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Orobanche grenieri (Orobanchaceae), a southwestern European species newly found in Asia

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We report occurrences of *Orobanche grenieri* (Orobanchaceae) in the Lesser Caucasus in southern Georgia (western Asia), and in Badakhshan in Tajikistan (central Asia). These localities are more than 3000 and 5000 km apart from its previously known distribution areas in Spain and France, respectively. We used morphological evidence as well as nuclear ribosomal ITS and plastid *rbc*L sequences to test the taxonomic assignment of the Georgian plants to *O. grenieri* and to determine their phylogenetic position. We list the features that differentiate *O. grenieri* from morphologically similar species, provide illustrations, a distribution map, and we propose an IUCN conservation status for the Georgian populations.

Introduction

The family Orobanchaceae is morphologically diverse and contains 2060 holo- or hemiparasitic species in 90 genera (McNeal *et al*. 2013). *Orobanche s. lato* is probably the largest taxonomic group of holoparasitic angiosperms and comprises ca. 200 species that parasitise the roots of various vascular plants (Pusch & Günther 2009). They are distributed almost worldwide, but the main centres of diversity are in the Mediterranean, western and central Asia, northern Africa and North America. A few of them are parasites of important crops (Joel *et al*. 2013).

Species of *Orobanche* are difficult to identify due to their frequently discolored herbarium material and reduced vegetative organs. Many of the species are rare, endangered or declining and have an unclear taxonomic circumscription. The distribution and host range of many species is still not sufficiently known (e.g. Piwowarczyk 2011, Frajman *et al*. 2013, Piwowarczyk *et al*. 2014).

Orobanche grenieri was previously known from the Palencia province in Spain and from several localities in France: Isère, Hautes-Alpes, Provence-Alpes-Côte d'Azur, Rhône-Alpes and the Languedoc-Roussillon regions (Schultz 1845, Carlón *et al*. 2005, Crouzet *et al*. 2009, Tison *et*

Fig. 1. Distribution of *Orobanche grenieri* based on field studies, photos and literature data.

al. 2014). Reports (mainly based on photographs) from Turkey (Anatolia), Ukraine (Crimea) and Italy (Province of Savona) require confirmation (http://www.farmalierganes.com/Otrospdf/publica/Orobanchaceae%20Index.htm#O_G; Fig. 1).

Orobanche grenieri (Fig. 2) parasitises the roots of *Lactuca* spp. only. It grows in sunny, rocky (limestone) and largely mountainous areas between 1100 and 1300(1500) m a.s.l. (Carlón *et al*. 2005). Apart from its flower morphology, this species is similar to *O. cernua*, which however has different hosts (mainly *Artemisia* spp.) (*see* Carlón *et al*. 2005; Table 1). The taxonomic relationships of *O. cernua* and *O. cumana* are unclear. The latter parasitises cultivated plants, mainly *Helianthus annuus*, but is often treated as a variety or taxonomic synonym of *O. cernua* (Chater & Webb 1972, Foley 2001), although other researchers recognize it as a separate species. The two taxa clearly differ in host preferences, morphological traits (Table 1), seed-oil fatty acid profiles (Pujadas & Thalouarn 1998, Pujadas-Salvà & Velasco 2000), seed morphology (Plaza *et al*. 2004) and at the molecular level

Table 1. The most important characters distinguishing *Orobanche grenieri*, *O. cernua* and *O. cumana* (after Pujadas-Salvà & Velasco 2000, Carlón *et al.* 2005, supplemented after our observations).

Character	*O. grenieri*	*O. cernua*	*O. cumana*
Stem	16–38(52) (cm), usually ± gracile	(10)15–32 (cm), usually ± thick	(35)40–65 (cm), ± gracile
Inflorescence	(4)7–20 (cm), dense, shorter than remaining stem	(4)6–17(23) (cm), dense, ± as long as remaining stem	(15)22–30(38) (cm), lax, longer than remaining stem
Scales	usually narrow-lanceolate	usually ovate or ovate-lanceolate	usually ovate or ovate-lanceolate ± broadly ovate-lanceolate
Bracts	± narrow, ovate lanceolate, ± acuminate	± broadly ovate, rarely acuminate	
Calyx (mm)	7–11	6–10	(5)7–9
Calyx segments	usually bidentate, ± narrow, ovate-lanceolate, ± filiform	usually bidentate, ovate, acute or subulate, rarely filiform	entire, rarely bidentate, entire, ovate-lanceolate
Corolla (mm)	(15)17–18	(13)15–18	(16)19–22
Corolla dorsal line	± regularly curved, upper part patent	geniculated, upper part decurved	curved forward, downward inflected
Corolla lower lip	with ± large and broad lobes, folds white, distinct	with ± small and narrow lobes, folds indistinct	with ± small and short lobes, folds indistinct
Corolla color	bluish-violet, basally yellowish-white	dark blue to violet, sometimes almost black at margins of lips	pale blue
Filaments	glabrous	glabrous	with sessile glandular hairs
Anthers	glabrous	glabrous or sparsely pubescent	hairy
Host	*Lactuca* spp. (mainly *L. viminea*)	*Artemisia* spp., rarely other Asteraceae	mainly *Helianthus*
Distribution	southwestern Europe (Spain, France), western and central Asia (Georgia, Tajikistan), Turkey*, Crimea*	mainly Mediterranean arid area to central Asia	mainly east Europe to central Asia, Mediterranean area

* localities that require confirmation.

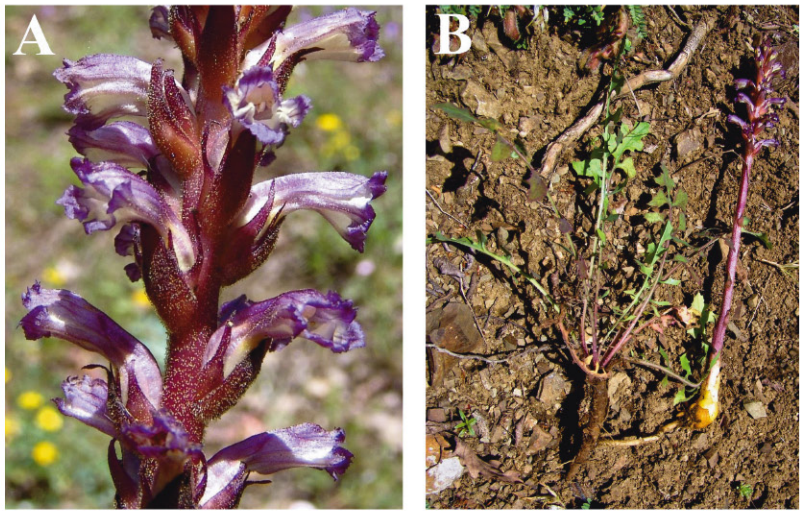

Fig. 2. *Orobanche grenieri* from Georgia. — **A**: Inflorescence. — **B**: Parasite connected with host *Lactuca* cf. *viminea*. Photos by R. Piwowarczyk taken on 31 May 2014.

(e.g., Román *et al*. 2003, Pineda-Martos *et al*. 2014 and the references therein).

This paper reports highly disjunctive populations of *O. grenieri* from western and central Asia (Georgia and Tajikistan, respectively). These are the first records of the species in Asia, representing the easternmost occurrences. We used morphological evidence as well as ITS and *rbc*L sequences from Georgian and European plants to test the taxonomic assignment of the Georgian population and to determine the phylogenetic position of *O. grenieri*.

Material and methods

Plant material

The specimens of *O. grenieri* were collected during field studies in Georgia (Lesser Caucasus) in May 2014. They were deposited in the herbarium of the Jan Kochanowski University in Kielce (KTC). The specimen from Tajikistan, which was labelled as *O. cumana*, was found in the herbarium in Saint Petersburg (LE; Fig. 3). The International Union for Conservation of Nature (IUCN) assessments follow the latest guidelines of the IUCN (http://jr.iucnredlist.org/documents/RedListGuidelines.pdf).

SPECIMENS EXAMINED: **Georgia**. Samtskhe-Javakheti district: Kortaneti (ca. 8 km NE Borjomi), a rocky slope above an asphalt road, 780–790 m a.s.l., 31 May 2014 *R. Piwowarczyk* (KTC) (Figs. 1 and 2). **Tajikistan**. Badakhshan: near the Shakdara River in the Roshtkala area, 22 June 1966 *R. Kamelin* (LE) (Figs. 1 and 3).

Phylogenetic analysis

DNA was extracted from freshly collected and silica gel-dried material with the exception of the specimen from Spain, which was a herbarium voucher. Total genomic DNA was isolated using a Plant & Fungi DNA Purification Kit (EURx) as described in the manufacturer's protocol.

The entire nuclear ribosomal ITS region (Internal Transcribed Spacer region: ITS1-5.8S-ITS2: internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2) of nuclear ribosomal DNA was amplified using the primers ITS 7A (Fuertes Aguilar *et al*. 1999) and ITS 4 (White *et al*. 1990). The DNA of the herbarium specimen (*O. grenieri* from Spain) was partly degraded and two pairs of primers were used for the amplification of the entire ITS sequence: ITS 7A and ITS C and the pair ITS B and ITS D (Blattner 1999). The plastid *rbc*L gene was amplified using the primers 1F and 1352R (Manen *et al*. 2004).

The PCR reactions were performed in a total volume of 25 μl and contained 1× concentration of Taq buffer with KCl (Thermo Scientific), 2 mM $MgCl_2$, 0.12 mM dNTPs (Thermo Sci-

Fig. 3. *Orobanche grenieri*. Specimen: Tajikistan, Badakhshan, near the Shakdara River, Roshtkala area on *Lactuca*, 22 June 1966, *R. Kamelin* (LE).

entific), 0.2 μM of each primer, 2 μg of bovine serum albumin (BSA), 1 U Taq DNA Polymerase (Thermo Scientific) and 20 ng of DNA template. Amplifications were performed using a T-100 Thermal Cycler (Bio-Rad) by applying a touchdown PCR protocol consisting of two stages. Stage 1 included an initial step of 5 min at 94 °C, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing at variable temperatures for 30 s and extension for 1 min at 72 °C. In this stage, the temperature of annealing was set to 62.5 °C in the first cycle followed by cumulative 0.5 °C decreases in the 29 subsequent cycles. Stage 2 included 10 cycles of 30 s at 94 °C, 30 s at 48 °C and 1 min at 72 °C and finally an extension of 10 min at 72 °C. The same program was used for all of the primers that were investigated.

PCR products were purified using a GeneMATRIX PCR/DNA Clean-Up Purification Kit (EURx) according to the manufacturer's protocol and sequenced on both strands at Genomed S.A.

Nuclear sequences of ITS and plastid *rbc*L (rubisco large subunit) were chosen to perform

the phylogenetic analysis. We sequenced specimens from Georgia and Spain. Additionally, nine sequences from GenBank were obtained for analysis (Table 2). DNA sequences were aligned using the ClustalW 2.1 software (Larkin *et al*. 2007) and then manually corrected and trimmed. Phylogenetic trees were generated using the Maximum Likelihood method in the Mega ver. 6.06 software (Tamura *et al*. 2013). *Orobanche coerulescens* from Austria was used as the outgroup.

Results

Distribution and ecology of *Orobanche grenieri* in Asia

Orobanche grenieri was found during field research in 2014 on the left bank of the Mtkvari River (Lesser Caucasus, Meskheti Range) which is a rocky slope above an asphalt road between the Borjomi-Kharaguli National Park and the Nedzvi Sanctuary (eastern edge) in Georgia. It grew in Transcaucasian mountain xerophytic vegetation along with *Carpinus*, *Juniperus*, *Pistacia*, at 780–790 m a.s.l. parasitising *Lactuca* cf. *viminea*. The population consisted of over 20 shoots.

The second locality was discovered in the herbarium materials in St. Petersburg (LE). The materials came from Tajikistan, Badakhshan, near the Shakdara River in the Roshtkala area, and were collected from *Lactuca*, probably more than 3000 m a.s.l. (originally identified as *Orobanche cumana*) (Figs. 1 and 3).

Phylogenetic and morphological studies

The morphological characters and results that were obtained using ITS and *rbc*L proved that the plants collected in Georgia and presumed to be *O. grenieri* were indeed closely related to *O. grenieri* found in Spain; they also clearly differed from *O. cernua* and *O. cumana* (Figs. 4 and 5). However, the Georgian and Tajikistanian specimens had a slightly more glandular pubescence on the bracts, corolla and stem than the Spanish plants, but in our opinion the similarity is strong enough to identify them as *O. grenieri*.

Table 2. List of taxa and sequences analysed.

Taxon	GenBank number		Origin	Voucher information or reference
	ITS	*rbc*L		
O. cernua	KP641332 KP641333	KP635273 KP635274	Georgia	*Piwowarczyk R.*, between Rustavi and Idumala, on *Artemisia*, 3 June 2014 (KTC)
O. cernua	EU655626*	–	Israel	unpublished
O. cernua var. *cernua*	–	AY582189*	Spain	Manen *et al.* 2004
O. cernua var. *cernua*	EU655625*	–	Spain	unpublished
O. cernua var. *cumana*	EU655627*	–	Israel	unpublished
O. cernua var. *desertorum*	EU655624*	–	Spain, Grand Canaria	unpublished
O. cumana	–	AF090349*	France	Delavault & Thalouarn 2002
O. coerulescens	AY209235*	–	Austria	Schneeweiss *et al.* 2004
O. coerulescens	–	AY582190*	Austria	Manen *et al.* 2004
O. grenieri	KP641335	KP635272	Georgia	*Piwowarczyk R.*, Kortaneti, 31 May 2014 (KTC)
O. grenieri	KP641334	KP635271	Spain	*Gómes Casares G.* and *Moreno Moral G.*, 7 Jun. 2004, Santibáñez de la Peña, Las Heras de la Peña (herb. Sánchez Pedraja Ó.)
O. grenieri	AY960728*	–	Spain	unpublished

* sequences obtained from GenBank.

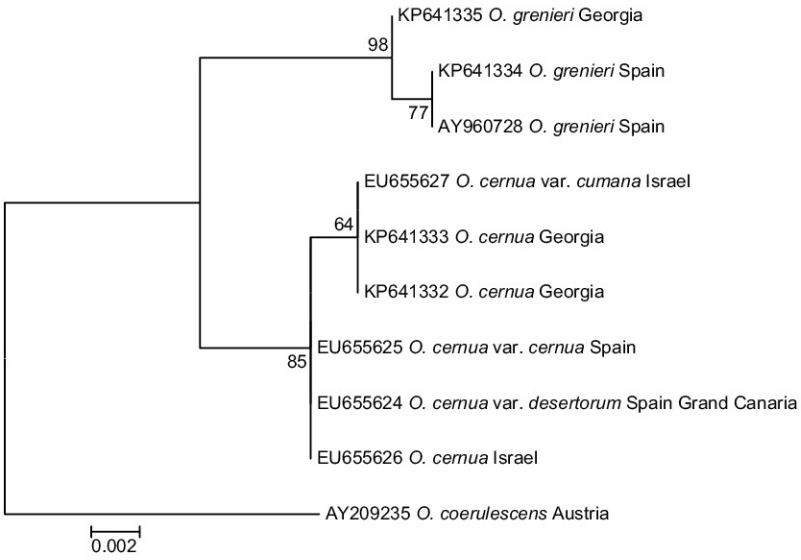

Fig. 4. Phylogram based on ITS sequences (Maximum Likelihood method). Bar indicates distance scale, numbers on nodes indicate bootstrap values.

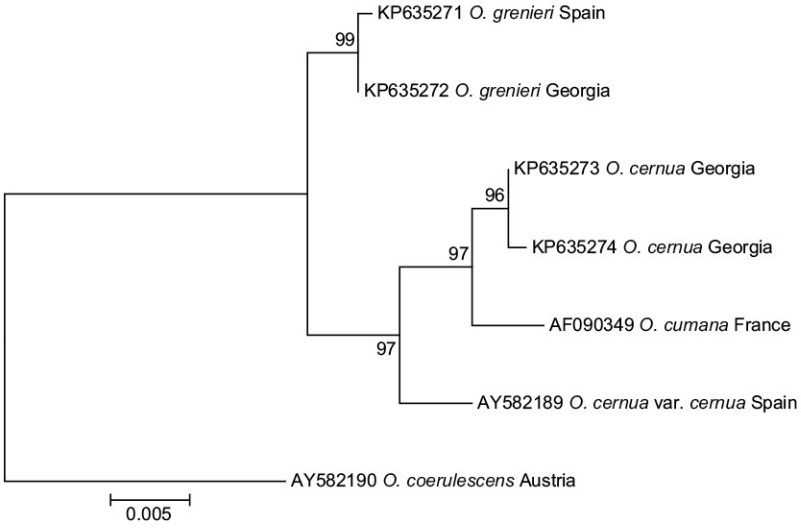

Fig. 5. Phylogram based on *rbc*L sequences (Maximum Likelihood method). Bar indicates distance scale, numbers on nodes indicate bootstrap values.

Discussion

Orobanche grenieri, which until now was known only from southwestern Europe, is much more widely distributed. It occurs in the Lesser Caucasus in southern Georgia (western Asia), and Badakhshan in Tajikistan (central Asia). This conclusion is supported by the morphological evidence as well as phylogenetic studies of the nuclear ribosomal ITS and *rbc*L sequences for the Georgian specimens. The new localities are more than 3000 and 5000 km, respectively, apart from the previously known distribution areas.

The locality of *O. grenieri* in Georgia is outside protected areas in the vicinity of the Borjomi-Kharaguli National Park. The locality is under threat due to the close proximity to the main road, and the population consists of only 20 individuals. Therefore, *O. grenieri* should be listed as endangered (http://jr.iucnredlist.org/documents/RedListGuidelines.pdf) in Georgia. The possible rarity of the species in Tajikistan requires more studies.

Caucasus is considered to be one of the 34 hotspots of biodiversity worldwide (Mittermeier *et al.* 2005). The distributions of *Orobanche*

and related genera and their hosts in this region are still not sufficiently well known (Rätzel & Uhlich 2004, Piwowarczyk 2015). Novopokrovskij and Tzvelev (1958) reported that *O. cernua* occurs in southern Crimea and is parasitic on *Lactuca viminea*. The Caucasian and Central Asian plants that were previously recognised as *O. cumana* along with other species from the section *Inflatae*, which are parasites on Asteraceae, should also be revised (Piwowarczyk *et al.* 2015).

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