



Disentangling phylogeography, polyploid evolution and taxonomy of a woodland herb (*Veronica chamaedrys* group, Plantaginaceae s.l.) in southeastern Europe

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ABSTRACT

Southeastern Europe is a centre of European biodiversity, but very little is known about factors causing the observed richness. Here, we contribute to fill this gap by reconstructing the spatio-temporal diversification of the cytologically variable and taxonomically intricate complex of *Veronica chamaedrys* (Plantaginaceae s.l.), growing in open forests, forest edges and grasslands, with flow cytometry, molecular markers (AFLPs, plastid DNA sequences) and morphometry. Our results show that both diploid and tetraploid cytotypes are widespread, but diploids predominate on the southern Balkan Peninsula. Plastid sequences suggest a first split into three main lineages in the mid-Pleistocene and a continuous diversification during the last 0.4 my. Two of the identified plastid lineages coincide with geographically distinct AFLP clusters. Altogether, the genetic data suggest forest refugia on the southern-most Balkan Peninsula (Greece), in Bulgaria, Istria (Croatia and Slovenia) and maybe the southeastern Carpathians (Romania). Morphometric and genetic data show little congruence with current taxonomy.

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1. Introduction

It is long and widely acknowledged that southeastern Europe, and especially the Balkan Peninsula, is a centre of European biodiversity (Turrill, 1929). One early recognised factor is the refugial character of the Balkan Peninsula with a high proportion of relic taxa, even if in many cases the claimed relic status still needs to be confirmed. Much less is, however, known about diversification processes and their spatio-temporal patterns on the Balkan Peninsula especially at lower taxonomic levels, i.e., within and among closely related species. This is partly due to the fact that in molecular studies the Balkan Peninsula is often neglected, such as in large-scale phylogeographical studies, where only a few (e.g., Taberlet, 1994; Santucci et al., 1998; Trewick et al., 2002) or no samples were included (Dumolin-Lapègue et al., 1997; Petit et al., 2002). Detailed studies focussing on the Balkan Peninsula are few and mostly deal with vertebrates (Podnar et al., 2004; Kryštufek

et al., 2007; Sotiropoulos et al., 2007; Ursenbacher et al., 2008), butterflies (Schmitt et al., 2006) or mountain plants (Frajman and Oxelman, 2007; Stefanović et al., 2008; Albach et al., 2009).

Southeastern Europe is recognised as a prime refugium for temperate European forest vegetation during the cold stages of the Pleistocene, together with the Iberian and Apennine Peninsulas (Comes and Kadereit, 1998; Taberlet et al., 1998; Gömöry et al., 1999; Hewitt, 2000; Hampe et al., 2003; Petit et al., 2003; Magri et al., 2006; Médail and Diadema, 2009). On the Balkan Peninsula, Pleistocene glaciation was restricted to the high massifs (Hughes et al., 2007; Milivojević et al., 2008), but as the climate was drier and more continental than at present (Horvat et al., 1974), survival of tree species was likely restricted to small areas with favourable conditions—“refugia within refugia”—as has been hypothesised for the Iberian Peninsula (Gómez and Lunt, 2007). These forest refugia were previously assumed to have been restricted to the southern tips of the Southern European peninsulas (Horvat et al., 1974; Hewitt, 2000), but recent studies found evidence for survival of tree species significantly further north than previously assumed (Stewart and Lister, 2001).

In southeastern Europe refugia have been suggested for numerous temperate tree species. Refugia of the beech (*Fagus sylvatica*)

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were suggested in Istria and adjacent areas on the Dalmatian coast, southern Bulgaria to northwestern Greece and maybe parts of the Carpathian arc (Magri et al., 2006). Possible refugia for the hornbeam (*Carpinus betulus*) were proposed in Romania and northern Greece (Grivet and Petit, 2003). Caucasian and European ash (*Fraxinus angustifolia* and *F. excelsior*) survived in at least two possible refugial areas, a western one in the Dinaric Alps and an eastern one stretching from the Rhodopes to the Carpathians (Heuertz et al., 2006). The hypothesis of several unconnected forest refugia during the Pleistocene is also supported by the understory vegetation that is much more diverse, regionally differentiated, and richer in endemics than in forests in central and northern Europe (Meusel and Jäger, 1992; Willner et al., 2009). However, the hypothesis of multiple forest refugia in southeastern Europe has never been tested with a herbaceous species in a phylogeographic framework.

A major force in plant evolution and diversification is polyploidy (Ramsey and Schemske, 1998, 2002; Wendel, 2000), which may in many cases be the result of secondary contact of populations differentiated in phases of allopatry, e.g., during restriction to different refugia (e.g., Petit et al., 1999). Polyploidy is recognised as an important mode of diversification by, for instance, promoting adaptation to new ecological niches or conferring reproductive isolation, which may eventually lead to speciation (Otto and Whitton, 2000). While allopolyploids may differ conspicuously from their diploid progenitors in morphology and physiology, autopolyploids that arise from the crosses within or between populations of a single species (Ramsey and Schemske, 1998) are often more difficult to distinguish on the basis of morphology alone (Levin, 1983, 2002). Recent cytogeographical studies not only indicate a higher incidence of autopolyploidy than previously thought, but also that autopolyploids often co-exist with their diploid parental populations (Husband and Sabara, 2004; Kron et al., 2007; Kolář et al., 2009). Despite the widely recognised importance of polyploidization in plant diversification and speciation, very little is known about its contribution to the high diversity on the Balkan Peninsula.

A good system to investigate diversification patterns on the Balkan Peninsula in the contexts of putative differentiation due to isolation in refugia and of polyploidy is the *Veronica chamaedrys* group (Plantaginaceae s.l.). Although it is widely distributed from western Europe to western Siberia, the Caucasus and Syria (Riek, 1935; Walters and Webb, 1972) and has a rather broad ecological amplitude (Dale and Causton, 1992a,b,c,d), it is a characteristic and widespread element of southeastern European forest vegetation, growing at forest margins and open forests dominated by, e.g., oaks, hornbeam or beech, in grasslands, thickets and hedges (Walters and Webb, 1972). This perennial herb is outbreeding (Goyder, 1983) with at least central European genotypes being self-incompatible (Albach, unpublished) and has the ability of clonal growth (Boutin and Harper, 1991). The *V. chamaedrys* group is a member of *V.* subgenus *Chamaedrys* section *Chamaedrys* subsection *Chamaedrys* (Albach et al., 2008) and comprises *V. ch.* subsp. *chamaedrys*, *chamaedryoides* and *micans* as well as *V. krumovii*, *V. micrantha*, *V. orbatica* and *V. vindobonensis* (Albach et al., 2004), whose phylogenetic relationships have not been resolved so far (Albach, 2006). With the exception of *V. micrantha*, which is endemic to the northwestern and central-western Iberian Peninsula (Martínez Ortega et al., 2009), all taxa occur in southeastern Europe and *V. ch.* subsp. *chamaedryoides*, *V. krumovii* and *V. orbatica* are restricted to that area. Apart from morphological differences concerning, among others, indumentum characters, these taxa do also differ karyologically. In particular, *V. ch.* subsp. *chamaedrys* is mainly tetraploid (Fischer, 1970, 1973b; Mirek and Fischer, 1986) with only a few diploids recorded from southern Austria (Fischer, 1973a), whereas *V. ch.* subsp. *chamaedryoides* and subsp. *micans*, as well as *V. krumovii*, *V. orbatica* and *V. vindobonensis* were suggested to be exclu-

sively diploid (Fischer, 1970, 1973b, 1974; Peev, 1972; Strid and Franzén, 1984; Mirek and Fischer, 1986). Since traditional cytotaxonomy, which is often based on a few chromosome counts only, may grossly underestimate the actual intricacy of polyploid complexes in general and of cytotype distribution patterns in particular (e.g., Suda et al., 2004), the association of ploidy level and taxonomy in the *V. chamaedrys* group remains to be tested, in particular if diploid and tetraploid taxa within the same genetic group are spatially segregated.

Here, we explore diversification patterns within the cytologically polymorphic *V. chamaedrys* group in southeastern Europe employing genetic (plastid sequences and AFLP fingerprints), ploidy level and morphometric data. Specifically, we want to assess the distribution of cytotypes in this region to test the hypothesis that polyploids are more frequent at higher latitudes, were range shifts of taxa due to climatic oscillations were more pronounced than in the South. We also want to infer mode and minimum number of polyploidization events to test (i) whether polyploids originated via autopolyploidy, as frequently observed in angiosperms (Otto, 2007), or via allopolyploidy, as possible after secondary contact of once geographically isolated diploid lineages (Petit et al., 1999) and (ii) whether polyploid cytotypes originated once or multiple times. Furthermore, we want to test whether the phylogeographical pattern of the woodland herb *V. chamaedrys* group agrees with those of tree species found in the same vegetation types. Finally, we want to assess whether and to which extent current taxonomy reflects genetically and/or morphometrically defined lineages.

2. Materials and methods

2.1. Plant material

In the summers of 2006 and 2007 the *V. chamaedrys* group was sampled in 121 sample sites (in the following referred to as “populations”). Leaf material was collected and immediately stored in silica gel. Voucher specimens are deposited at the Faculty Centre of Biodiversity, University of Vienna, Austria (herbarium WU; voucher numbers given in Table 1). Plants were determined by Manfred A. Fischer based on Fischer (1970, 1973b, 1974, 1991), Mirek and Fischer (1986) and Peev (1972, 1995) as well as on personal experience. A detailed description of taxa delimitation as used in our study (in particular subsuming *V. orbatica* and *V. ch.* subsp. *chamaedrys* var. *eglandulosa*, Table 1) is given in Appendix 1, an overview over the characters used for determination in Appendix 2.

2.2. Flow cytometry

DNA ploidy levels were estimated for five individuals per population. Flow cytometry was conducted with silica gel dried material following the protocol of Baranyi and Greilhuber (1996) with propidium iodide staining using a CyFlow ML (Partec GmbH, Münster, Germany) equipped with a green laser (Cobolt Samba 532 nm, Cobolt AB, Solna, Sweden) and using *Pisum sativum* cultivar ‘Kleine Rheinländerin’ as internal standard.

2.3. Molecular methods

Total genomic DNA was extracted from silica gel dried tissue (ca. 10 mg) of one individual per population. In eight populations with mixed cytotypes, one individual per cytotype was analysed and will in the following be referred to with a separate population identifier: this concerns populations 38/39, 42/43, 45/46, 52/53, 78/79, 86/87, 95/96 and 108/109 (Fig. 1 and Table 1). Extraction followed the CTAB-protocol of Doyle and Doyle (1987) with a

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