Orobanche species and population discrimination using intersimple sequence repeat (ISSR)

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Summary

Orobanche species are commonly identified using morphological characteristics. In many cases, the distinction of closely related species is difficult, and a molecular tool is more suitable to differentiate them. In this study, genomic polymorphism between morphologically distinct species was investigated through amplification by polymerase chain reaction (PCR) of intersimple sequence repeat (ISSR) regions. Five primers were used to study genetic variation in the morphologically distinct species *O. hederae* and *O. amethystea*, as well as the closely related species *O. cernua* and *O. cumana*. For the first two species, all the primers detected genetic polymorphism. Anchored primers allowed the identification of more specific molecular markers than non-anchored tri- and tetranucleotide primers. Genetic polymorphism was investigated among three *O. hederae* populations using the two types of primer. One non-anchored and two anchored primers detected intraspecific variation, which was not correlated with the geographical location of those populations. The primer $(GATA)_4$ detected polymorphism between five specimens each of *O. cernua* and *O. cumana* species collected from different countries, permitting these two closely related species to be clearly differentiated. This study demonstrated that ISSR markers can be highly reliable for precise identification of *Orobanche* species.

Keywords: broomrape, taxonomy, molecular markers.

Introduction

Orobanche spp. (broomrapes) are root parasitic angiosperms devoid of leaves and chlorophyll and entirely dependent on their host for reduced carbon. Some species cause severe damage to crop plants in the Mediterranean area and in south-eastern Europe (Parker, 1994). The most noxious belong to the sections *Trionychon* (*O. aegyptiaca* Pers. and *O. ramosa* L.) and *Orobanche*, also known as *Ospreleon* (*O. cumana* Warll., *O. cernua* Loefl., *O. crenata* Forskal. and *O. minor* Sm.). The lack of clear morphological differences to distinguish between species leads, in many cases, to difficulties in identification. Moreover, as reported by Musselman (1994), the taxonomy of the *Orobanche* is further complicated by inherent variability and the influence of the host plant. It is only in recent years that the usefulness of molecular markers has been investigated as a means of characterizing and discriminating the different species more precisely. The random amplified polymorphic DNA (RAPD) technique is often used to discriminate between closely related species or to detect variability within an *Orobanche* population (Paran *et al.*, 1997). Polymorphic amplification products were found to be useful in distinguishing between species such as *O. cumana*, *O. aegyptiaca* or *O. cernua* (Joel *et al.*, 1998a). According to Roman & Rubiales (1999), polymorphic RAPD bands also allow the detection of small genetic differences among individuals within a population. Using this technique, Gagne *et al.* (1998) demonstrated low levels of genetic variability within *O. cumana* populations from Bulgaria, Romania, Turkey and Spain. *Orobanche* seeds have also been used for species identification by RAPD and sequence-characterized amplified region (SCAR) techniques by Portnoy *et al.* (1997) and Joel *et al.* (1998b). In other studies, the plastid genome of some parasitic plants was carefully investigated for either its size or its gene content (de Pamphilis & Palmer, 1990; Wolfe *et al.*, 1992; Thalouarn *et al.*, 1994). Four *Orobanche* species

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(*O. fasciculata* Mult., *O. corymbosa* Rydh., *O. ramosa* and *O. cumana*) were investigated for their *rbcL* nucleotide sequence by Wolfe & de Pamphilis (1997). More recently, the authors demonstrated the use of the *rbcL* plastid pseudogene as a molecular tool for *Orobanche* identification (Benharrat *et al.*, 2000).

In plant and animal genomes, some regions are highly variable and contain simple sequence repeats (SSR) also known as microsatellites. In plants, microsatellites are abundant and spread throughout the genome. They have been used to investigate genetic variations in both cultivated species such as tomato (Vosman & Arens, 1997) as well as natural populations such as *Penstemon* spp. (Wolfe *et al.*, 1998). Microsatellites have also been used as genetic markers of inter- and intraspecific variations (Soranzo *et al.*, 1999). Although microsatellites are widely used in the genetic analysis of many plant species, no information on *Orobanche* spp. is available yet.

In this study, a rapid technique was applied using a small amount of DNA from plant material where identification based on morphological characteristics was difficult. This method is a derived technique of microsatellite characterization based on amplification by polymerase chain reaction (PCR) of intersimple sequence repeat (ISSR) regions primed by a single oligonucleotide corresponding to part of the microsatellite motif. In these conditions, only sequence regions flanked by two identical and inverted microsatellites will be amplified.

The aim of the present study was to associate new molecular characters, as ISSR, to closely related species belonging to the same section such as *O. hederae* Duby and *O. amethystea* Thuill. or *O. cumana* and *O. cernua*, which are currently discriminated using morphological characters. In addition, the technique was used to distinguish between different populations belonging to the same species, *O. hederae*.

Materials and methods

Specimen collection

Orobanche hederae and *O. amethystea* were collected from natural populations (respectively fixed on *Hedera helix* Duby and *Eryngium campestris* L.) in the west and centre of France. In total, 100 individuals were sampled (50 specimens of each species). The interspecific variation study was conducted on 10 different specimens of each of these species, and the replicability was checked five times. The study of intraspecific variation was focused on *O. hederae* specimens collected in Nantes and Blois, two locations 250 km apart. Within Nantes, samples were collected from two separated populations 500 m apart (Nantes A and B).

Orobanche cumana and *O. cernua* were collected from infested sunflower (*Helianthus annuus* L.) fields in Spain (three specimens of *O. cumana* and four specimens of *O. cernua*), Bulgaria (one specimen of *O. cumana*), Israel (one specimen of *O. cernua*) and Romania (one specimen of *O. cumana*).

DNA extraction

Approximately 100 mg of frozen tissue (shoot and inflorescence) was ground in a liquid nitrogen precooled mortar. Total DNAs were extracted from the powdered samples using a Nucleon Phytopure DNA extraction kit (Amersham, Buckinghamshire, UK). According to the manufacturer's protocol, after breaking of the cell wall and cell lysis in reagent (potassium SDS), chloroform and Nucleon Phytopure resin (containing free boric acid) are added. The polysaccharides are bound by the resin particles. After centrifugation, the DNA-containing upper phase is transferred to a fresh tube, and DNA is precipitated with cold isopropanol. DNA is recovered as a pellet by centrifugation at a minimum of 4000 *g* for 5 min, washed with cold 70% ethanol, dried, dissolved in TE buffer and stored at −20 °C until use.

PCR amplification

DNA variations were studied using five primers, $(CAA)_5$, $(GACA)_4$, $(GATA)_4$, $(CA)_6RG$ and $(CTC)_4RC$, synthesized at ESGS, Cybergene Group, Evry, France (Table 1). PCR amplifications were performed in a 25-μL mixture containing 20 ng of template DNA, 200 μM each dNTP, 4 μM ISSR motif primer, 2.5 μL of 10× buffer (Appligene, Illkirch, France) and 0.5 U of *Taq* polymerase (Appligene) in a PCR thermal cycler (Perkin-Elmer Cetus, Norwalk, USA). Thermal cycling was as follows: a first denaturing step (94 °C, 1 min) followed by 35 cycles each including a denaturing step (94 °C, 1 min), an annealing step [TM + 2 °C $(GATA)_4$ for 1 min; TM + 4 °C $(GACA)_4$ for 1 min; TM + 12 °C $(CAA)_5$ for 1 min; TM + 1 °C $(CA)_6RG$ for 1 min and TM−5 °C $(CTC)_4RC$ for 1 min], as well as an extension step (72 °C, 4 min). The last cycle consisted of an extension step of 5 min at 72 °C. The reaction mixture

Table 1 List of the ISSR primers used in this study

Primer code	Primer sequence
$(CAA)_5$	5′-CAA CAA CAA CAA CAA-3′
$(GACA)_4$	5′-GAC AGA CAG ACA GAC A-3′
$(GATA)_4$	5′-GAT AGA TAG ATA GAT A-3′
$(CA)_6RG$	5′-CAC ACA CAC ACA RG-3′
$(CTC)_4RC$	5′-CTC CTC CTC CTC RC-3′

was then stored at 4 °C. Replicability of amplification was checked five times.

Electrophoresis

Aliquots of PCR products (10 μL) were mixed with 2 μL of loading buffer [50% glycerol (*V*/*V*), 0.25% bromophenol blue (p/*V*) and 0.25% xylene cyanol (p/*V*)] and electrophoresed on 1.5% agarose gel. After staining with ethidium bromide, band patterns were visualized under a UV transilluminator.

Southern hybridization

Southern hybridizations of ISSR gels were performed using a probe labelled by random-primed incorporation of digoxygenin (DIG)-labelled deoxyuridine triphosphate. The probe was prepared by excising the 1600-bp polymorphic band provided by the tetranucleotide primer $(GACA)_4$ and specific to *O. hederae* specimens. This amplification product was purified using a QIA quickgel extraction kit from Qiagen (Courtaboeuf, France). The *O. amethystea* $(GACA)_4$ amplification pattern, separated on 1.5% agarose gel, was blotted on nylon membrane and hybridized with the 1600-bp *O. hederae* probe. Detection was conducted using a DIG luminescent kit (Roche Diagnostics, Meylan, France).

Results

Interspecific variations among Orobanche *species belonging to the subsection* Minores

To check for genetic variation between *O. hederae* and *O. amethystea*, four different primers were used: $(GACA)_4$, $(CAA)_5$, $(CA)_6RG$ and $(CTC)_4RC$. For each of the four primers, a polymorphic banding pattern was observed (Table 2). The tri- and tetranucleotide primers $(CAA)_5$ and $(GACA)_4$ provided amplification patterns (Fig. 1 and Table 2) of *O. hederae* with three specific polymorphic bands (1200, 1400 and 1600 bp). In order to confirm this interspecific polymorphism, a probe for the 1600-bp product was synthesized. Southern hybridization experiments confirmed that this polymorphic band is specific to *O. hederae*, as no hybridization could be observed with the *O. amethystea* specimens (data not shown). The di- and trinucleotide repeat motifs with an anchoring sequence of two nucleotides [$(CA)_6RG$ and $(CTC)_4RC$] provided several specific markers for each of the two species, as *O. hederae* and *O. amethystea* could be differentiated by eight specific and reproducible polymorphic bands (Table 2). For example, the $(CA)_6RG$ primer gave two distinct PCR products of 250 and 300 bp specific to *O. hederae*, whereas *O. amethystea* was characterized by two other distinct bands of 650 and 900 bp (Fig. 1). In the PCR fingerprint profiles obtained using $(CTC)_4RC$, three *O. amethystea* molecular

Table 2 Interspecific polymorphism revealed by intersimple sequence repeat (ISSR)

Primers	Molecular markers (size, bp)	Species
$(CAA)_5$	1400	*O. hederae*
$(GACA)_4$	1200, 1600	*O. hederae*
$(CA)_6RG$	250, 300	*O. hederae*
	650, 900	*O. amethystea*
$(CTC)_4RC$	900	*O. hederae*
	2100, 850, 300	*O. amethystea*
$(GATA)_4$	270, 590	*O. cernua*
	250, 300	*O. cumana*

Orobanche hederae and *O. amethystea* are characterized by one, two or three molecular markers.

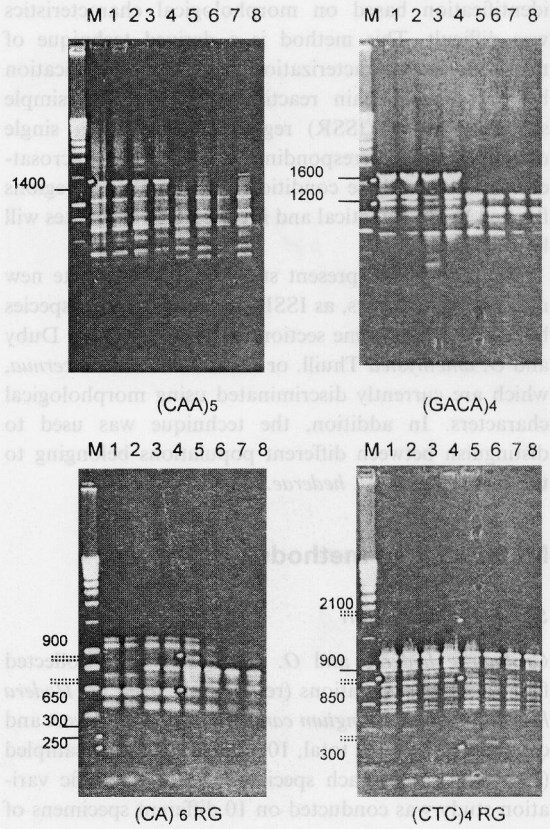

Fig. 1 Interspecific variations among *O. hederae* and *O. amethystea* using four primers, $(CAA)_5$, $(GACA)_4$, $(CA)_6RG$ and $(CTC)_4RC$, which provide specific markers for each species. M, molecular marker (Smart Ladder, Eurogentec); lanes 1–4, *O. hederae*; lanes 5–8, *O. amethystea*. Solid lines, *O. hederae* specific markers; dotted lines, *O. amethystea* specific markers.

markers were obtained, two of which were strong and very easily observed (300 and 850 bp), while the third was faint but present in all specimens of *O. amethystea*. A 900-bp molecular marker was characteristic of *O. hederae* (Table 2).

Interspecific variations among Orobanche *species belonging to the subsection* Coerulescentes

Orobanche cumana and *O. cernua* are closely related and difficult to distinguish using morphological or behavioural characteristics alone. To discriminate between these species, a $(GATA)_4$ primer was used, giving rise to bands specific for *O. cumana* and *O. cernua* (Table 2). Two bands of 250 and 300 bp (Fig. 2) were observed for the five *O. cumana* specimens. Two sharp bands (270 and 590 bp) appeared on the agarose gel for the five *O. cernua* specimens. No individual variation was observed even when experimental conditions generated the appearance or disappearance of non-replicable bands.

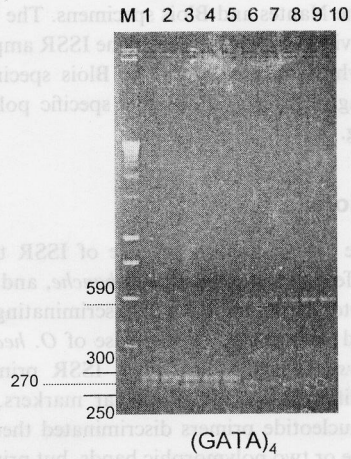

Fig. 2 Interspecific variations among *O. cumana* and *O. cernua* using $(GATA)_4$. M, molecular marker (Smart Ladder, Eurogentec); lanes 1–5, *O. cumana*; lanes 6–10, *O. cernua*. Lanes 1–3 and 6–9 correspond to samples collected in Spain; lane 4, in Bulgaria; lane 5, in Romania; lane 10, in Israel. Solid lines, *O. cumana* specific markers; dotted lines, *O. cernua* specific markers.

Intraspecific variation among O. hederae *populations*

Genetic variability among three *O. hederae* populations was investigated using four ISSR primers, $(CAA)_5$, $(GACA)_4$, $(CA)_6RG$ and $(CTC)_4RC$. With $(GACA)_4$, there was no variation among the *O. hederae* specimens collected in Nantes (A and B) and Blois; however, the three other primers provided polymorphic band patterns (Fig. 3). The PCR profiles generated using the $(CAA)_5$ primer allowed the differentiation of *O. hederae* specimens into two groups. Specimens originating from Nantes A presented two polymorphic bands (1700 and 1800 bp), and specimens collected in Nantes B and Blois were characterized by a 1750-bp band. This genetic polymorphism within *O. hederae* populations was confirmed by the $(CA)_6RG$ primer, which gave a distinct specific band (800 bp) in specimens from Nantes B and Blois. These two primers, $(CAA)_5$ and $(CA)_6RG$, displayed an ISSR genetic marker shared by two geographically distant populations. With the fourth primer, $(CTC)_4RG$, genetic differentiation was observed

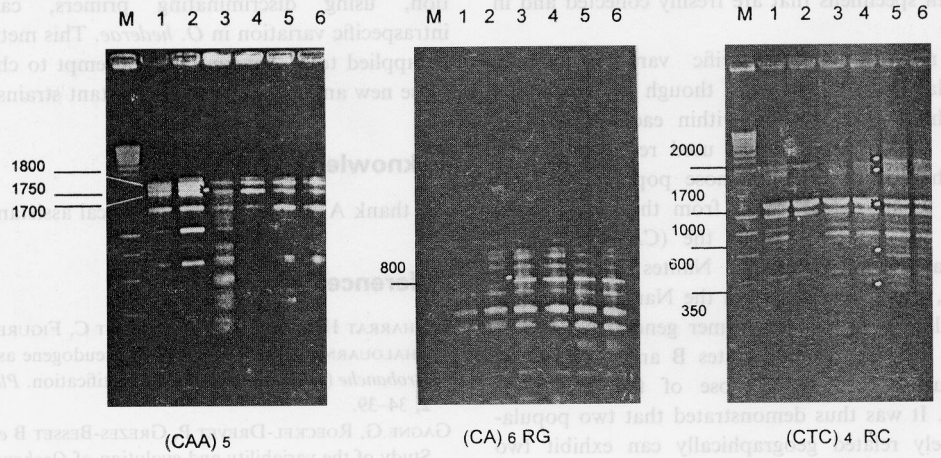

Fig. 3 Intersimple sequence repeat (ISSR) fingerprints of three *O. hederae* populations using the primers $(CAA)_5$, $(CA)_6RG$ and $(CTC)_4RC$. M, molecular marker (Smart Ladder, Eurogentec); lanes 1 and 2, Nantes A population; lanes 3 and 4, Nantes B population; lanes 5 and 6, Blois population.

between the Nantes and Blois specimens. The Nantes A and B individuals presented the same ISSR amplification pattern, which differed from the Blois specimens, the latter being characterized by five specific polymorphic bands (Fig. 3).

Discussion

This is the first report on the use of ISSR to display genetic differences among the *Orobanche*, and it clearly demonstrates their usefulness in discriminating between species and populations. In the case of *O. hederae* and *O. amethystea*, each of the four ISSR primers used revealed different specific molecular markers. The tri- and tetranucleotide primers discriminated these species by only one or two polymorphic bands, but primers with anchoring sequence revealed more polymorphism. All these ISSR primers revealed six polymorphic bands specific to *O. hederae* and five to *O. amethystea*. Although the subsection *Minores* is known as a complex group in the *Orobanche* genus, the ISSR amplification method appears to be an effective tool for discriminating between the species. Whereas molecular studies based on restriction fragment length polymorphism (RFLP) or *rbcL* sequence have not been sufficient to discriminate *O. hederae* from *O. amethystea* (Benharrat *et al.*, 2000), the ISSR patterns obtained did provide specific markers for each species. This method is of potential taxonomic interest because the results obtained are in agreement with *Orobanche* classification based on morphological characters (e.g. *O. hederae* and *O. amethystea*, which can be distinguished by their stigma colour). The ISSR method provides a means of species determination that can be used at any time, in contrast to identification keys based on morphological characteristics, which are only usable with specimens that are freshly collected and in flower.

With regard to intraspecific variation among the populations studied, even though no individual polymorphism was detected within each population, three out of the four primers used revealed distinct polymorphic bands between those populations. This difference seems not to result from the geographical origin of the populations, as the $(CA)_6RG$ primer revealed a band common to Nantes B and Blois specimens, which was absent in the Nantes A individuals. Similarly, the $(CAA)_5$ primer generated patterns that were identical in the Nantes B and Blois populations but differed from those of the Nantes A specimens. It was thus demonstrated that two populations closely related geographically can exhibit two different PCR patterns. These results demonstrate the potential usefulness of the ISSR method for discrimination between populations.

Compared with previous results with RFLP (Benharrat *et al.*, 2000), the ISSR method demonstrated a higher discriminating power for the study of inter- and intraspecific variation. In addition, where ISSR and RAPD have been compared in studies of genetic variability, many researchers have found that the former produces more reliable and reproducible bands (Nagaoka & Ogihara, 1997; Qian & Hong, 2001). This is on account of the primer annealing temperature, which is higher than the theoretical temperature of primer TM in ISSR amplification. Moreover, as microsatellites are frequent and widely distributed throughout the genome, the ISSR targets are abundant. It has been shown that the ISSR technique is more economical than the RAPD (Yang *et al.*, 1996), SCAR and AFLP methods. Compared with SSR markers, where the flanking regions of the SSR motifs have to be known in advance, ISSR amplification takes advantage of the fact that no prior sequence information is required, and the results are therefore obtained more rapidly.

In this study, we have shown clearly, using the $(GATA)_4$ primer, how two closely related species (*O. cumana* and *O. cernua*), which are involved in high yield losses on sunflower, can be easily discriminated using ISSR markers. Results obtained for *O. cumana* and *O. cernua* showed that the low number of bands and the patterns obtained were easy to interpret, in contrast to a study made on these two parasites using RAPD markers (Paran *et al.*, 1997), in which there were over 20 bands, and the patterns obtained were similar for both species.

Orobanche cumana is now the main pest on sunflower (Gagne *et al.*, 1998) and, during the last 10 years, some virulent new populations have appeared that are impossible to discriminate using morphological characteristic alone. This study showed how ISSR amplification, using discriminating primers, can detect intraspecific variation in *O. hederae*. This method could be applied to *O. cumana* in an attempt to characterize these new and economically important strains.

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