Polymorphism in peroxidase isozymes detected in *Phelipanche* (*Orobanchaceae*) from Bulgaria

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Abstract. Five species of the *Orobanchaceae* family in Bulgaria are examined: *Phelipanche purpurea*, *Ph. mutelii*, *Ph. oxyloba*, *Ph. ramosa*, and *Orobanche cumana*. The results of electrophoretic survey in describing peroxidase isozymes have shown ten specific loci. The species are characterized by specific bands of anodic and cathodic migration. The approach could be used for better understanding of the taxonomic relationships in *Orobanchaceae*.

Key words: *Orobanche*, peroxidase, *Phelipanche*

Introduction

Broomrapes (*Orobanchaceae*) are parasites on many crops and wild plants. Their seedlings attach to the root system of the host plant, establishing a connection with the vascular system of the host via a specialized organ – haustorium, and deprive the host of water, mineral nutrients and metabolites. Although the main interest in *Orobanchaceae* has been its control, there are some other important areas of practical concern in this parasitic weed. Its adaptation, from a physiological viewpoint, as an obligate parasite, its origin and evolution out of green plants, and its population structure also present some aspects for consideration (Roman & al. 2002).

Seven species of genus *Phelipanche* (Pomel) Sojak are known in the Bulgarian flora, included in two genera, namely: *Phelipaea* Desf. (Velenovsky 1891, 1898) and *Orobanche* sect. *Trionychon* Wallr. (Stojanov & Stefanov 1925; 1948; Georgiev 1937; Stojanov & al. 1967; Kozhuharov 1992; Delipavlov 1995; Cheshmedziev 2003). Of these, *Ph. mutelii* (Shultz) Pomel, *Ph. ramosa* (L.) Pomel, *Ph. nana* (Reut.) Sojak, *Ph. oxyloba* (Reut.) Sojak, *Ph. arenaria* (Borkh.) Pomel, and *Ph. purpurea* (Jacq.) Sojak have been confirmed, while the status of *Ph. aegyptiaca* (Pers.) Pomel remains uncertain (Stoyanov 2005a). *Orobanche* L. s.str. contains about 19 species in Bulgaria (Stojanov & Stefanov 1925, 1948; Georgiev 1937; Stojanov & al. 1964; Kozhuharov 1992; Delipavlov 1995; Cheshmedziev 2003).

Phelipanche is divided into two sections: *Arenariae* (Andary) Teryokhin and *Phelipanche* (Teryokhin 1997). However, species delimitation in the second section is controversial. *Phelipanche oxyloba* differs from *Ph. ramosa* by the shape of the lower lip lobes (Chater & Webb 1978), and from *Ph. mutelii, Ph. ramosa* and *Ph. nana* by the bract-to-calyx ratio and by the shape of corolla lobes (Kozhuharov 1992). Teryokhin (1997) treats *Ph. oxyloba* and *Ph. nana* as subspecies of *Ph. ramosa* and *Ph. mutelii* as a separate species. On the other hand, *Ph. mutelii* and *Ph. nana* are often accepted as subspecies of *Ph. ramosa*, while *Ph. oxyloba* is treated as a separate species (Chater & Webb 1978; Musselman 1994).

The morphological and anatomical features of *Orobanche* and *Phelipanche* species are predominantly influenced by fluctuations in the environmental conditions. Therefore, such molecular markers as

isozymes are potentially useful for taxonomic purposes.

Knowledge of genetic diversity in parasitic plants is still limited and needs to be expanded (Verkleij & al. 1994). Isozymes have been used to investigate genetic variability among populations of *O. crenata* from Syria and Spain (Verkleij & al. 1991a, b). These data suggest that molecular markers may be a suitable method for identification of pathogenic groups in parasite populations (Roman & al. 2002). Generally, allozyme markers are often used to investigate systematic problems, or to measure the levels of variation within and among natural plant populations (Hamrick & Godt 1990). Although nowadays new, DNA-based molecular techniques are used, isozymes still represent a powerful tool for evaluation of gene variability within and between populations of plants (Zeidler 2000).

Peroxidase profiles have been used as taxonomic markers and measures of interpopulation variability in the *Sphagnum subsecundum* complex, where the lack of morphological discontinuities renders the separation of taxa based only on these features controversial and pointless (Krzakowa & Melosik 1999). Eleven enzymes have been used to describe isozyme variation between *Elytrigia ×litorea* and its parents *E. repens* and *E. junceiformis* (Angelov 2003).

The aim of this study is to shed light on the relationships between different taxa of *Phelipanche* collected in Bulgaria with the help of morphological data and electrophoretically detected peroxidase patterns.

Materials and methods

Living plant material from different accessions was collected at different locations and from different host plants in Bulgaria during the years 2003-2005 (Table 1). Six species of *Phelipanche* have been investigated: two of sect. *Arenariae* (*Ph. arenaria* and *Ph. purpurea*), and four of sect. *Phelipanche* (*Ph. ramosa, Ph. nana, Ph. mutelii* and *Ph. oxyloba*). One species of genus *Orobanche* s.str. – *O. cumana* Wallr. has been taken for comparative purposes in case of future studies. After washing the samples and cutting of pieces from the subterranean parts of flowering specimens, they were ground in 0.1 n HCl, 0.2 M Tris, 0.005 M L-cistein, 0.5 M sucrose, pH = 4. Polyvinylpyrrolidone (PVP) 0.1 % was added to the extraction buffer to bind polyphenols. After straining of extracts through a nylon sieve and centrifugation at 6000 rpm for 15 minutes, the detached supernatant was kept frozen at –18 °C to retain activity for a few months. The electrophoresis was carried out in 15 % horizontal starch gel (pH = 8.4) for two hours, at 120 V (Stoyanova 1987). All procedures for the extraction and separation of peroxidase isozymes were carried out at +4 °C. The staining protocols followed an earlier described method (Stoyanova 1987). The developed peroxidase patterns were photographed with a digital camera (Daisy PhotoClip DM334) in two replicates: one hour after pouring in the substrate solution (1 % benzidin-dihydrochlorid, pH = 4.7), followed by adding of 0.1 % H_2O_2, and overnight at +4 °C.

Results and discussion

Variation of peroxidase (PO.1.11.1.7) izozymes in *Phelipanche* species is presented by the characteristic band patterns of different electrophoretic mobility and intensity (Fig.1). Specific peroxidase izozymes require different times to develop. Therefore, we use two photographs for documentation of the peroxidase patterns: one hour after the staining and overnight. As the peroxidase pattern of *Ph. arenaria* has developed very poorly, it will not be discussed.

Ten major peroxidase components have been assigned according to their mobility (Fig. 1). Each species is characterized by specific bands of anodic and cathodic migrations. Two of the bands (4′ and 9′) are very close to the respective major bands (4 and 9). The total number of major PO loci is 10, while the number of alleles is 22 (Table 2). PO-4 (PO-4′) is present in *Ph. mutelii* (incl. al-

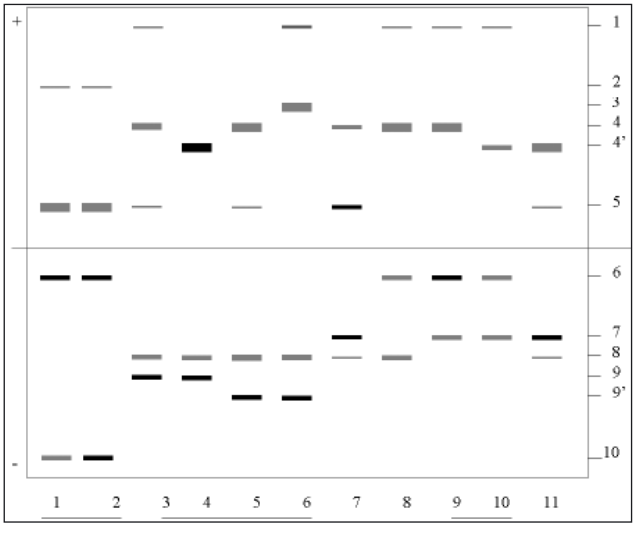

Fig. 1. Zymograms of electrophoretically detected peroxidase phenotypes in *Phelipanche* and *Orobanche* species: **1-2**, *Ph. purpurea*; **3-6**, *Ph. mutelii* (4 albinistic specimens); **7**, *Ph. oxyloba*; **8**, *Ph. nana*; **9**, *Ph. ramosa* var. *monoclonos*; **10**, *Ph. ramosa* var. *ramosa* f. *cyanea*; **11**, *Orobanche cumana.*

binistic specimens), *Ph. oxyloba, Ph. nana,* both taxa of *Ph. ramosa*: *Ph. r*. var. *monoclonos* (Wallr.) Delip. and *Ph. r.* var. *ramosa* f. *cyanea* G. Beck, as well as in *O. cumana*. PO-5 is observed in *Ph. purpurea*, *Ph. mutelii, Ph. oxyloba,* and in *O. cumana*; PO-6 in *Ph. purpurea*, *Ph. nana* and both taxa of *Ph. ramosa*; PO-8 in *Ph. mutelii* (incl. albinistic specimens), *Ph. oxyloba* and *Ph. Nana,* as well as in *O. cumana*; PO-7 in *Ph. oxyloba* and both taxa of *Ph. ramosa*. The fastest migrating component PO-1 has been found in *Ph.nana,* both taxa of *Ph. ramosa* and in *Ph. mutelii* (with the exception of albinistic specimens).

The detected differences confirm the earlier observed differences between Bulgarian *Phelipanche* species on the basis of seed morphology (Stoyanov 2005b). The most prominent differences are between *Ph. purpurea* – the rare alleles PO-2 and PO-10 have been confined for *Ph. purpurea* – and the group of branched species (*Ph. ramosa*, *Ph. nana*, *Ph. mutelii,* and *Ph. oxyloba*) and are thus in agreement with the separation of two sections *Arenariae* and *Ramosae*. On the other hand, in section *Ramosae* differences have been observed between *Ph. ramosa, Ph. mutelii* and *Ph. oxyloba*, while those between *Ph. ramosa* and *Ph. nana* have been less pronounced, thus suggesting treatment of both taxa as subspecies of *Ph. ramosa* s.l. A difference between *Ph. ramosa var. monoclonos* (SOA 56934) and var. *ramosa* f. *cyanea* (SOA 56938) has been observed in the presence/absence of PO-4 and PO-4'. Intraspecific polymorphisms has been found in *Ph. mutelii*, PO-3 appearing in some plants but not in the albinistic specimens. More than one allele has been detected in *Ph. purpurea* (PO-10) and in *Ph. mutelii* (PO-1, PO-4, PO-5). Although the present study was not aimed at describing the max-

Table 1. ***Phelipanche*** **species collected in Bulgaria.**

Region[1]: MGRS location[2], location description, altitude[3] / host[4] / date	SOA voucher	Sample[5]
I ***Ph. ramosa***		
A **var. *monoclonos***		
18: LG-26, IPGR Sadovo, 140 m / L. / 29.07.2004	56934	A9, B14
B **var. *ramosa* f. *cyanea***		
13: GL-39, near the frontier-post of Ilinden, 550 m /?/ 17.06.2005	58127	B16
14.1: GM-20, near Popovi Livadi, 1200 m / ? / 18.06.2005	58128	B15
14.2: GM-13, near Dobrinishte, 840 m / N. / 1.09.2004	56933	A11
15: GM-04, between Razlog and Bansko, 825 m / N. / 31.08.2004	56938	A10
		A12
II ***Ph. nana***		
15: FM-78, over Doupnitsa, 535 m / ? / 19.06.2005	58129	B13
III ***Ph. mutelii***		
17.2: LG-52, near Komouniga, 400 m / N. / 14.07.2005	58130	B7
17.3: LF-67, between Makaza pass and Orlitsa, 600 m / N. / 14.07.2005	58131	B8
17.3: LF-99 near Rogach, 272 m / N. / 14.07.2005	58132	B4
17.3: LG-62, over Chernoochene, 564 m / N. / 14.07.2005	58133	B5
17.3: LG-71, under Perperikon fortress, 380 / N. / 14.07.2005	58134	B6
17.3: MF-28, near Siv Kladenets, 194 m / N. / 15.07.2005	58135	B9
17.3: MF-29, near Svirachi, 235 / N. / 14.07.2005	58136	B3
18: LG-15, near Kouklen, 300 m / L. / 14.09.2004	56936	A4, A6
18: LG-15, near Kouklen, 300 m / N. / 14.09.2004	56937	A5
(Albinistic specimens):		
17.3: LF-67, between Makaza pass and Orlitsa, 600 m / N. / 14.07.2005	58137	B10
17.3: LF-99, near Rogach, 272 m / N. / 14.07.2005	58138	B11
IV ***Ph. oxyloba***		
18: LG-25, over Asenovgrad, 400 m / ? / 4.05.2003	56540	A8, B12
V ***Ph. arenaria***		
18: KG-86, near Trivoditsi, 200 m / A. mil. / 1.06.2005	58139	B1
VI ***Ph. purpurea***		
5.3: NH-63, Pomorie pass 200 m / A. nob. / 17.06.2004	56900	A3, B2
18: LG-24, near Martsiganitsa chalet, 1240 m / A. cl. / 26.06.2004	56897	A1
	56935	A2
VII ***Orobanche cumana***		
18: LG-26, fields near Sadovo, 140 m / H. / 25.06.2004	56939	A7, A13

1 – Floristic regions according to *Flora R Bulgaricae*: **5.3** – Balkan Range (*Eastern*); **14.2** – Pirin Mts (*Northern*)**; 15** – Rila Mts, **17.2** – Rhodopi Mts (*Central*); **17.3** – Rhodopi Mts (*Eastern*); **18 –** Thracian Lowland.

2 – The coordinates are given in 10×10 UTM squares and stored as MGRS codes

3 – Altitude (meters a.s.l.).

4 – Host plant abbreviations: *A. – Achillea***;** *A. cl. – A. clypeolata***;** *A. mil. – A. millefolium***;** *A. nob. – A. nobilis***;** *H. – Helianthus***;** *L. – Lycopersicon esculentum***;** *N. – Nicotiana tabacum***.**

5 – Sample number.

Table 2. Peroxidase phenotypes observed in *Phelipanche* species as described by the number of loci and number of alleles per species.

Plant species	Number of loci	Number of alleles
Phelipanche purpurea	PO-2	1
	PO-5	1
	PO-6	1
	PO-10	2
Ph. mutelii	PO-1	2
	PO-3	1
	PO-4	2
	PO-5	2
	PO-8	2
	PO-9	1
	PO-9′	1
Ph. mutelii – albinistic specimens	PO-4′	1
	PO-8	1
	PO-9	1
Ph. oxyloba	PO-4	1
	PO-5	1
	PO-7	1
	PO-8	1
Ph. nana	PO-1	1
	PO-4	1
	PO-6	1
	PO-8	1
Ph. ramosa var. *monoclonos*	PO-1	1
	PO-4	1
	PO-6	1
	PO-7	1
Ph. ramosa var. *ramosa* f. *cyanea*	PO-1	1
	PO-4′	1
	PO-6	1
	PO-7	1
Orobanche cumana	PO-4′	1
	PO-5	1
	PO-7	1
	PO-8	1

imum number of alleles, some readable differences between *Phelipanche* species have been observed. Although there was a number of loci specific for the *Phelipanche* species, some of them also appeared in *O. cumana*.

The plants of three species used in this study have been collected from different host plants (Table 1) and thus the different isozyme spectra could correlate with the different host plants. Differences detected in PO-10 for *Ph. purpurea* could be associated with the different host plant, but the results for *Ph. mutelii* and *Ph. ramosa* have shown no relationship between peroxidase isozyme spectra and the host plant species. Therefore, variation in isozymes could be more firmly bound to different taxonomic entities.

Conclusions

The results of an electrophoretic survey of peroxidase isozymes in *Phelipanche* species from Bulgaria revealed the presence of ten specific loci. The sections and species are characterized by specific bands of anodic and cathodic migration. Intraspecific differences were found in both varieties of *Ph. ramosa* and in the albinistic forms of *Ph. mutelii*. The minor differences between *Ph. ramosa* and *Ph. nana* suggest their treatment as subspecies of one species only. This approach can provide further evidence for better understanding of the taxonomic relationships between the *Phelipanche* species.

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