who try to understand the evolutionary processes that shape the history of species. Nevertheless, evolutionary histories involving radiations or complex processes such as hybridization, introgression and/or polyploidization, complicate phylogenetic reconstruction (Naciri and Linder, 2015). This, together with a lack of morphological differences and uncertainties over reproductive isolation among polyploids and their diploid progenitors, has resulted in taxonomic biases within polyploid complexes (Soltis et al., 2007; Barley et al., 2013). Here, the importance of the species concept is fundamental. The biological concept of species proposed by Mayr (1942) is difficult to apply when working with closely related species in which hybridization and introgression are common. Most

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[*] Corresponding author at: Departamento de Botánica, University of Salamanca, E-37007 Salamanca, Spain.
E-mail address: nelidam@usal.es (N. Padilla-García).

[1] Present address: Department of Molecular Evolution and Plant Systematics & Herbarium (LZ), Institute of Biology, Leipzig University, Leipzig, Germany.

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plant taxonomists have traditionally relied on morphology to delimit species boundaries (i.e., morphological species concept), whereas others adopted in the last decades a concept based on genetic differences and monophyly (i.e., genetic and phylogenetic species concepts). However, in species groups with frequent hybridization and polyploidization, the general lineage concept (de Queiroz 2005, 2007) may be more appropriate. According to this concept, species are defined as separately evolving metapopulation lineages, which can be identified by different properties accumulated by that species during the process of speciation (e.g., reproductive isolation, morphological or genetic differences, monophyly, etc.). This general lineage concept has been broadly adopted and promoted the development of an integrative taxonomic approach in which multiple and complementary methods are integrated to delimit species boundaries (Dayrat, 2005). This approach argues that taxonomic inference should be based on congruence across different types of characters and analyses. When results from different sources of data are incongruent, caution to delimit species is preferable since taxonomic conclusions may have significant implications (Carstens et al., 2013). For example, regarding conservation and sustainable use of natural resources, in accordance with the Convention on Biological Diversity (https://www.cbd.int/gti/importance.shtml), taxonomy is necessary for effective decision making, because it provides basic understanding about the components of biodiversity. In a world where wild species are increasingly under threat, the conservation status of a taxon can only be correctly evaluated under the light of a clear taxonomic framework (Mace, 2004). The identification and preservation of evolutionary processes is also essential in conservation programs, especially for endangered, rare and endemic species.

In the present study, complementary methodologies are used to address the taxonomic challenges of a study group with a complex evolutionary history, *Veronica* subsection *Pentasepalae* Benth. This subsection is a monophyletic lineage within *V.* subgenus *Pentasepalae* (Benth.) M.M. Mart. Ort., Albach & M.A. Fisch. (Rojas-Andrés et al., 2015). It has a recent origin (mean crown age 2.8 Mya., Meudt et al., 2015) and is composed of ca. 20 closely related perennial species distributed in Eurasia and North Africa. Interestingly, the group comprises five species and three subspecies endemic to single countries or sometimes only a small area within one country. Some of them are included in regional, national and/or international Red Lists (Peñas de Giles et al., 2004; Cabezudo et al., 2005; Alcántara de la Fuente et al., 2007; Petrova and Vladimirov, 2009; Bilz, 2011; International Union for Conservation of Nature, 2016), although there is a clear lack of information for numerous species that have not yet been carefully evaluated. The most important diversification center of *V.* subsection *Pentasepalae* is the Balkan Peninsula. The group is characterized by the presence of a pentapartite calyx with the fifth sepal being significantly smaller, by a capsule usually rounded at the base, and a base chromosome number of $x = 8$. However, although the group is well defined within *Veronica* (Albach et al., 2008), the existence of morphologically intermediate forms within the subsection due to overlapping morphological character states makes *V.* subsection *Pentasepalae* one of the taxonomically most complicated groups within the genus (Albach and Meudt, 2010). Since Bentham described *V.* subsection *Pentasepalae* in 1846, numerous taxonomic treatments have been proposed (for a historical review of monographs and Floras, see Rojas-Andrés and Martínez-Ortega, 2016), and several studies based on morphological, karyological or molecular data have tried to elucidate the evolutionary history of the group (e.g., Martínez-Ortega et al., 2000, 2004, 2009; Andrés-Sánchez et al., 2009). In the most recent molecular study, Rojas-Andrés et al. (2015) used nuclear and plastid DNA sequence data to perform a phylogenetic analysis of the subsection. Despite the contributions of that study to the understanding of the evolutionary history of the group, a high degree of incongruence was found between the ITS and plastid DNA trees, probably caused by hybridization and incomplete lineage sorting (ILS). Hence, some questions about the monophyly and the relationships among species remained unresolved. Such questions are unlikely to be answered using a few loci alone, especially considering the prevalence of hybridization and polyploidization in the genus (Albach and Chase, 2004).

The variety of ploidy levels in the subsection, ranging from diploid to decaploid (data previous to 2008 summarized by Albach et al., 2008; Rojas-Andrés et al., 2015), indicate that polyploidy has been a crucial process in the evolution of the group. Polyploidy or whole-genome duplication (WGD) is a frequent mechanism of evolution and speciation in flowering plants (Stebbins, 1950; Grant, 1971; Soltis et al., 2004, 2009, 2015; Mayrose et al., 2011; Kellog, 2016). Despite ongoing research regarding the distinction between the types of polyploids (Levin, 2002; Soltis et al., 2010; Husband et al., 2013; Doyle and Sherman-Broyles, 2017), two main categories are generally recognized based on their origin: (i) autopolyploids that arise within a species, via intraspecific hybridization and duplication of similar genomes (homologous) and (ii) allopolyploids formed by interspecific hybridization and chromosome doubling of genomes that are more or less divergent (homeologous). The prevalence of different types of polyploids in nature has been intensively discussed (Müntzing, 1936; Stebbins, 1947; Lewis, 1980; Parisod et al., 2010), and recent studies suggest a parity in the incidence of autopolyploidy and allopolyploidy (Barker et al., 2016 but see Doyle and Sherman-Broyles, 2017). The differentiation between these processes is fundamental to evaluate the importance of polyploidization and hybridization in plant evolution. In this context, the diploid-polyploid complex *Veronica* subsection *Pentasepalae* is an excellent model to gain deeper insights into the contribution of these mechanisms to the evolutionary history of angiosperms.

The aim of this study is to clarify the phylogenetic relationships of *V.* subsection *Pentasepalae* by analyzing the nuclear genome using Amplified Fragment Length Polymorphism (AFLP). In addition to its use in phylogeographic studies, the AFLP technique is now widely used to infer phylogenetic relationships and to identify hybridization and polyploid events in recently evolved polyploid non-model groups (Meudt, 2011; Reberning et al., 2012; Himmelreich et al., 2014; Zozomová-Lihová et al., 2014). Compared to the previous study by Rojas-Andrés et al. (2015), in addition to using AFLPs, a molecular tool for which markers are distributed throughout the genome, we expand the study to include for the first time all taxa currently recognized within the subsection. Also, we added individuals that are difficult to identify to species and of different ploidy level from mixed-ploidy populations. AFLPs were generated to address the following specific points: **(i)** The role of auto- and allopolyploidization processes in the evolutionary history of *V.* subsection *Pentasepalae*; **(ii)** The implications of these processes in species delimitation and classification; and **(iii)** A review of the taxonomic status of the taxa within *V.* subsection *Pentasepalae*.

2. Materials and methods

2.1. Plant material

Samples were collected in the field during 2009–2015 except for one population of *V. satureiifolia* and one population of *V. tenuifolia* subsp. *fontqueri* that were collected in 2002. Localities, initial taxonomic assignment, and further information about samples are given in Table S1. Fresh leaf material was collected and stored in silica gel. For *V. krylovii*, three individuals were included of which two were selected from herbarium material and one from a cultivated specimen in the Botanical Garden of Oldenburg (Germany). *Veronica orientalis,* which belongs to *V.* subsection *Orientales* (Wulff) Stroh of *V.* subgenus *Pentasepalae* was chosen as outgroup. The complete data-set comprises 243 individuals from 95 populations (outgroup included) covering the geographic distribution of each taxon (Fig. 1). From each location, 2–3 individuals were included, except for populations with mixed-ploidy levels. In these exceptional cases, two individuals per cytotype were analyzed whenever possible. Initial plant identification was based on