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**Published on:** 09 Apr 2019 - bioRxiv (Cold Spring Harbor Laboratory)

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1 **Development of specific molecular markers to distinguish and quantify broomrape species in a**
2 **soil sample from infected field**

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14 **Keywords:** Plant parasite, *Phelipanche aegyptiaca, Orobanche* spp., ribulose-1,5-
15 bisphosphate carboxylase (*rbcL*), Internal transcribed spacer (*ITS*).

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17 **Conflict of interest:** The authors have declared no conflict of interest

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23 **Abstract**


24 Broomrapes (*Orobanche* and *Phelipanche*) are obligate holoparasites that cause heavy damage to
25 numerous crops, reducing the yield and its quality. The parasite develops in the soil and exerts the
26 greatest damage prior to its emergence; therefore the majority of field loss may occur before diagnosis
27 of infection. Because of the parasite tiny seed size (200 to 300 µm) and dormancy for several decades
28 in the field, it is very difficult to diagnose the parasite by conventional methods. Therefore, to restrict
29 the parasite seeds spread and contamination to other commercial fields, development of DNA-based
30 molecular markers to identify and quantify broomrape species in a soil sample is much needed. In this
31 study, we developed a specific molecular marker (RbcL-M) based on *rbcL* (large subunit of the
32 ribulose-bisphosphate carboxylase) gene from *Orobanche crenata* to differentiate between *Orobanche*
33 *crenata* and *Orobanche cumana.* Likewise, a specific marker (ITS100) based upon unique sequences
34 in the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA of *Phelipanche*
35 *aegyptiaca* to quantify three species of the parasite (*P. aegyptiaca*, *O. crenata* and *O. cumana*) in a
36 soil sample was developed. Genomic DNA was extracted from soil samples artificially infested with
37 broomrape seeds or tissue of *P. aegyptiaca*, *O. cumana* and *O. crenata* and subjected to PCR analysis.
38 RbcL-M marker successfully amplified a PCR product (1300bp) when *O. crenata* seeds or tissues
39 (collected from several locations in Israel) were added to the soil samples. The same marker amplified
40 a PCR product (1000bp) when *O. cumana* seeds or tissues were added to the soil samples. RbcL-M
41 marker did not amplify soil samples with seeds or tissues of *P. aegyptiaca* or any soil-borne DNA.
42 Furthermore, using ITS-100 marker and Real-Time PCR analysis, allowed quantitative diagnostic of
43 the parasite in a soil sample from infected sunflower field. As expected the universal internal control
44 primer (UCP-555) amplified a PCR product (555bp) when genomic DNA extracted from soil samples
45 with or without broomrape tissues. The development of an efficient, simple and robust molecular
46 marker to detect and distinguish between broomrape species, has a significant insights on assessment
47 the level of infestation and planning eradication program to the parasite in a field crop.


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49 **INTRODUCTION**

50 Broomrapes (*Orobanche* and *Phelipanche* spp.) are member of Orobanchaceae family (C. Parker,
51 1993), is an underground root parasite mostly affecting agricultural crop plants (McNeal et al., 2013).
52 These parasitic plants are unable to synthesize their photosynthetic assimilate due to lack of
53 chlorophyll; and totally depend on host plants for the germination and growth by feeding through
54 haustorium (Westwood et al., 2010). The Orobanchaceae family contains the largest number of
55 parasitic species that form haustoria in roots, for example *Orobanche, Phelipanche* species (Yoshida
56 et al., 2016). Haustoria are special organ of parasitic plant which invades host tissue and serve as
57 connecting bridge that allows the parasite to obtain water, mineral and nutrient from host (Joel and
58 Losnergoshen, 1994; Westwood et al., 2010).The natural habitats of broomrapes are the warm and
59 temperate regions of the northern hemisphere, including the Mediterranean region (C. Parker, 1993),
60 however branched broomrape *O. ramosa* is native to central and south-western Europe, and
61 considered as a major threat to agricultural crops. They cause extensive damage by reducing the yield
62 of all economically important crops belongs to Solanaceae, Fabaceae, Brasicaceae, Asteraceae and
63 Apiaceae family (Aly, 2007; C. Parker, 1993; Joel, 2006). In Israel the significance of infested fields
64 has increased dramatically during the last decade, causing heavy crop damage or even total yield
65 losses.

66 In general, parasitized crops suffer from reductions in total biomass at the greatest expense to their
67 productive tissue (Joel, 2006). Broomrapes cause many problems for agriculture farmers because of
68 their tiny seed size (200-300µm) which makes them difficult to detect in harvested crop seeds and in
69 agriculture soil (Joel, 1987). Moreover, small broomrape germinates and grows only in the presence
70 of a susceptible host. In addition, small broomrape seeds can survive more than a decade in the soil
71 (Habimana et al., 2014). Therefore, identification and quantification of broomrape seeds in a soil
72 sample urgently required and will be helpful in prevention of crop damages by these parasites (Joel et
73 al., 1996). The conventional methods of detection and differentiation such as seed coat morphological
74 feature (Joel, 1987), Random amplified polymorphic DNA technique (RAPD) (Katzir et al., 1996;
75 Paran et al., 1997), Intersimple sequence repeat (ISSR) (Benharrat et al., 2002) and high resolution

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76 melting analysis (Rolland et al., 2016) has some limitation to distinguish and quantify broomrapes
77 exist in the soil. Recent progress in sequencing broomrape plastid genome, which was made available,
78 providing new insight to develop molecular marker for specific broomrape species identification
79 (Cusimano and Wicke, 2016; Wicke et al., 2013). Genetic or DNA based molecular marker such as
80 internal transcribe spacer (ITS), plastidial ribulose bisphosphate carboxylase (Rubisco) large subunit
81 (*rbcL*) and maturase K (*matK*) are among the most widely used molecular markers in evolutionary,
82 taxonomy, phylogenetic and diversity identification of many organisms including *Orobanche*, have
83 been established in recent years (Agarwal et al., 2008; Park et al., 2008; Schneeweiss et al., 2004).

84 In order to develop a complementary methodology for mapping parasitic plants in the field, a
85 procedure includes: a known geographical information systems (GIS) for soil sampling that will
86 characterize the spatial variation in the field (Eizenberg et al., 2012), and at the same time, use of
87 molecular markers to identifying broomrapes in a soil sample. Those molecular markers would assist
88 detection and diagnosis of broomrapes species and population level in the soil. In this study we
89 developed a simple and easy method based on *rbcL* gene to differentiate between three broomrape
90 species *O. crenata, O. cumana* and *P. aegyptiaca*, the most common and destructive weed in Israel.
91 We also developed a specific molecular marker to quantify the parasite seeds in soil sample in an
92 infected sunflower field.

93 **MATERIAL AND METHODS**

94 **Plant material**

95 Seeds, shoots and inflorescences of three broomrape species (*P. aegyptiaca, O. cumana and O.*
96 *crenata*) were collected from different Israeli locations (Golan heights, Havat Eden- Emic Betshan
97 and Jordan valley respectively). Seeds were harvested from freshly inflorescences and stored at 4°C
98 while shoots and inflorescences were dried and stored at −80°C until used. Two hundred milligrams
99 of parasite plant seeds/tissue were ground manually in presence of liquid nitrogen with the help of
100 pestle and mortar. Total genomic DNA was extracted using the GeneGET Plant genomic DNA
101 extraction kit according to the manufacturer instructions (Thermo Scientific).

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102 **ITS and *rbcL* sequences and primers design**

103 The internal transcribed spacer (ITS) regions of *Phelipanche aegyptiaca* (GenBank-Accession No.
104 AY209327) was used to design ITS100 marker. The PCR product yielded a 100bp fragment in length.
105 UCP-555 was used as universal internal control primer, which amplifies a region of the small subunit
106 of nrDNA (555 bp) from a wide variety of microorganism such as protists, fungi, and plants. The
107 length of the *rbcL* sequences was 1211bp and 1290bp for *O. cumana* (GenBank: AF090349.1) and *O.*
108 *crenata* (GenBank: AY582191.1) respectively. Sequence similarities between broomrape species
109 were done using Clustal Omega software. Design of primers for the qPCR detection of broomrape
110 species was based on the alignment of the ITS of nuclear ribosomal DNA regions. All primers were
111 designed with Gene Runner software. PCR amplifications of *rbcL* sequences were performed with the
112 RbcL-M-F and RbcL-M-R primers using PCR Ready Mix (BioLabs, Israel), and 50ng DNA-template.
113 The following PCR conditions were used: denaturation for 5 min at 95°C; 32 cycles with 30 second at
114 95°C, 30 second at 55°C, 1.30 min at 72°C; and final elongation for 10 min at 72°C.

115 **Soil sampling and extraction of Genomic DNA**

116 Design and mapping the soil samples in the sunflower tested field were performed according to
117 (Eizenberg et al., 2012). Soil samples were collected from the infected field on May and counting the
118 inflorescences was performed on July. Each sample includes 500gm of a soil sample that was
119 introduced into one-litter container, mixed thoroughly with 1.5L of (13.3 M $CaCl_2$) solution and kept
120 at room temperature for overnight. The next day, the upper phase including the organic materials and
121 broomrape seeds were collected, dried on Whatmann paper and the parasite seeds were obtained by
122 filtration through 50 and 100 mesh. Genomic DNA was extracted by PowerSoil kit (MO BIO Lab.
123 Inc., Loker Ave West, Carlsbad, CA). according to the manufacturer's instructions (Sagova-
124 Mareckova et al., 2008).

125

126 **Quantitative real time PCR of soil sample from the sunflower fields**

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127 The quantitative real time PCR was performed using PerfeCTa® SYBR® Green Fast Mix®, ROX™
128 (Quanta biosciences) and ABI-Prism 7000 Real-Time PCR Detection System (Applied Biosystems)
129 according to the manufacturer's protocol. The qPCR was performed in quantitative reaction with final
130 volume of 10 µl including: 100ng DNA, 5 µl of SYBR Green Fast Mix- ROX and 500nM of each
131 ITS-100 primers. *O. cumana* actin was used as endogenous control and the relative gene expression
132 level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Two standard curves were
133 prepared: The first curve included standard points from *O. cumana* seeds ranging from 0.001 to 10 ng
134 DNA per tube, were made using 1:10-fold serial dilutions of broomrape DNA and were used for
135 qPCR assay. The second curve was generated by mixing different amounts of genomic DNA from *O.*
136 *cumana* (0.1 to 100 ng) with total genomic DNA (10 µg) extracted from 250 mg soil sample. Each
137 point on the standard curve was assayed in triplicate.

138 **Viability evaluation of the parasite seeds by Tetrazolium test**

139 Tetrazolium test was performed as described earlier (Lopez-Granados and Garcia-Torres, 1999;
140 Thorogood et al., 2009). Briefly, 50-100 seeds were hydrated between paper towel sheets for 2-3
141 days. After pre-conditioning, they were placed in flasks, covered with a 1% solution of 2,3, 5
142 triphenyl tetrazolium chloride (TZ) and incubated at 40°C for 2h. After that, the TZ solution was
143 discarded; and the seeds were washed thoroughly with sterile water. Seeds were directly evaluated for
144 viability under the microscope using magnification (40X and 100X) and classified in two categories:
145 viable seeds – orange to brown light coloured or non-viable seeds –yellow to white colour.

146 **RESULTS**

147 **Design of *rbcL* specific marker to differentiate between *O. cumana* and *O. crenata***

148 In our previous study, we reported amplification of ITS region using ITS350 primer; which
149 specifically detect broomrape in soil sample (Aly et al., 2012). However, ITS350 marker does not
150 differentiate between broomrape species because of the high sequence similarity among ITS regions.
151 Therefore, we compared and analysed the *rbcL* gene sequences from these species using Clustal
152 Omega and designed new molecular marker to differentiate broomrape species such as *O. cumana* and

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153 *O. crenata* (Fig. S1). To distinguish between the above broomrape species, probes differing at least by
154 five-base mismatches or more were designed. Based on sequence alignment, a molecular marker
155 (RbcL-M) was designed using *rbcL* gene from *O. cumana* (GenBank accession number: AF090349.1)
156 Soil samples were artificially contaminated with seeds or shoots of *P. aegyptiaca*, *O. crenata* and *O.*
157 *cumana* and subjected to PCR analysis. By PCR amplification using RbcL-M, we were able to
158 differentiate between *O. crenata* and *O .cumana* according to the length of the PCR products. For *O.*
159 *crenata* the PCR product size was 1300bp compared to 1000bp for *O. cumana*. However, RbcL-M
160 marker failed to amplify any PCR product while soil samples were artificially contaminated with *P.*
161 *aegyptiaca* seeds or shoots (Fig. 1a). As expected, the universal internal control primers amplified a
162 PCR product (555bp) from soil samples with or without broomrape shoots or seeds (Fig. 1b).

163 **Development of specific ITS100 marker to detect seeds of three broomrape species in a soil**
164 **sample**

165 To analyse the number of the broomrape seeds present in a soil sample from infected field, we
166 designed ITS100 primers from the highly conserved region of ITS350 region (Aly et al., 2012). To
167 determine the specificity of the marker to each broomrape specie, we first extracted genomic DNA
168 from soil samples (250mg) containing (0, 1, 10, 25, 50 100 and 250 seeds) of *P. aegyptiaca*, *O.*
169 *crenata* or *O. cumana* and subjected to PCR analysis. ITS100 primers successfully amplified a PCR
170 product (100bp) when *P. aegyptiaca*, *O. crenata* or *O. cumana* were mixed with soil sample, except
171 soil sample without *Orobanche* seeds. However, a PCR product (555bp) was amplified in all samples
172 including soil with or without broomrape seeds by using universal control primer UCP-555 (Fig. 2a).

173 In addition, the specificity of the markers generated in this study were evaluated by testing DNA
174 extracted from sunflower, carrot and tomato leaves and roots corresponding to possible respond to the
175 host plants. Fortunately, the qPCR assay always yielded negative results for these plant species (data
176 not shown). Next to quantify *Orobanche*, we generated standard curve using *O. cumana* genomic
177 DNA and ITS100 marker utilizing qPCR procedure. *O. cumana* genomic DNA was purified and serial
178 dilutions (1:10 fold) ranging from 0.001 to 10 ng/µl were used for qPCR assay. The standard curve

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179 for *O. cumana* exhibited a slope of -1.28 and ($R^2$) of 0.9994 (Fig. 2b). The same type of assay was
180 performed with *O. cumana* genomic DNA extracted from soil sample (250 mg) mixed with the
181 parasite seeds (0.1, 1, 10 and 100 mg seeds) and subjected to qPCR. The standard curve for *O.*
182 *cumana* seeds mixed with soil sample, exhibited a slope of -1.332 and ($R^2$) of 0.987 (Fig. 2c).

183 **Prediction and quantification of *O. cumana* seeds in sunflower field**

184 Previously, we have developed a protocol that allows extraction of genomic DNA from few tiny seeds
185 of *Orobanche* spp. in a soil sample and we were able to subject the sample DNA to a rapid PCR-assay
186 (Aly et al., 2012). Here, we extended the protocol, allowing for the identification and distinguishing
187 between soil-borne and parasite seed species that are collected from a field soil sample. Following
188 confirmation and validation of the ITS100 marker specificity to *O. cumana* seeds, geo-statistics model
189 for soil sampling that characterized the spatial variation in the field, was performed according to
190 (Eizenberg et al., 2012) in a sunflower field located at Havat Eden, Emic Betshan– Israel, which was
191 infected with *O. cumana* (Fig. 3a and b). Five soil samples (spot number 2,4,6,8 and 10) were
192 collected from a plot of 1 acre according to the illustration (Fig. 3c). Genomic DNA was extracted
193 from each soil sample and subjected to qPCR using ITS100 primers to predict and quantify *O.*
194 *cumana* DNA in naturally infected field. Then, genomic DNA from soil samples was extrapolated
195 with Ct values to quantify the number of seeds in each sample according to the generated Ct values
196 (Fig. 2b and c). The results showed variable quantity of *O. cumana* seeds in the soil sample ranged
197 from 2 to 22 parasite seeds (Fig. 3d) in 250mg soil sample processed originally from 1kg soil sample
198 from the infected field. Sample no. 6 showed the highest density of *O. cumana* seeds (22 seeds) in the
199 sunflower infected field as compared to the other samples. Sample no. 6 was collected from a highly
200 infected area as was shown by the geo-statistics model proposed by (Eizenberg et al., 2012).
201 Concomitantly, we monitored and counted the parasite inflorescences in adjacent to the position were
202 the soil samples were taken. Our results revealed that average density of 0.6 inflorescences /$m^2$, in a
203 total area of 1acre was recorded. Inflorescences counts in the selected area in the field were also
204 variable ranged from 2 to 15 and were less than the seed numbers found in the soil sample when
205 qPCR was applied (Fig. 3d).

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206 **Evaluation viability of the parasite seeds in sunflower infected field**

207 To determine viability of broomrape seeds in a sunflower infected field, a tetrazolium test (TZ)
208 (Lopez-Granados and Garcia-Torres, 1999; Thorogood et al., 2009) was conducted. According to TZ
209 test, viable parasite seeds tends to have orange to brown colour because of its metabolite activity,
210 while non-viable tends to have yellow to faint colour (Fig. 4a). To evaluate the parasite seed viability,
211 two treatments were assayed: soil samples (organic matter only) collected from sunflower field
212 located at Havat Eden, Emic Betshan, naturally infested with *O. cumana* and pure *O. cumana* seeds (1
213 mg) prepared from an old seed stock. Our results indicate that the highest percentage (% of total) of
214 viable *O. cumana* seeds were found in soil samples collected from the sunflower field (83%) as
215 compared to 25% found in the old seed stock (Fig. 4b).

216 **DISCUSSION**

217 Parasitic weeds are often exert their greatest damage prior to their emergence; therefore, the majority
218 of field loss may occur before diagnosis of infection. Early detection of infection by visual inspection
219 is not possible. This hampers early control strategy efforts and the detection methods used by crop
220 scientists are laborious and time consuming. Most of the methods developed to detect broomrape
221 seeds in soil samples, need several steps such as seed purification (flotation, filtration and binocular
222 microscope observation (Kroschel, 2001). Despite of the great advances in genome sequencing
223 approaches that has already been achieved, our understanding towards genomic evolution of
224 Orobancheaceae is still incomplete due to aberrant evolution of plastid genome of holoparasite (Park
225 et al., 2007). Available DNA sequence data are insufficient to differentiate closely related species,
226 such as the weedy parasites *O. cumana, O. crenata* and *P. aegyptiaca*.

227 Genetic or DNA based marker techniques such as ITS or *rbcL* are routinely being used in
228 evolutionary, taxonomical, phylogenetic and diversity studies (Agarwal et al., 2008; Park et al., 2008;
229 Schneeweiss et al., 2004). Diagnosis and early identification of the parasite species in the field by soil
230 sampling is of great importance to farmers and it is particularly needed due to the host-parasite
231 specific interaction, the difficulty to detect the tiny parasite seeds by conventional methods in

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232 contaminated fields and because seeds will only germinate and grow in the presence of a susceptible
233 host. The gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase
234 (Rubisco; *rbcL*) is retained as a pseudogene in *Orobanche* and *Phelipanche* (Wicke et al., 2013). In
235 the present work, we designed sets of primers targeting the plastid *rbcL* gene sequence from *O.*
236 *cumana* to differentiate between three broomrape species (*O. cumana, O. crenata* and *P. aegyptiaca*)
237 the most common and harmful species in field crops in Israel. Another sets of primers were designed
238 to target ITS sequences (Schneeweiss et al., 2004) to quantify the parasite seeds potentially present in
239 a soil sample to help for seed bank assessment in the field. We found that RbcL-M primers (marker)
240 were useful to distinguish between DNA from *O. cumana* and *O. crenata* producing different
241 amplicon sizes (a specific PCR product about 1300bp for *O. crenata* and 1000bp for *O. cumana*).
242 Subsequent BLASTn with previously published broomrape genomic data (Cusimano and Wicke,
243 2016) reconfirmed the differences between the two species. In contrast, DNA from *P. aegyptiaca*
244 failed to produce PCR amplicon suggesting possibly that *Phelipanche* species completely lost *rbcL*
245 gene (Delavault et al., 1995; Leebens-Mack and de Pamphilis, 2002).To allow differentiation between
246 *P. aegyptiaca* from other species, a specific molecular marker was recently designed (Aly et al.,
247 2019), (GenBank accession numbers MK637618-637624).
248 To diagnose and quantify broomrape species in a seed stock (Dongo et al., 2012), different types of
249 nuclear and plastid DNA markers have been proposed. (Schneeweiss et al., 2004) were the first to
250 present molecular phylogenetic analysis using nuclear ITS sequences. ITS-based markers were also
251 used to detect *P. aegyptiaca* seeds in a soil sample (Aly et al., 2012) and quantify contamination of
252 *O. ramosa* and *O. cumana* in crop seed lots (Dongo et al., 2012). Here we developed ITS100 marker
253 that was based on primers consisting of unique sequences in the internal transcribed spacer (ITS)
254 regions of the nuclear ribosomal DNA (nrDNA) of *O.crenata*. ITS-100 marker was used with qPCR
255 assay to quantify *O. cumana* seeds in a soil sample from sunflower field located at Havat Eden, Emic
256 Betshan – Israel.
257 We were able to detect 0.1 mg *O. cumana* seeds in 250 mg soil sample (wt ⁄ wt). A detection threshold
258 of 0.1 mg broomrape seeds in 20g seed samples was previously reported (Dongo et al., 2012). For
259 detection, mapping and quantifying *O. cumana* seeds in the field, we used geographical information

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260 systems (GIS) for soil sampling and other advanced technologies for parasitic weed mapping and field
261 history data storage (Eizenberg et al., 2012) followed by qPCR assay. Soil samples (500gr) were
262 collected from the sunflower field, organic material was extracted from each sample ending with
263 250mg then, genomic DNA was extracted and subjected to qPCR using ITS100 primers. This method
264 allowed specifically detecting and quantifying the DNA of *O. cumana* in a total DNA extract from
265 sunflower soil sample. Accordingly, the results of this assay can be also expressed as the number of
266 parasite seeds per kilogram of soil following extrapolation with the standard curve was prepared. Our
267 results indicate that samples collected from highly infected area (Fig. 3c) according to the geo-
268 statistics model proposed by Eizenberg et al. (2012), were with agreement with our qPCR assay
269 (sample no. 6 showed the highest density of *O. cumana* seeds (22 seeds) in the sunflower infected
270 field as compared to the other samples. However, no correlation was found between qPCR assay
271 (parasite seed number) compared to number of the parasite inflorescences collected from the same
272 location in the sunflower infected field. An explanation for that may be related to the viability of *O.*
273 *cumana* seeds, genomic DNA from a soil sample will contain viable and non-viable parasite seeds,
274 add to that, we cannot exclude the presence of some related parasite seeds in the same sample
275 therefore, we counted more parasite seeds using the qPCR assay. Specificity of the qPCR assay was
276 tested against several possible contaminants of soil-borne pathogens by using universal internal
277 control primer UCP-555 (White, 1990) or harvested crop seeds like sunflower and tomato using
278 ITS100. No amplification was observed, confirming the specificity of the marker.
279 The powerful of broomrape-infected field to distribute and contaminate the neighbouring non-infested
280 fields depends on the soil seed-bank and viability of the parasite seeds. Viability of broomrape seeds
281 in the sunflower infected field was determined by tetrazolium test. Our results showed the highest
282 percentage of viable broomrape seeds were found in soil samples collected from the sunflower field
283 (83%) as compared to 25% found in an old seed stock. We assume that the highest count recorded in
284 the sunflower field was due to the release and distribution of the fresh parasite seeds by the newly
285 parasite inflorescences showed up through the crop growth. We have to take in consideration that we
286 used an old seed stock (10 years old) that doesn't represent newly harvested seed stocks. Our
287 experience with seed germination of *O. cumana* with germination stimulant (GR) could reach more

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288 than 90%. Additionally, we cannot exclude the presence of some related parasite seeds in the same
289 sample from previous growth seasons.
290 In this study, we provide a simple, fast and non-expensive approach to distinguish and quantify
291 broomrape seeds exist in a soil sample from a crop field. Molecular markers would assist accurate
292 detection and population level of *Phelipanche* and *Orobanche* spp. in a soil sample and offer
293 numerous advantages over conventional phenotype based alternatives, as they are stable and
294 detectable in all tissues regardless of growth, differentiation or development. These methods could be
295 helpful in precision agriculture, in which they provide answers routinely questioned by the farmers:
296 are there parasite seeds in my crop field? What species? and how much seeds are exist in a soil
297 sample?

298 **ACKNOWLEDGMENTS**

299 Results of this research were supported by the Chief Scientist of the Ministry of Agriculture and Rural
300 Development - Israel, grant No.132-1499-10 (MEZAMALEKET). VKB is grateful to the ARO-
301 Volcani Center, Agricultural Ministry of Israel for providing the Postdoctoral fellowship.

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390 **Figure legends**

391 **Fig. 1.** **(a)** Specificity of RbcL-M marker and PCR amplification patterns in three broomrape species
392 (*P.aegyptiaca, O.cumana and O.crenata*). Soil samples (250 mg) were artificially contaminated with
393 broomrape seeds (100) or 10 mg shoots of *P.aegyptiaca, O.crenata and O.cumana* and subjected to
394 PCR analysis using RbcL-M primers. Genomic DNA was extracted and 100 ng were used for PCR
395 amplification. A PCR product of 1300bp was obtained from *O.crenata* seeds and shoots, A PCR
396 product of 1000bp was obtained from *O.cumana* seeds and shoots. However, RbcL-M marker failed
397 to amplify PCR product with soil sample alone or with soil contaminated with *P.aegyptiaca* seeds or
398 shoots. **(b)** The same samples were subjected to PCR detection using universal internal control
399 primers (UCP-555). Arrows indicates the PCR product sizes.

400 **Fig. 2.** PCR amplification patterns of ITS100 primers in *O.cumana* seeds **(a)** gDNA was extracted
401 from soil samples (250 mg) containing the parasite seeds (1, 10, 25, 50, 100 and 250 seeds). For each
402 sample, 100 ng gDNA were used for PCR amplification. The same samples were subjected to PCR
403 detection using universal internal control primers (UCP-555). Arrows indicates the PCR product sizes
404 **(b)** Standard curves generated for qPCR to quantify *O.cumana* seeds in a soil sample. standard curve
405 generated by plotting the value of threshold cycle value (Ct) against log of the amount of template
406 DNA (ng/µl) from *O.cumana.***(c)** Detection and quantification of *O. cumana* template DNA (ng) from
407 known amount of the parasite seeds mixed with extracted soil sample DNA (10 µg). Each point on the
408 standard curve was assayed in triplicate.

409 **Fig. 3.** Quantification of *O.cumana* seeds in an infected sunflower field by qPCR and manually
410 counting the inflorescences adjacent to the soil sample. **(a and b)** represents a section of the sunflower
411 infected field. Red arrows show *O.cumana* inflorescences. **(c)** Representing a (GIS) model (Eizenberg
412 et al., 2012) for soil sampling and characterize the spatial variation in sunflower field infected with
413 *O.cumana* in Havat Eden. Five soil samples (2, 4, 6, 8, 10 ) of 500gm were collected from the infected
414 field on May. Each sample was collected from a depth of 0 to 20 cm in a total area of 1x1 meter. The
415 blue spots indicate location of the sample in the infected field. Red color in the plot represents high

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416 density of the parasite inflorescences. Infectivity was also monitored by counting the inflorescences
417 on July and was restricted to the total area 1x1 meter of the five selected soil samples. **(d)**
418 quantification of *O. cumana* seeds in a soil sample by qPCR (black bars) and monitoring the parasite
419 inflorescences counts in fields (Grey bars). The data are the means of three soil samples or biological
420 replicates. Vertical lines indicate SD of three independent measurements.

421 **Fig. 4.** Evaluation the viability of *O.cumana* seeds by Tetrazolium test. **(a)** Tetrazolium test was used
422 to visually differentiate between metabolically active tissue from (cleaned non-viable seed (a-1), non-
423 viable seed with organic matter from an infected sunflower field (a-3), and active tissues from
424 (cleaned viable seed (a-2), viable seed with organic mater from an infected sunflower field (a-4).
425 Images were acquired using fluorescence microscope using 40X and 100X magnification. **(b)** The
426 same test was also performed to evaluate percentage of viability of *O.cumana* seeds in soil samples
427 from the infected field (83%) as compared to viability of the seeds from cleaned old seed bank (25%).
428 The data are the means of three separate experiments with vertical lines indicating SD.

**Fig. 1**

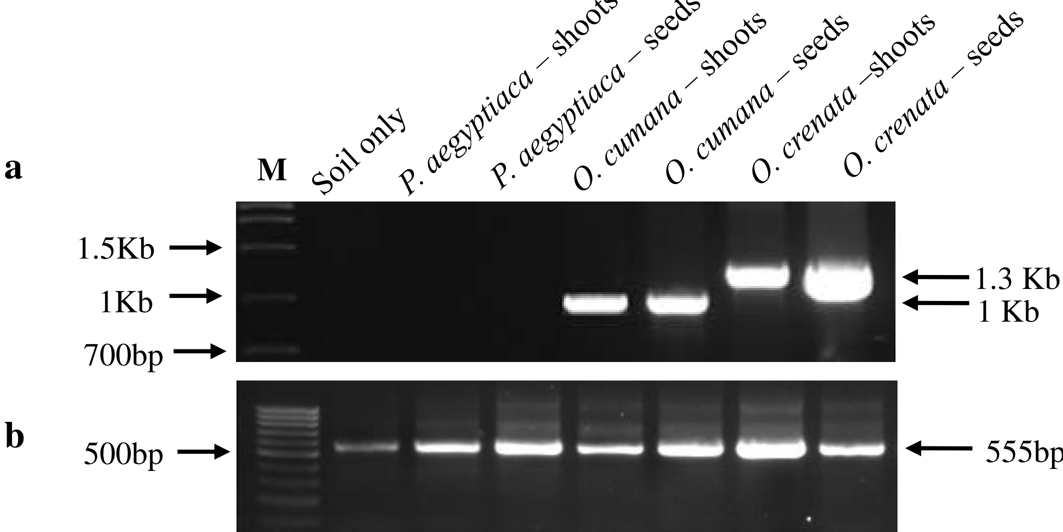
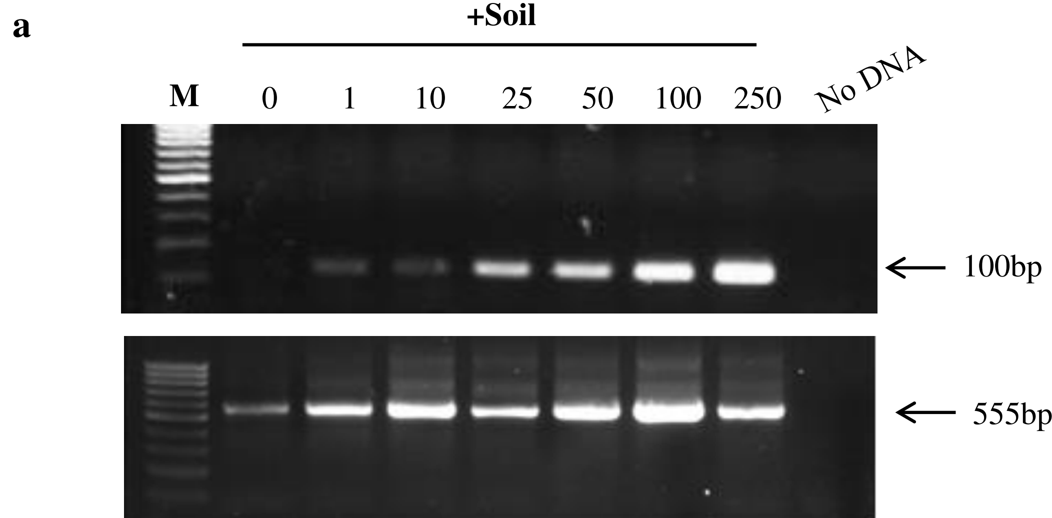
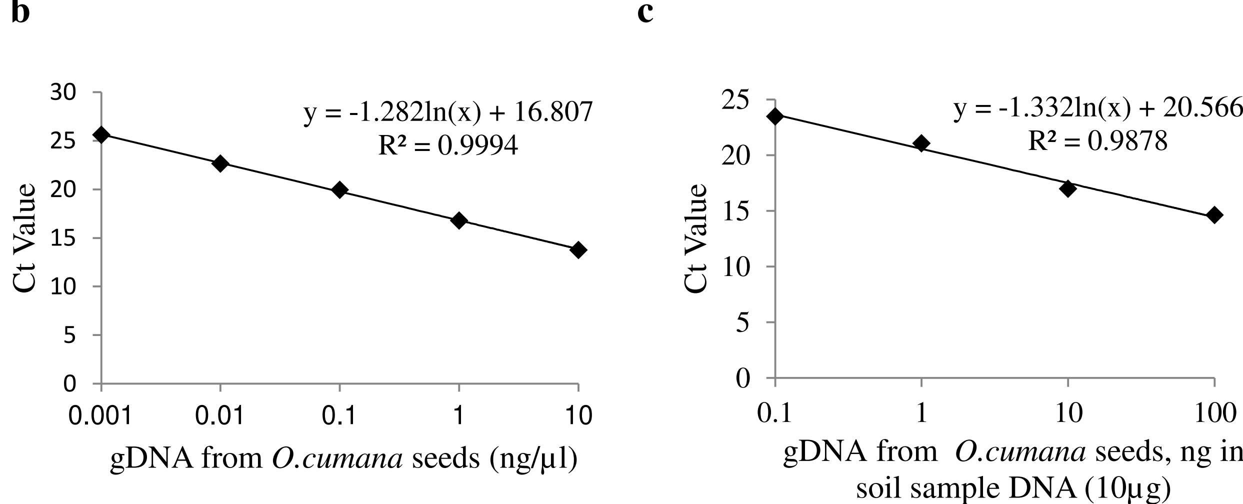


**Fig. 2**



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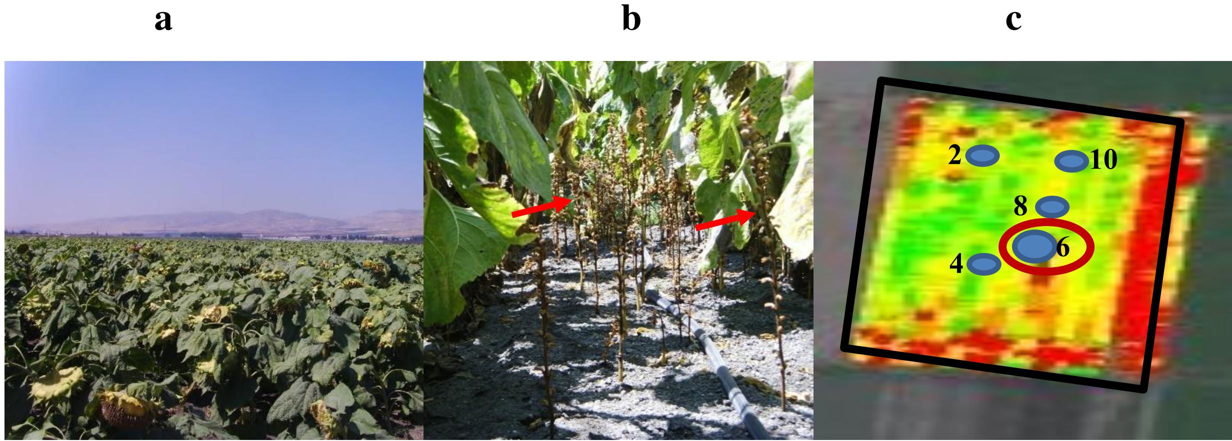


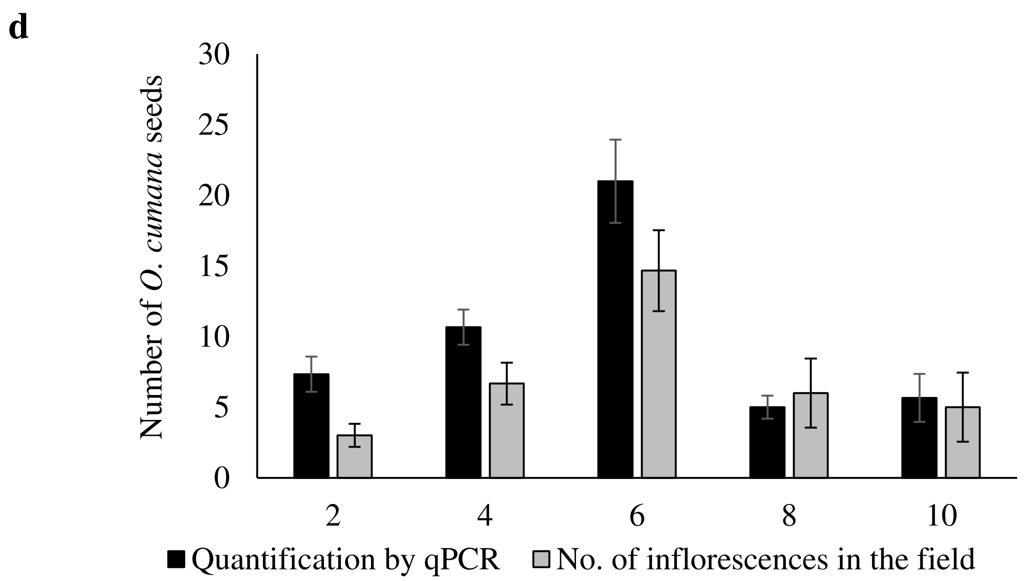
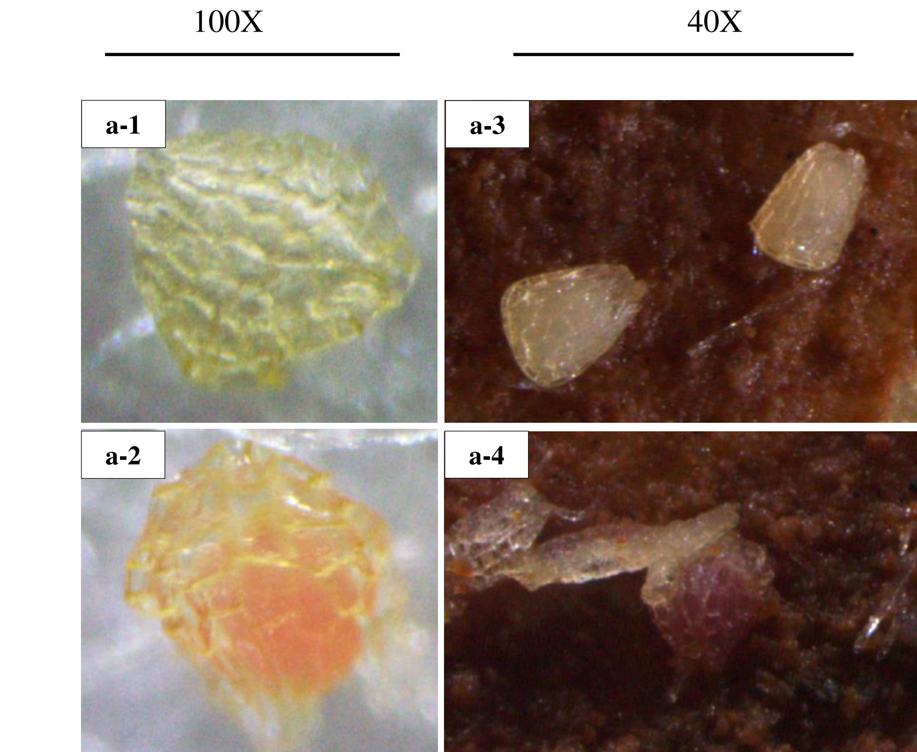
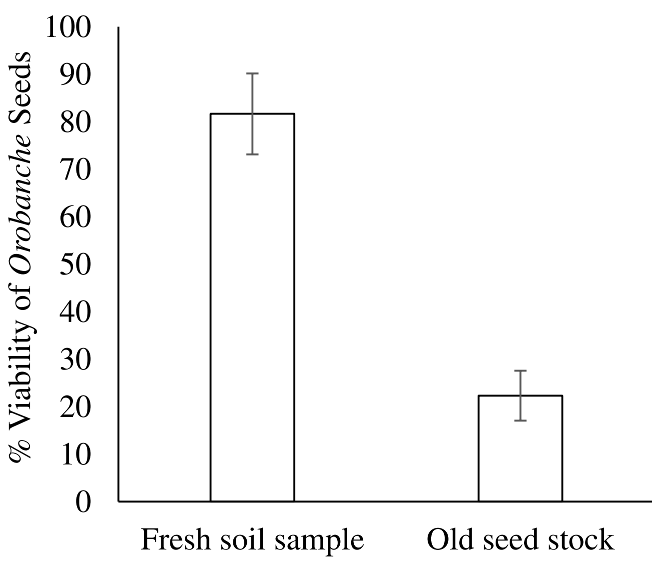



**Fig. 4**

**a**




**b**



Supplementary Information

# Development of specific molecular markers to distinguish and quantify broomrape species in a soil sample from infected field

Radi Aly[1]*, Vinay K. Bari[1], Avishai Londner[1], Jackline Abu Nassar[1], Leena Taha-Salaime[1], Eizenberg Hanan[1] and Ran Lati[1]

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**Fig. S1.** Multiple sequence alignment of *O.cumana* and *O.crenata rbcL* gene using online tool CLUSTAL Omega

```
                      RbcL-M-F
O.cumana        ------------------------------------------------------------ 0
O.crenata       ATGTCACCACAAACAGAAACTAAAGCAAGTGTTGGATTCAAAGCGGGTGTTAAAGATAAA 60

O.cumana        ------------------------------------------------------------ 0
O.crenata       GAGTACAAATTGACTTATTATATTCCTGAATACAAAACTAAAGATACTGATATTTTAGCA 120

O.cumana        ------------------------------------------------------------ 0
O.crenata       GCATTTCGAGTAACTCCTCAACCTGAAGTTCCGCCTGAACAGGGGCTGCAGTAGCTGCCG 180

O.cumana        ------------------------------------------------------------ 0
O.crenata       AATCTTCTATTGGTACATGGACAACTGTGTGGACTGATGGACCCTTGATCGTTACAAAGG 240

O.cumana        ------------------------------TATCGAGTA----GACCTTGTGTTGTTATC 26
O.crenata       TCGATACTACCACATCACATCGAGCCCAGTTCTGGAGAAACAGATCAATATATTTGTTAT 300
                                              * * *** *       *  * * **  *
                                                                RbcL-M-F
O.cumana        AGAATTTTTAATTCATAAGTTTTAGGGAGAAATTTATGTCACC--------ACAAACAGA 78
O.crenata       ATAGCTTACCCTTTAGAC-TTTTTTGAGGAAGGTTCTGTTACTAACATGTTTACTTCCAT 359
                * *  **     ** * *  ****  *  ***  ** *** **             *
O.cumana        AACTAAAGCAAGTGTTGGATTCAAAGTCCTGCGTGATCTATGTCTGGAATATCTGCGAAT 138
O.crenata       TATAGGAAATATATTTGGATTTAAAGCCCTGCGTGCTCTACGTCTGGAAGATCTACGAAT 419
                 *    *   *   ******* **** ******** **** ******** **** *****
O.cumana        CCCTCCTGCTTATATTAAAA---TTTTCCAAGGCCCGCTCCATGGTATTCAAGTTTAAAG 195
O.crenata       TCCTCCTGCTTATATTAAAATTATTTTCCAAAGCCCGCCCCATGAGATTAAAGTTGAAAT 479
                 ******************    ******** ****** *****   *** ***** ***
O.cumana        AGATAAATTAAACAAGTATGGTCGTCCCCTGTTGGGATGTATTATTAAACCTAAA----T 251
O.crenata       AGATAAATTGAACAAGTATGGTCGTCCCCTGTTGGGATGTACTATTAAATAAACCTAAAT 539
                ********* ****************************** *******    *      *
O.cumana        TGGGGTTATCCACTAAAAACTATGGGGGAACAGTTTATGAATGTCTTCGCGGTGGACTTG 311
O.crenata       TAGGATTATCCGC---------------------------------------TAAAAACC 560
                * ** ****** *                                       *  *
O.cumana        ATTGTACCAAAGATGATGAGAACGTAAATTCCCAGCCATTTATGCGCTGGAGAGATCGTT 371
O.crenata       ATGGGAGAACAGTTGATGAGAACGTAAACTCCCAGCCATTTATGCGCTGGAGAAATCGTT 620
                ** * *  * ** *************** ************************ ******
O.cumana        TTTTATTTTGTGCTGAAGCAATTTATAAATCACAGGCTGAAACAGGCGAAATTAAAGGCC 431
O.crenata       TCTTATTTTGTGCCGAAGCAATTTATAAAACACAGGCTGAAACAGGCGAAATCAAAGGTC 680
                * *********** *************** *********************** ***** *
O.cumana        ATTACTTGAATACTACTGCAGGTACATGCGAGGAAATGATCAAAAGAGCTGTCTTTGCTA 491
O.crenata       ATTACTTGAATACTACTGCAGGTACATGTGAGGAAATGATCAAAAGAGCTGTTTTTGCCA 740
                **************************** *********************** ***** *
O.cumana        GAGAATTGGGAGTTCCTATTATAA--ATGTACGACTACTTAACAGTAGGATTCACTGCAA 549
O.crenata       GAGAATTGGGAGTTCCTATTATAATAATGCACGACTACTTAACAGGAGGATTCACTTCAA 800
                ************************  *** *************** ********** ***
O.cumana        ATACTAGCTTGGCTCATTATTGCCGTAATAATGTCC-----TACTTCTCACCGTGCAATG 604
O.crenata       ATACTAGCTTGGCTCATTATTGCCATAATAATGGCCTGGCCTACTTCTTCACCTGCAATG 860
                ************************ ******** **     *******   * *******
O.cumana        CATGTAGTTATTGATAGACAGAAGAACCATAGTATACATTTCCGTTGTACTAGCTAAAGC 664
O.crenata       CATGCAGTTATTTATAGACAGAAGAACCATGGTATACATTTCC-GTGTACTAGCTAAAGC 919
                **** ******* ***************** ************  ***************
O.cumana        GTTACGTATGTCTGGTGGAGATCATATTCACTCTAGGACCGTAGTAGGTAAACTTGAAGG 724
O.crenata       ATTACGTATGTCTGGTGGAGATCATATTCACTTTGGGACTGCAGTAGGTAAACTTGAAGG 979
                 ******************************** * **** * ******************


O.cumana        AGAAAGAGACATTACTTTGGGCTTTGTTGATTTATTGCGTGATGATTTTATTAA-AAAGA 783
O.crenata       AGAAAGAGACATTACTTTTGACTTTGTTGATTTATTGCGTGATGATTTTATTGAAAAAGA 1039
```

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                 ****************** * ******************************* * *****
O.cumana         TCGAAGTCACGGTATTTATTTTACTCAAGATTGGGTTTCTTTACCAGGTGTTTTTACTGT  843
O.crenata        TCGAAGTCGCGATATTTATTTCACCCAAGATTGGGTTTCTCTACCAGGTGTTATTCCTGT  1099
                 ******** ** ********* ** *************** *********** ** ****
O.cumana         GGCTTCAGGGGGTATTCACGTTTGGCATATGCCTTCATCT-GACGGAGATCTTTGGGGAT  902
O.crenata        GGCTTCAGGGGGTATTCACGTTTGGCATATGCCTTCCCTGACCGCGAGATCTTTGGGGAT  1159
                 ************************************         ***************
O.cumana         GATTCCGTACTACAGTTTGACGGAGGAACTTTAGGACATCCTTAGCGTAATGCACCAGGC  962
O.crenata        GATTCCATACTACAGTTTGGCGGAGGAACTTTAGGACATCCTTGGGGTAATGCACCAGGT  1219
                 ****** ************ *********************** * *************
O.cumana         GCTCTGTAGCTAATCGAGTAGCTATAGAAGCATGTGTACAAGCTCGTAATGAAGGATGTA  1022
O.crenata        GC--TGTAGCTAATCGAGTAGCTATATAAGCATATGTACAAGCTCGTAATGAAGGACGTG  1277
                 **  ********************** ****** ********************** **

                                                                 RbcL-M-R
O.cumana         ATCTTGCTACTGAGGGGAATGCAATTATACGCGAGGCTAGGAAATGGAGCCCTGAACTAG  1082
O.crenata        ATCTTGCTGCTGAGGGTAATTATA-----CGTGAGGCTAGCAAACGGAGTCCTGAACTAG  1332
                 ******** ******* ***    *      ** ******** *** **** **********
                                                                  RbcL-M-R

O.cumana         TTGCTGTTTGTGAGGTATGTAAAGATATCAAATTTGAGTTTAAAGCAGTCGATACTTTGG  1142
O.crenata        TTGCTG------------------------------------------------------  1338
                 ******
O.cumana         GTAAGTGTAAGTAAGATAACATTACTCTTCATTCTCTTAATTGAATTTCAATTAAATTCG  1202
O.crenata        ------------------------------------------------------------  1338

O.cumana         GCTTAATC   1210
O.crenata        --------   1338
```

**Table S1. List of oligo's used in this study**

| Primer's name | Sequence 5 ⟶ 3' | Use in the study |
|---|---|---|
| UCP555-F | GTAGTCATATGCTTGTCTC | Universal internal control |
| UCP555-R | GGCTGCTGGCACCAGACTTGC | |
| RbcL-M-F | ATGTCACCACAAACAGAAAC | Molecular marker to identify *O.cumana* and *O.crenata* |
| RbcL-M-R | CAGCAACTAGTTCAGGCTCC | |
| ITS100-F | CAACGGATATCTCGGCTCTC | qRT-PCR to quantify broomrape |
| ITS100-R | TTGCGTTCAAAGACTCGATG | |
| ACT1-F | ATGGGCCAGAAAGATGCATATGTT | Housekeeping gene used for qRT-PCR expression normalization |
| ACT1-R | GTGTGATGCCAAATTTTCTCCATGT | |