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Albanian violets of the section Melanium, their morphological variability, genetic similarity and their adaptations to serpentine or chalk soils

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This manuscript is dedicated to our friend M. Geoffrey Yates, Lewes, G.B. who passed away on August 12, 2014.

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ABSTRACT

Violets of the section Melanium from Albanian serpentine and chalk soils were examined for their taxonomic affiliations, their ability to accumulate heavy metals and their colonization by arbuscular mycorrhizal fungi (AMF). The sequence analysis of the ITS1-5.8S rDNA-ITS2 region showed that all the sampled six Albanian violets grouped between *Viola lutea* and *Viola arvensis*, but not with *Viola tricolor*. The fine resolution of the ITS sequences was not sufficient for a further delimitation of the Albanian violets within the *V. lutea*–*V. arvensis* clade. Therefore, the Albanian violets were classified by a set of morphological characters. *Viola albanica*, *Viola dukadjinica* and *Viola raunsiensis* from serpentine soils as well as *Viola aetolica* from a chalk meadow were unambiguously identified, whereas the samples of *Viola macedonica* showed high morphological variability. All the violets, in both roots and shoots contained less than or similar levels of heavy metals as their harboring soils, indicating that they were heavy metal excluders. All the violets were strongly colonized by AMF with the remarkable exception of *V. albanica*. This violet lived as a scree creeper in shallow serpentine soil where the concentration of heavy metals was high but those of P, K and N were scarce.

Introduction

Serpentine soils worldwide are specifically characterized by their low organic carbon content and low water holding capacity, their high Mg/Ca ratio, their scarce availability of the essential elements P, N and K and their high concentrations of either Ni or Cr and Fe (Karataglis et al., 1982; Brady et al., 2005; Amir et al., 2007; Kazakou et al., 2010; Ho et al., 2013). In Europe, the largest serpentine areas occur on the Balkan Peninsula, in Serbia, Macedonia, Bosnia, Croatia, Northwest and Central Greece and last but not least Albania (Vasić and Diklić, 2001; Stevanović et al., 2003; Psaras and Constantinidis, 2009). However the distribution of serpentine soils is patchy, since they are interspersed within other, often high chalk habitats. Edaphic isolation resulted in the development of a richness of local endemic species on serpentine soils. Among these, several are monotypic obligate serpentine genera with species such as *Halacsya sendtneri*, *Leptoplax emarginata* or *Paramoltkia doerfleri* (Stevanović et al., 2003) which are tertiary relicts because large areas of the Balkans were not covered with ice during the glacial epochs. Still nowadays, floristic composition and speciation in serpentine areas is inadequately known in parts of the Balkan range, especially in Albania (Stevanović et al., 2003), due to the difficult logistics and, until recently, political inaccessibility of the areas.

Among the 30 families of serpentine endemics (Stevanović et al., 2003), members of the Violaceae play a prominent role. Diverse *Viola* species, mainly of the section Melanium (=the pansies), were found on the Balkans, either on serpentine or in chalk areas,

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however, often with unresolved taxonomic affinities (Becker, 1926; Markgraf, 1931; Erben, 1985; Mullaj et al., 2010; Meyer, 2011). Little is known about the adaptation of these violets to the harsh, often adverse soil conditions in the Balkans.

Violets are known to settle in extreme habitats. The endemic violet (*Viola cheiranthifolia*) forms a unique community on gravel of the Pico del Teide in Tenerife at altitudes between 2500 and 3500 m and is thus the highest upward climbing cormophyte of the Canary Islands (Lemus et al, 2009). Central Europe harbors two very locally restricted endemic violets which occur exclusively on heavy metal soils. The blue zinc violet lives only in lead ditches and their surrounding heaps in an area of some 1 km × 1.5 km at Blankenrode close to Paderborn in Eastern Westfalia, Germany. Its yellow counterpart occurs on former zinc mining areas between Aachen, Germany and Liège, Belgium. Our previous investigations, both molecular (Hildebrandt et al., 2006) and morphological (Kuta et al., 2012), showed that the two zinc violets are close relatives of the alpine *Viola lutea* and should, therefore, be classified as *V. lutea* ssp. *westfalica* and *V. lutea* ssp. *calaminaria*, respectively.

Metallophytes such as the zinc violets could be either heavy metal accumulators or excluders (Baker, 1981; Bothe, 2011). Although some contradictory claims have been published (Jędrzejczyk et al., 2002; Bizoux et al., 2004), our repeated measurements (Hildebrandt et al., 1999; Kaldorf et al., 1999; Słomka et al., 2011b, in agreement with Ernst, 1982) showed that both zinc violets contain less heavy metals than the soils which harbor them. In addition, the concentrations of heavy metals (Zn, Pb, Cu, Cd) were lower in shoots than in roots, in contrast to the situation in heavy metal accumulators. Both zinc violets are heavily colonized by arbuscular mycorrhizal fungi (AMF) in their natural habitats (Hildebrandt et al., 1999; Słomka et al., 2011b). The fungi deposit the heavy metals in their cell walls or vacuoles thereby avoiding toxic levels coming into contact with the plant cells (Kaldorf et al., 1999; Słomka et al., 2011b). Similarly, heartsease (*Viola tricolor*) forms ecotypes on heavy metal heaps such as in Bukowno (Southern Poland), in the German Harz Mountains (Northern Germany), in Bad Bleiberg (Southern Austria) or Cave del Predil (Northern Italy). Heartsease from these habitats contains less amounts of heavy metals than the surrounding polluted metal soils and is heavily colonized by AMF in most instances, but surprisingly not at extreme adverse soil conditions. This latter finding has been described for sites at Bukowno and at Bad Bleiberg (Słomka et al., 2011b; Bothe et al., 2013). At the latter site, the concentration of P in the heavy metal soil was high, and then the violets saved the expenditure to form a symbiosis with AMF (Bothe et al., 2013).

The few studies on the adaptations of plants to heavy metal stress in serpentine soils are scattered in the literature (Brady et al., 2005). In the case of field scabious (*Knautia arvensis*), serpentine populations showed a significant lower AMF colonization than plants from non-polluted sites (Doubková et al., 2011), although the degree of AMF colonization of a plant does not generally correlate with its fitness above a threshold value of about 20% colonization (Feldmann et al., 2009). Worldwide, some serpentine violets, e.g. *Viola cuneata* and *Viola howelli* from North America (Reeves et al., 1983), *Viola yubariana* and *Viola sacchalinensis* var. *alpina* of Hokkaido in Japan (Horie et al., 2000) and some few species within the genera *Hybanthus* and *Rinorea* of the Violaceae (Severne and Brooks, 1972; Cole, 1973; Brooks and Wither, 1977) were reported to be accumulators of heavy metals, particularly of Ni, but not so much as the strongly accumulating Brassicaceae of the genera *Thlaspi, Bornmuellera* or *Alyssum*. For the Balkans, *Viola vourinensis* of the serpentine Mt. Vourinos in Northern Greece was reported to accumulate Ni, although not so strongly as *Centaurea thracica* (Psaras and Constantinidis, 2009). For Albania, hyperaccumulators such as *Alyssum* spp. have also been described (Shallari et al., 1998), but members of the Violaceae have not yet been investigated.

The present study with six *Viola* taxa of the section Melanium collected from Albanian serpentine or chalk soils addressed the following questions: (a) Do molecular and morphological characters match when elucidating the taxonomic affinity of this group of violets? (b) Are the *Viola* specimens collected from field sites colonized by AMF? (c) Are these violets accumulators or excluders of heavy metals at the sites where they thrive?

Materials and methods

Site descriptions and plant characteristics of the violets collected in Albania

The sampling sites in Albania are indicated in a map (Fig. 1).

- *Viola raunsiensis* W. Becker & Košanin
  04 June 2012, N 41°58′82″, E 20°17′46″ 1021 m, above Kapit village, along the road from Surroj village to Qafa e Komit, on serpentine gravel, perennial, with many shoots originating from the aboveground head of the roots according to Erben (1985), gravel creeper. It could be a hybrid of *Viola dukadjinica* and *V. epirota* (Erben, 1985). However, compared to *V. dukadjinica*, *V. raunsiensis* has blue flowers, multifloral shoots and the leaflets of the stipules are separated like fingers of a hand (=palmately compound). *V. raunsiensis* is close to *Viola allchariensis*, but the former is completely glabrous (Valentine et al., 1968) and has a much longer spur.
- *Viola* cf. *macedonica* Boiss. & Heldr. Zeba mountain
  04 June 2012, N 41°59′19″, E 20°16′20″, 1750 m, on top of Zeba mountain, open field sites in the upper timber line between *Pinus heldreichii* and *Pinus leucodermis*, on chalk. The taxonomic affiliation is somewhat uncertain. It could be related to *Viola schariensis*. This latter species has been described from Šar Planina in Macedonia at altitudes between 2100 and 2400 m, and forms completely heterogeneous hybrid populations with *Viola latisepala* and *Viola ivonis* at lower altitudes (Erben, 1985). Recently *V.* cf. *macedonica* was described for neighboring sites (Meyer, 2011). The long petiole of the leaves and the dark red violet flowers, typical for *V. schariensis*, are not present in samples collected from the Zeba mountain which suggests that these specimens are close to *V. macedonica*. However, in contrast to *V. macedonica* (Valentine et al., 1968), the lateral petals are with veins.
- *V. macedonica* Boiss. & Heldr. Munella mountain
  06 June 2012, N 41°57′62″, E 20°04′10″, 1370 m, at a slope on the Munella mountain, under a serpentine summit next to a chalk rock (termed A). Plants were found on the steep slope (around 50% declination) in gravel consisting of a mixture of serpentine and chalk. All characters (Erben, 1985) are typical for this species: shoots are 10–30 cm long, with more than one flower per shoot, peduncles are long, stipules of the lower leaves are short, are palmately compound, and acute. Typically, the upper petals often off-white and then change to blue. Such distinctly different colors of the upper petals were seen in the samples from Munella mountain. The lower petals often remained bright yellow with few or no veins. Lateral petals are without veins (Valentine et al., 1968). This species was always regarded as the Albanian substitute of *V. tricolor* (Schmidt, 1963; Raus, 1986).

  A second sample of *V. macedonica* (termed B) was collected some 500 m apart from the first stand.