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Host-specific races in the holoparasitic angiosperm *Orobanche minor*: implications for speciation in parasitic plants

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• *Background and Aims Orobanche minor* is a root-holoparasitic angiosperm that attacks a wide range of host species, including a number of commonly cultivated crops. The extent to which genetic divergence among natural populations of *O. minor* is influenced by host specificity has not been determined previously. Here, the host specificity of natural populations of *O. minor* is quantified for the first time, and evidence that this species may comprise distinct physiological races is provided.

• *Methods* A tripartite approach was used to examine the physiological basis for the divergence of populations occurring on different hosts: (1) host–parasite interactions were cultivated in rhizotron bioassays in order to quantify the early stages of the infection and establishment processes; (2) using reciprocal-infection experiments, parasite races were cultivated on their natural and alien hosts, and their fitness determined in terms of biomass; and (3) the anatomy of the host–parasite interface was investigated using histochemical techniques, with a view to comparing the infection process on different hosts.

• *Key Results* Races occurring naturally on red clover (*Trifolium pratense*) and sea carrot (*Daucus carota* ssp. *gummifer*) showed distinct patterns of host specificity: parasites cultivated in cross-infection studies showed a higher fitness on their natural hosts, suggesting that races show local adaptation to specific hosts. In addition, histological evidence suggests that clover and carrot roots vary in their responses to infection. Different root anatomy and responses to infection may underpin a physiological basis for host specificity.

• *Conclusions* It is speculated that host specificity may isolate races of *Orobanche* on different hosts, accelerating divergence and ultimately speciation in this genus. The rapid life cycle and broad host range of *O. minor* make this species an ideal model with which to study the interactions of parasitic plants with their host associates.

Key words: Parasitic plant, *Orobanche*, speciation, divergence, host-specificity, host-specific races.

INTRODUCTION

Approximately 4000 species of angiosperm are parasitic and rely on other plants for water and solute acquisition (Kuijt, 1969). Parasitism is taxonomically and geographically widespread, occurring in 17 families in most major biomes (Barker *et al.*, 1996; Press and Phoenix, 2005). Parasitic plants can be classified as hemiparasites, which are photosynthetic, or holoparasites, which are completely devoid of chlorophyll and thus obligately dependent on their host plants for their nutrition. Parasitic angiosperms have significant ecological effects on plant community structure by altering the competitive balance between host and non-host species (Press and Graves, 1995; Press and Phoenix, 2005). In spite of this, our understanding of the evolution of parasitic plants is rudimentary. This is largely because the evolutionary shift to heterotrophy has been associated with the degeneration of taxonomically useful morphological features and genome size, which has impeded efforts to resolve phylogenetic relationships among parasitic plants (Young *et al.*, 1999; Nickrent *et al.*, 2004; Davis *et al.*, 2007). However, recent advances in molecular systematics have given new insights into the evolutionary relationships between highly reduced

parasites and their photosynthetic ancestors. For example, Davis *et al.* (2007) used mitochondrial, nuclear and plastid gene sequence data to show that *Rafflesia* (Rafflesiaceae), which famously produces the largest flower on earth, has undergone an incredible 73-fold increase in flower diameter following divergence from its closest ancestor within the spurge family (Euphorbiaceae). Each of the 17 species of *Rafflesia* is specific to one or two of the 95 species of *Tetrastigma* vine (Vitaceae) in south-east Asia (Nais, 2001). Isolation of *Rafflesia* populations on host vines with differing ecologies and distributions, followed by genetic divergence, may have driven speciation in this genus. Analogous to this, host-associated habitat isolation has driven divergence among many insect species (Funk *et al.*, 1995; Hawthorne and Via, 2001; Schwarz *et al.*, 2005).

Similar studies of host-driven speciation in parasitic plants are, however, rare. Many parasitic plants have a broad potential host range (Press and Graves, 1995), but even host generalists show distinct patterns of host specificity at a local level (Thorogood and Hiscock, 2007; Thorogood *et al.*, 2008). Therefore host specificity may have been an underestimated driver of genetic divergence, and ultimately speciation among parasitic plants.

The broomrape genus (*Orobanche*; Orobanchaceae) includes over 150 species of root-holoparasites with a centre

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of diversity in the Mediterranean Basin (Barker *et al.*, 1996). A number of *Orobanche* species have undergone host shifts from their native hosts to crop species (Román *et al.*, 2007), and are now a severe obstacle to food production in certain parts of the world (Parker and Riches, 1993). Current estimates suggest that approx. 1·2 % of the world's arable farmed land may be infested with *Orobanche* seed (Sauerborn, 1991). However, our understanding of the interactions between *Orobanche* and its hosts lags behind that of host–parasite associations of the related genus of hemiparasites, *Striga*, which are pernicious weeds that infect cereal crops in the semi-arid tropics of the Old World (Parker and Riches, 1993; Barker *et al.*, 1996). A variety of physical, cultural, chemical and biological methods have been investigated as potential control approaches against parasitic weeds such as *Orobanche* and *Striga*, all with limited success (Fernández-Aparicio *et al.*, 2008). Recently, a number of *Orobanche* spp. have been classified as emerging pests, most notably *O. foetida* on faba bean in Tunisia (Kharrat *et al.*, 1992) and Morocco (Rubiales *et al.*, 2005). In the light of this, an enhanced understanding of the evolution, cell biology and physiology of host specificity in this genus is currently crucial. As far as is known, no previous studies have investigated such aspects of the host specificity of natural populations of *Orobanche*, which may be a proxy for understanding the evolution of this genus from both an ecological and an agricultural standpoint.

Orobanche minor is known to infect hundreds of species in taxonomically diverse families from the Ranunculaceae to the Poaceae, but with a clear preference for the Fabaceae and Asteraceae (Rumsey and Jury, 1991). Despite the broad host range of *O. minor*, strains are known to only infect certain hosts (Musselman and Parker, 1982); however, the extent of race formation in this species has not been ascertained. *Orobanche minor* appears to be a largely self-pollinated species (Jones, 1989). Shifts to self fertility are characteristic of recently colonized populations (Antonovics, 1968; Larson and Barrett, 1998); therefore selfing may have acted as an isolating mechanism in this species, where gene flow between host-specific populations could potentially disrupt adaptive gene complexes. Thus, isolation by host specificity may drive patterns of genetic divergence among populations, reinforced by inbreeding. Previously, molecular markers were used to identify genetic divergence between two races of *O. minor*: *O. minor* var. *minor*, which grows preferentially on red clover (*Trifolium pratense*) as well on a range of native and alien exotic hosts, and *O. minor* ssp. *maritima*, which grows almost exclusively on sea carrot (*Daucus carota* ssp. *gummifer*) (Thorogood *et al.*, 2008). Here, a three-way approach to quantify the host specificity of these races on their natural hosts, red clover and sea carrot, is described with a view to understanding the physiological basis for their divergence. Specifically, the following were examined: (*a*) the early stages of infection of these putative host-specific genetic races using 'rhizotron' bioassays, in which host and parasite are cultivated together in reciprocal 'choice' experiments; (*b*) the fitness of parasites cultivated on clovers and carrots as measured by biomass; and (*c*) the anatomy of the host–parasite interface using histochemical techniques. Due to the rapid life cycle of *O. minor* (approx. 16 weeks), and its broad host range, this species represents an excellent model with which to study host–parasite interactions using an experimentally tractable holoparasite.

MATERIALS AND METHODS

Plant material

Plants of red clover (*Trifolium pratense*) were raised from seeds collected from a population infested with *Orobanche minor* var. *minor* in Somerset (ST5471) on calcareous grassland. Sea carrot (*Daucus carota* ssp. *gummifer*) plants were raised from seeds collected from a population in Dorset (SY8179) infested with *O. minor* ssp. *maritima* on south-facing sea cliffs. For each host species, seeds from ten individuals were pooled and sown in trays, then repotted after 6 weeks for use in rhizotron and pot experiments. Seeds from *O. minor* var. *minor* and *O. minor* ssp. *maritima*, identified to be genetic races (Thorogood *et al.*, 2008), were also collected from populations at the sites described above. For each race, seeds from ten individuals were pooled prior to inoculation. Both host and parasite seeds were stored at 4 °C until use.

Rhizotron bioassay study of early infection

Early stages of parasite infection were observed by cultivating host plants with their parasite associates in rhizotron bioassays. Rhizotrons consisted of Petri dishes (10 cm diameter) filled with sand in which a hole was cut at each end, one to allow the host plant to grow though, and the other for drainage (Fig. 1). Host plant root systems were spread out on two layers of glass-fibre filter paper (Sigma-Aldrich), and the rhizotrons then sealed with light-excluding polyethene to prevent root greening (Keith *et al.*, 2004). Rhizotrons were maintained for 3 weeks under a 16-h day-length regime in a controlled environment growth cabinet, operating with a photon flux density of 900 µmol m^{-2} s^{-1} at 21 °C, to allow the root systems to develop. Rhizotrons were then inoculated with parasite seed in a reciprocal experimental design in which ten clovers and ten carrots were each inoculated with seed from both their 'alien' and 'natural' parasite races, respectively. *Orobanche* seed was pre-conditioned for 2 weeks at 20 °C in the dark, and then surface-sterilized with 70 % ethanol for 2 min followed by 10 % commercial bleach and 0·1 % Tween 20 for 5 min, followed by six rinses with double-distilled water (ddH$_2$O). *Orobanche* seed was immersed in distilled water and 100 mg (dry seed weight) inoculated on the filter paper of the rhizotron by pipette. Inoculated rhizotrons were then transferred to a controlled environment growth cabinet (conditions as described above) and watered weekly. Thirty days after inoculation, rhizotrons were observed under a Nikon binocular microscope, and the following stages of infection recorded: (*a*) number of *Orobanche* seeds germinated; (*b*) number of germinated seeds attached to host roots; (*c*) number of parasite tubercles present (Fig. 2A). Due to the difficulties associated with counting large numbers of tiny parasite seeds, a 5 × 5 mm grid marked on a transparent acetate sheet was placed on each rhizotron and germination and attachment counts carried out in three randomly chosen grid-squares for each replicate, in which both host root and parasite seeds were present. Attempts to artificially germinate
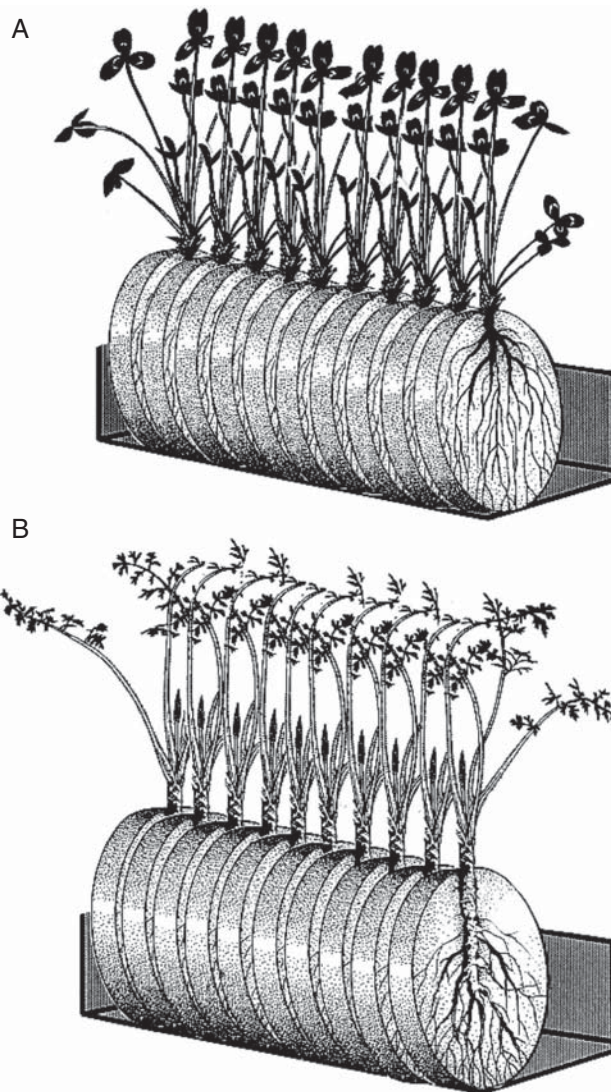
FIG. 1. Rhizotron bioassays in which host plants were inoculated with alien and natural *O. minor* races. Using a reciprocal infection design, ten clover plants (A) and ten sea carrot plants (B) were each inoculated with *O. minor* var. *minor* and *O. minor* ssp. *maritima*.
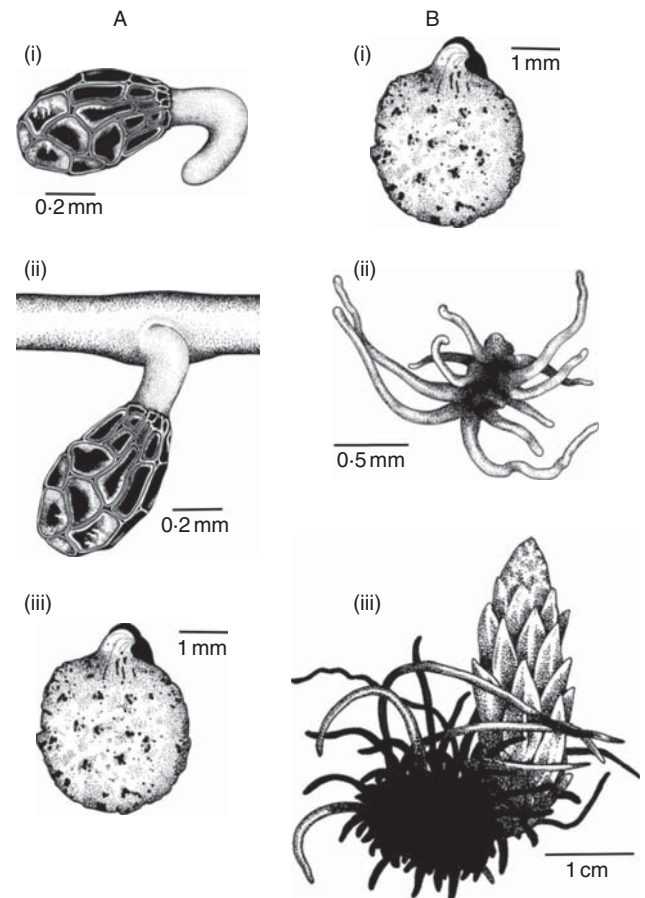

FIG. 2. Stages of *O. minor* development. (A) Stages recorded from rhizotron bioassays: (i) seed germination, (ii) seedling attachment to host root, (iii) tubercle. (B) Stages recorded from pot-cultivated host–parasite interactions: (i) tubercle, (ii) 'spider', (iii) floral shoot.

seeds to manipulate on host roots were unsuccessful, due to the lack of responsiveness of *O. minor* ssp. *maritima* to the germination inducer GR-24, which to date is the most universal germination stimulant for parasitic plant seeds.

Pot study of parasite and host growth and development

Fifty seedlings each of red clover and sea carrot cultivated previously for 6 weeks in seed trays were transplanted into 8×8 cm pots and maintained in controlled-environment growth cabinets (conditions as described above) for a further 6 weeks to develop. After this period, weaker individuals were discarded, and ten of the remaining plants selected at random to infect with *Orobanche* seed for each treatment. Using a reciprocal infection design, ten clovers and ten carrots were each inoculated with alien and natural parasite races, respectively. Ten clovers and ten carrots were also kept as uninfected controls. To infect host plants, 12×12 cm pots were filled to a depth of 10 cm with a substrate of $40 : 40 : 20$ sharp sand, compost and garden lime (to simulate the calcareous substrates on which both host species naturally occur), and 100 mg of dry *Orobanche* seed added. A single host plant was then added to each pot, and the remainder of the pot filled with the substrate. Pots were then arranged in a randomized design, and watered weekly. Every 2 weeks, plants were watered additionally with Vitafeed 111 (Vitax Ltd) at 0.5 g L^{-1} (half the recommended dose). After 16 weeks, the first parasites formed floral buds, and plants were harvested: host shoots (including stems, leaves and flowers) were separated from root balls, which were carefully washed under running water over a 2-mm-meshed sieve (Barker *et al.*, 1996). Parasites were then dissected from the host roots and classified into three developmental stages: (*a*) tubercles; (*b*) 'spiders' and (*c*) floral shoots (Fig. 2B); attempts to classify developmental stages further appeared to be subjective, and were therefore abandoned. Host and parasite material were then dried to constant weight at $70\,°C$ for 7 d prior to weight determination.

Data analyses

The data were subjected to Student's *t*-test or analysis of variance (ANOVA) as appropriate using SPSS statistical software for Windows. Differences between individual means were tested for significance using the *post-hoc* Tukey's multiple comparison test. Proportion data from the rhizotron studies were transformed using arcsine square root transformation of the raw data ($y = \arcsin\sqrt{x}/100$, where x = percentage germination) prior to statistical analysis (Fenández-Aparicio *et al.*, 2007).

Histological studies

Orobanche tubercles attached to the corresponding host root were dissected from rhizotrons 50 d after inoculation. Five samples per treatment were fixed in FAA (50 % ethanol + 5 % formaldehyde + 10 % glacial acetic acid in ddH$_2$O) for 48 h at 4 °C (Pérez-de-Luque *et al.*, 2005). Fixed samples were dehydrated through an ethanol series (50 %, 75 % overnight, 80 %, 100 %, 100 %; 2 h each). Samples were then taken through a Histo-Clear^{TM} (Raymond A. Lamb) ethanol series (30 %, 50 %, 80 %, 100 %, 100 %; 0·5 h each) and then saturated in paraffin (VWR International) in a vacuum oven (Hearson, UK). Thin sections (10 μm) were cut with a rotary microtome (Leica) and mounted on microscope slides in a water bath at 60 °C. Paraffin was removed from sample material with Histo-Clear^{TM} (5 min twice) and rehydrated through an ethanol-water series (100 %, 100 %, 80 %, 70 %, 50 %, 3 0 %, ddH$_2$O; 5 min each). Toluidine blue was used as a general stain for parasite and host tissue; sections were immersed in 0·5 % toluidine blue in 0·05 M phosphate buffer, pH 7·2 for 60 s (Hiscock *et al.*, 2002). Sections were then dehydrated through an ethanol–water series, immersed in Histo-Clear^{TM} twice and mounted with DPX (Fisher, UK). Sections were observed using a light microscope (Leica DMLB) under bright field illumination (magnification ×100 to ×400) and photographed using an SLR camera (RICOH photo micro).

RESULTS

Early stages of infection on sea carrots and red clovers

Orobanche minor races showed evidence of host preference even during the initial stages of infection (Table 1 and Fig. 3). Seeds of both *O. minor* var. *minor* and *O. minor* ssp. *maritima* showed high levels of germination on sea carrots (60·56 % ± 3·50 and 59·30 % ± 3·43, respectively), which were not significantly different on this host ($P = 0·99$). Levels of germination on clovers were significantly lower than on carrots for both *O. minor* var. *minor* and *O. minor* ssp. *maritima* (28·10 % ± 7·23 and 4·80 % ± 1·99, respectively; $P < 0·01$). Attachments (Fig. 2A) were observed in all treatments other than *O. minor* ssp. *maritima* on clover roots though this may be a direct consequence of poor germination on this host, which was significantly lower than for all other treatments ($P < 0·01$). However, levels of attachment among the parasite races were not equal on all hosts; the highest level of attachment was observed in *O. minor* var. *minor* infecting sea carrot (3·29 % ± 1·05 of germinated

TABLE 1. *Relative proportions of the three classified stages of parasite development observed in rhizotron bioassays*

Treatment ($n = 10$)	% seeds germinated (± s.e.)	% seedlings attached (± s.e.)	% tubercles (± s.e.)
O. minor ssp. *maritima* on sea carrot	59·35 ± 3·43	0·87 ± 0·30	0
O. minor var. *minor* on sea carrot	60·56 ± 3·50	3·29 ± 1·05	0·22 ± 0·16
O. minor ssp. *maritima* on red clover	4·80 ± 1·99	0	0
O. minor var. *minor* on red clover	28·10 ± 7·23	1·17 ± 0·43	0

Values are expressed as a percentage of the total number of seeds counted. Measurements were taken 30 d after inoculation.
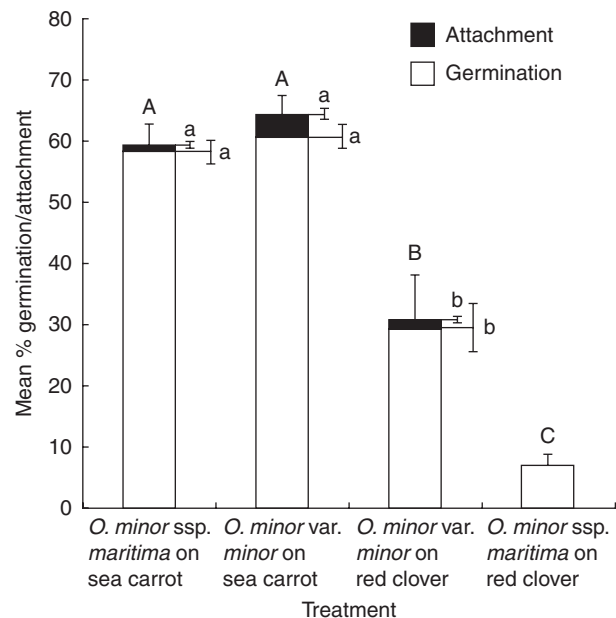

FIG. 3. Percentage germination and attachment of *O. minor* var. *minor* and *O. minor* ssp. *maritima* seedlings, reciprocally cultivated on sea carrots and clovers in rhizotrons. Percentages calculated from data pooled from ten rhizotrons per treatment; results presented as means ± s.e. Columns sharing the same letter are not significantly different (ANOVA, $P > 0·01$): upper-case letters are the column totals; lower-case letters refer to the components within each column.

seedlings; Table 1). Indeed, this race showed a higher level of attachment on sea carrot than on red clover (the natural host for this race) which may be a result of the higher level of germination on the sea carrots. Tubercles (Fig. 2A) were only produced by *O. minor* var. *minor* infecting sea carrot roots at the time of data collection (0·22 % ± 0·16 of germinated seedlings), suggesting a higher rate of infection on this host compared with other treatments.

O. minor *infectivity on pot-grown sea carrots and red clovers*

Orobanche minor races showed quantitative differences in infectivity on pot-grown hosts (Fig. 4). The largest mean

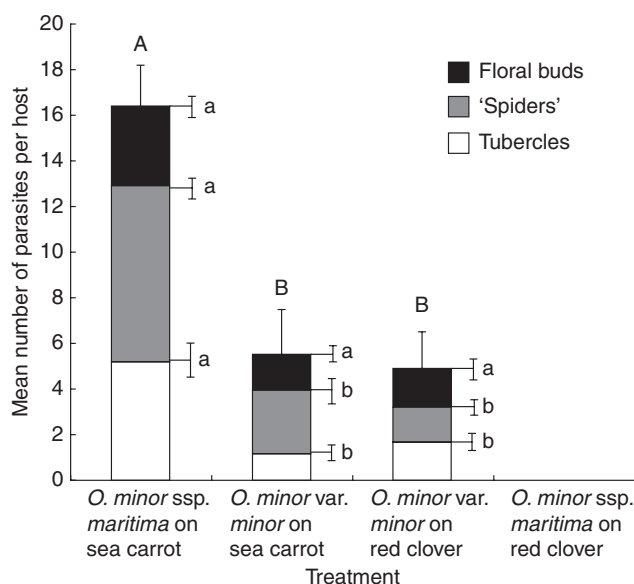FIG. 4. Mean number of *O. minor* plants per host cultivated in pots, classified as tubercles, 'spiders' and floral buds. Results presented as means ± s.e. (*n* = 10). Columns sharing the same letter are not significantly different (ANOVA, *P* > 0·05): upper-case letters are the column totals; lower-case letters refer to the components within each column.

number of plants per host was observed in sea carrots infected with *O. minor* ssp. *maritima* (*P* < 0·01). Sea carrots and red clovers infected with *O. minor* var. *minor* yielded similar numbers of plants (*P* = 0·97), whereas clovers inoculated with *O. minor* ssp. *maritima* showed no evidence of infection. In all treatments where infection was observed, mature *Orobanche* plants produced floral buds, indicating completion of the parasite life cycle on that host. *Orobanche minor* races also showed quantitative differences in biomass accumulation on different hosts, irrespective of the total number of plants attached (Table 2 and Fig. 5). The highest accumulation of biomass was observed for *O. minor* races cultivated on their natural hosts (*O. minor* var. *minor* on red clover and *O. minor* ssp. *maritima*

on sea carrot), which did not differ significantly (177·3 and 203·1 mg host^{-1}; *P* = 0·39). The lowest accumulation of parasite biomass was observed for *O. minor* var. *minor* on sea carrot – the alien host for this race, though this was not significantly lower than the other treatments (65·5 mg host^{-1}; *P* = 0·24). Qualitative differences were also observed among the different treatments; 'spiders' (Fig. 2B) attached to sea carrots induced a significant distension of the host root, such that the diameter of the host root above the point of attachment was approximately four times the diameter of the corresponding root below the point of attachment (Fig. 7C). Such tissue hyperplasia was absent or less pronounced in clover roots in response to infection. In addition, 'spiders' frequently produced secondary haustoria on carrot roots (Fig. 7D) that were never observed on clover roots, suggesting differences may occur in the modes of nutrition and perhaps the life history of races infecting different hosts.

Host biomass

Host plants generally showed little reduction in total dry weight in response to *O. minor* infection (Table 2 and Fig. 6). Red clovers suffered a significant decrease in biomass (−10·5 %; *P* < 0·05) in response to infection with *O. minor* var. *minor*. This reduction in host biomass was only partially compensated for by *Orobanche* biomass (1·32 g unaccounted for). However, sea carrot showed only a small reduction in biomass in response to infection with either *O. minor* var. *minor* or *O. minor* ssp. *maritima* that was not significantly different from uninfected controls (−0·25 and −2·74 %, respectively; *P* = 0·859). The allometric relationship between host shoot and root dry weight was not affected by parasitism in clovers or carrots (Table 2).

Histological observations of haustorial morphology

Orobanche minor ssp. *maritima* produced haustoria on sea carrot roots, and *O. minor* var. *minor* produced haustoria on both sea carrot and red clover roots, and no evidence of host

TABLE 2. *Biomass values obtained from pot-cultivated host plants and* O. minor *races in dry weight, showing the relative abundance of three developmental stages of parasite infection (A) and the partitioning of biomass between host shoots and roots (B)*

(A) Mean parasite biomass

Treatment (*n* = 10)	Tubercles (mg host^{-1}; ± s.e.)	'Spiders' (mg host^{-1}; ± s.e.)	Floral buds (host^{-1}; ± s.e.)
O. minor ssp. *maritima* on sea carrot	16·50 ± 0·002	60·00 ± 0·002	126·6 ± 0·012
O. minor var. *minor* on sea carrot	0·40 ± 0·001	27·70 ± 0·003	37·0 ± 0·006
O. minor ssp. *maritima* on red clover	–	–	–
O. minor var. *minor* on red clover	6·40 ± 0·002	21·10 ± 0·004	149·8 ± 0·030

(B) Mean host biomass

| Treatment (*n* = 10)	Shoot (g; ± s.e.)	Roots (g; ± s.e.)	Shoot : root
Sea carrot infected with *O. minor* ssp. *maritima*	8·39 ± 0·40	6·32 ± 0·18	1·33
Sea carrot infected with *O. minor* var. *minor*	8·55 ± 0·17	6·31 ± 0·13	1·35
Uninfected sea carrots	8·69 ± 0·27	6·44 ± 0·26	1·35
Red clover infected with *O. minor* ssp. *maritima*	–	–	–
Red clover infected with *O. minor* var. *minor*	6·88 ± 0·26	5·80 ± 0·10	1·19
Uninfected red clover	7·96 ± 0·39	6·22 ± 0·19	1·28

Biomass was harvested 16 weeks after inoculation.
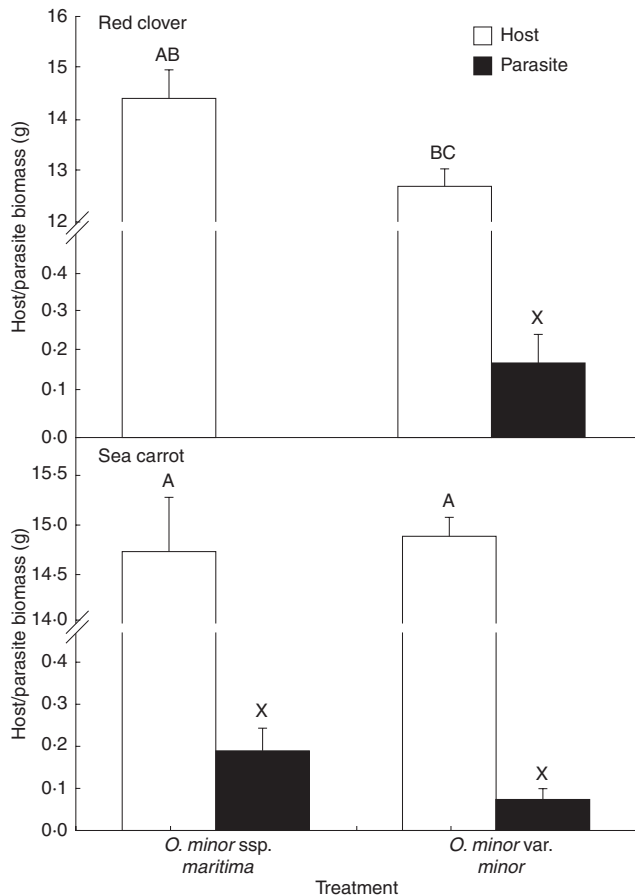
FIG. 5. Host and parasite biomass harvested from pot-cultivated red clovers and sea carrots. Note the split scale; results presented as means ± s.e. (*n* = 10). Bars sharing the same letter (ABC = hosts; X = parasites) are not significantly different (ANOVA, *P* > 0·01).

resistance was observed. Haustoria on both host species were large and highly differentiated, producing interfacial parenchyma and a concentration of haustorial xylem strands at the host–parasite interface, suggesting vascular conduits were present (Fig. 7E). Morphological differences were also observed; penetration of the haustorium through the clover root cortex resulted in little disruption of host cortical cells, or distension of the host root. The endophyte (internal haustorial cells) penetrated the vascular core of the host root (Fig. 7F). Penetration of carrot roots by haustoria produced by both *O. minor* var. *minor* and *O. minor* ssp. *maritima* also resulted in vascular continuity between host and parasite tissues, but produced significant distortion of host tissues. The large endophyte engulfed the host root cortex, resulting in significant destruction of cortical cells proximal to the haustorium, and a distal hyperplasia of the root cortex (Fig. 7G). In addition, infection of carrot roots resulted in extensive differentiation of secondary xylem at the host–parasite interface (Fig. 7H).

DISCUSSION

Orobanche minor sensu lato is not a strict host generalist, and genetic races appear to show physiological adaptations to their
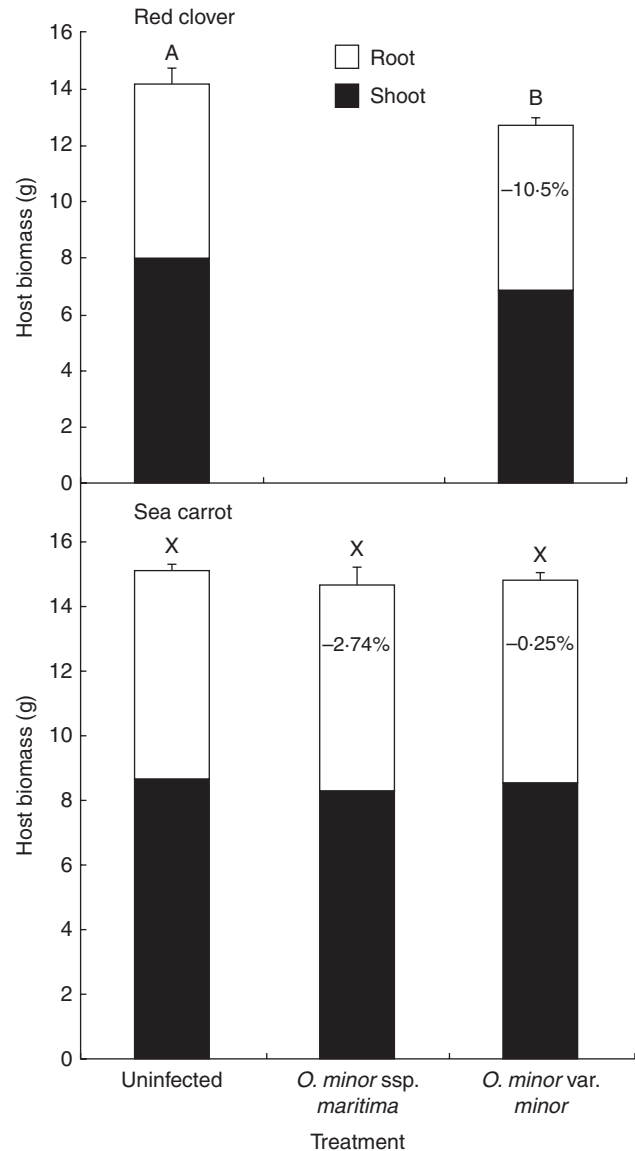

FIG. 6. Mean host biomass from pot-cultivated red clovers and sea carrots, separated into shoot and root tissue. Numbers in columns represent percentage reduction in total host biomass compared with controls. Results presented as means ± s.e. (*n* = 10). Columns sharing the same letter are not significantly different (A + B: *t*-test, *P* > 0·05; X: ANOVA, *P* > 0·05).

local host associates. Patterns of host specificity may have isolated populations of *O. minor* on hosts with particular ecological niches, and driven their genetic divergence, as suggested by a recent study by Thorogood *et al.* (2008). The current data show that *O. minor* var. *minor* can infect both red clover and sea carrot, but with a degree of preference towards clover, the commonest host for this taxon. However *O. minor* ssp. *maritima* showed a high degree of host specificity towards sea carrot, and did not develop on red clover beyond the stage of germination. Germination of *O. minor* ssp. *maritima* seed on clover was significantly lower than for *O. minor* var. *minor*, suggesting that low induction of germination may be an important component of the host specificity of this race. Germination in response to host-derived chemical
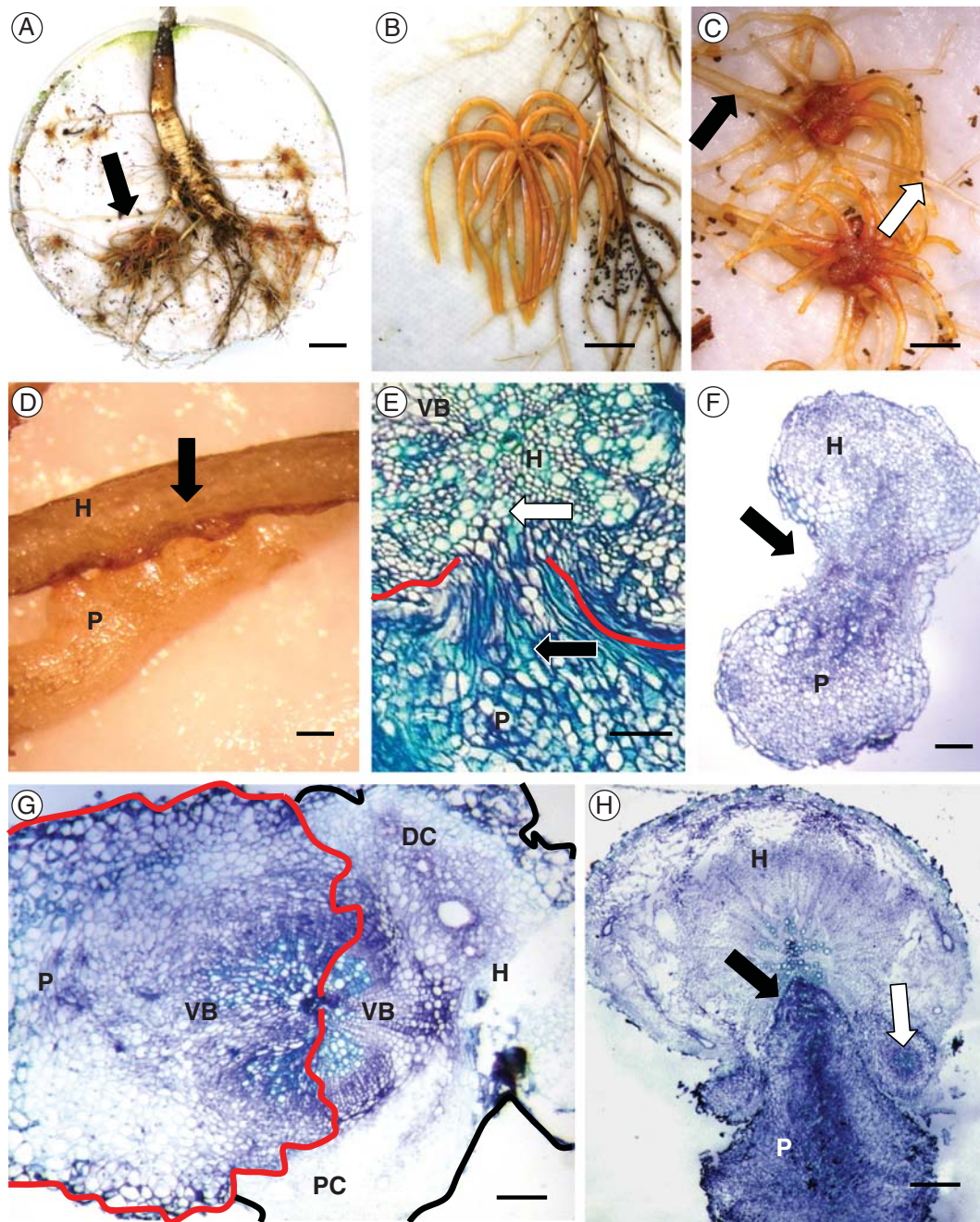
FIG. 7. (A) Rhizotron-propagated root system of a sea carrot infected with *O. minor* 'spiders' (arrow); (B) 'spider' of *O. minor* var. *minor* on clover root; (C) 'spiders' of *O. minor* ssp. *maritima* on carrot roots (note the host root is distended before the point of parasite attachment, indicated by a black arrow, relative to post-attachment); (D) secondary haustoria of *O. minor* subsp. *maritima* parasite (P) attached to host root (H); (E) haustorial interface of *O. minor* on clover root with host (H) xylem elements (white arrow) and a concentration of parasite (P) xylem elements (black arrow) resulting in vascular connectivity (red lines mark the host–parasite interface); (F) haustorium of *O. minor* var. *minor* (P) attached to clover root (H) with vascular connectivity (arrow) at host–parasite interface; (G) *O. minor* ssp. *maritima* haustorium (P) attached to carrot root (H) showing connectivity of vascular bundles (VB) and massive distension of distal host cortical parenchyma (DC) relative to proximal host cortical cells (PC). Parasite tissue is marked in red, host tissue is marked in black; (H) *O. minor* var. *minor* haustorium (P) attached to host carrot root (H) with parasitic endophyte (black arrow) and secondary parasite vascular tissue differentiation. Scale bars: (A) 1 cm; (B) 4 mm; (C) 3 mm; (D) 0·5 mm; (E) 50 μm; (F) 200 μm; (G) 200 μm; (H) 200 μm.

cues, followed by rapid attachment is believed to be a cause of host selection (Press and Phoenix, 2005; Phoenix and Press, 2005). In addition, attempts to artificially induce germination of seeds using the strigol analogue GR-24 during the course of these experiments were successful for *O. minor* var.

minor, yet seeds of *O. minor* ssp. *maritima* were unresponsive (data not shown), indicating distinct biochemical differences between these taxa in their mechanisms of host detection. Chemical features of host phenotypes have previously been shown to affect parasite specificity, and potentially facilitate

genetic differentiation of host-specific races in the dwarf mistletoe (*Arceuthobium vaginatum*; Snyder *et al.*, 1996). Differences in infection rates of *O. minor* races on red clover and sea carrots may also be an indirect result of differences in host root-mass, as well as biochemistry. Spread, size and growth rate of root systems vary between species, and potential hosts with spreading and fast-growing roots may be more likely to encounter parasite seeds (Keith *et al.*, 2004). The root systems of pot-cultivated sea carrots and red clovers yielded similar values in biomass; however, in rhizotrons, carrot roots had a greater spread. Higher germination of both races on sea carrot may therefore have been a consequence of increased root availability, and of a subsequently greater production of germination stimulants in this host.

Data from pot-cultivated hosts were largely congruent with the results from the rhizotron bioassay study. Interestingly, sea carrots infected with *O. minor* ssp. *maritima* yielded a significantly higher density of established plants than other treatments, yet the biomass accumulated by the parasites was not significantly different from that of red clovers infected with *O. minor* var. *minor*, suggesting that a finite biomass is available to the parasite, regardless of their abundance. Similar results were obtained from experiments in which tobacco plants inoculated with different densities of *O. cernua* seed yielded the same parasite biomass (Hibberd *et al.*, 1998). The largest biomass accumulation was observed in treatments in which host plants were infected with the races of *O. minor* with which they naturally co-occur. This reinforces evidence from the rhizotron bioassay study, which suggests that parasite races show patterns of adaptation to their local hosts. Similarly, *O. minor* ssp. *maritima* showed no evidence of infecting red clover, indicating that this race shows little or no development on this species beyond the stage of germination. The treatment with the largest number of infections in the pot-growth experiment was *O. minor* ssp. *maritima* on sea carrot; however, in the rhizotron bioassay experiment, *O. minor* var. *minor* showed a greater infectivity on this host. This disparity may suggest a time lag in infectivity by *O. minor* ssp. *maritima* on its natural host, or that pots provide a more accurate simulation of natural host–parasite interactions. Plants used for rhizotron bioassays were also younger and smaller than pot-grown plants. Evidence suggests that variation in source–sink relationships in host plants is age-dependent, and may affect the infectivity of parasitic plants; older hosts have a greater source strength but also have a larger sink strength, obstructing the parasite's access to resources (Cameron *et al.*, 2005). This should be a minor limitation of the present study since rhizotrons were only used to measure the early stages of infection. Interestingly, total host biomass, and biomass partitioning between shoot and root showed very little reduction or change in response to infection for each treatment, in contrast to previous studies, in which the biomass of host plants was reduced by up to 50 % due to infection by parasitic plants (Barker *et al.*, 1996; Hibberd *et al.*, 1998; Labrousse *et al.*, 2001; Vasey *et al.*, 2005). However, it is known that the relationship between parasitic angiosperms and their hosts is variable, and infection may result in negligible effects in terms of host growth and physiology (Cameron *et al.*, 2005). Indeed the effect of *Orobanche* parasitism on its

host plants in particular is known to vary (Labrousse *et al.*, 2001).

It has been suggested previously that parasitic plants have a higher fitness on leguminous hosts, probably as a consequence of their high nitrogen content (Seel and Press, 1993; Radomiljac *et al.*, 1998, 1999). In the present study, *O. minor* ssp. *maritima* was unable to infect red clover, and *O. minor* races performed better on the hosts on which they naturally occur. Therefore populations of *O. minor sensu lato* probably show patterns of host specificity regardless of the nitrogen content of potential hosts. Similarly, Jiang *et al.* (2008) have established that nitrogen fixation does not influence the quality of leguminous hosts for the root hemiparasite *Rhinanthus minor*, and that haustorial anatomy and host resistance may in fact underpin host preference. Parasites may also show a preference towards deep-rooted hosts with improved access to water (Pate *et al.*, 1990); sea carrot occurs on sunbaked sea cliffs and produces tap roots, which may confer advantages over co-occurring potential host species. Most species of *Orobanche* parasitize a narrow range of perennial hosts and are potentially perennial themselves, while a few have shifted to annual hosts in anthropogenically disturbed ecosystems. Therefore host-specialization may be associated with predictable resources (perennial hosts) and host-generalism with unpredictable resources (annual hosts), a pattern often found in animal parasites (Schneeweiss, 2007). *Orobanche minor* ssp. *maritima* parasitizes sea carrot, which is perennial, and is therefore potentially perennial itself. Furthermore, secondary haustoria were observed arising from this parasite on sea carrot, which could potentially give rise to multiple foci of infection, and confer a perennial life history to this race of *O. minor*. Secondary haustoria have recently been observed arising from the roots of *O. aegyptiaca* on the tubers of *Solanum tuberosum*, and are believed to serve the parasite in acquiring additional nutrients from neighbouring roots (Joel, 2007).

Orobanche minor var. *minor* often parasitizes red clover which is probably short-lived, and occurs on disturbed grassland. In the present study, *O. minor* var. *minor* showed no evidence of producing secondary haustoria on red clover, indicating the life history of *O. minor* varies on different hosts. Host root anatomy may also contribute to the evolution of physiological races of parasite; for instance, thicker carrot roots may contain larger vascular bundles for efficient host–parasite solute transfer, but also require significant energy expenditure to penetrate. Parasites attached to carrot roots induced conspicuous distention on the host root. Previous studies have also identified host root distension at the point of *Orobanche* attachment (Dale and Press, 1998). While such a response was also observed on clover roots, the degree of swelling at the point of infection was substantially less. Observations of the host–parasite interface suggest that the significant swelling response to infection seen in carrot roots may be a result of a proliferation of host root cortical cells causing a hyperplasia of root tissue. The unusually large differentiation of the haustorial cells of the parasite endophyte may cause disruption to the host root cortex, since cells appeared to be crushed in response to haustorial penetration. Deformation of the host root cortex has also been observed in sunflower roots infected by *O. cumana* (Labrousse *et al.*,

2001). Similarly, Cameron *et al.* (2006) report parasite-induced host root deformation, and cellular resistance to haustoria in the root hemi-parasite *Rhinanthus minor*. In susceptible hosts, haustoria encompassed the host root, crushed the cortex, and formed a penetration peg containing differentiated secondary xylem. In contrast, haustoria on resistant hosts showed poor development, and failed to encompass the host root. Variation in root anatomy and host susceptibility to infection may lead to differential parasite fitness on particular hosts, which is likely to have played a major role in the evolution of physiological races of parasitic plants, and coevolution with their hosts.

The present data support previous research which suggested that *O. minor* may comprise host-specific strains (Musselman and Parker, 1982). Characterization of host-specific races among natural populations of *Orobanche* will be a useful tool in understanding patterns of speciation in parasitic plants. It has been well documented that host specificity can drive the synchronous speciation of parasites with their host associates, a relationship referred to as Fahrenholz's rule (Fahrenholz, 1913; Hafner and Nadler, 1988; Hafner and Page, 1995). Divergence followed by isolation on hosts with differing ecologies has been established as a probable route to speciation in insects (Drès and Mallet, 2002), e.g. leaf beetles (*Ophraella* spp.; Funk *et al.*, 1995), aphids (*Acyrthosiphon* spp.; Hawthorne and Via, 2001), stick insects (*Timema* spp.; Nosil *et al.*, 2002) and tephritid fruitflies (*Rhagoletis* spp.; Schwarz *et al.*, 2005). It has also been hypothesized that host-driven divergence may have played an important role in the speciation of parasitic plants (Zuber and Widmer, 2000; Thorogood *et al.*, 2008; de Vega *et al.*, 2008). For instance, host specificity may have preceded genetic divergence among hemiparasitic mistletoes (*Viscum album*): Zuber and Widmer (2000) used chloroplast DNA and nuclear DNA internal transcribed spacer markers to elucidate relationships among morphologically cryptic subspecies of mistletoe parasitizing different hosts. More recently, the root holoparasite *Cytinus hypocistis* (Cytinaceae) which shows distinct patterns of host specificity at a local level, has been shown to form regional patterns of divergence on different lineages of host in the Cistaceae (Thorogood and Hiscock, 2007; de Vega *et al.*, 2008). While studies on speciation in parasitic angiosperms are scarce, it is likely that host-driven divergence may be an underestimated driving force in the evolution of this poorly understood group of plants.

CONCLUDING REMARKS

Orobanche minor is a host generalist, attacking hosts from taxonomically disparate families (Rumsey and Jury, 1991). Using reciprocal-infection experiments, further incremental evidence that genetic divergence among populations of *O. minor* could be a consequence of host specificity (Thorogood *et al.*, 2008) has been provided by showing that *O. minor* comprises physiological races. Races of *O. minor* are morphologically cryptic, yet plants raised on sea carrot and red clover showed distinct host preferences and a higher fitness, measured in biomass, on their natural host associates. These races showed host specificity even at the level of germination, and

histological observations using sea carrot and red clover suggest that host responses to infection vary between species. Nevertheless, the present study only investigated the interaction between host and parasite over a small time scale. Further work will determine the differential fitness of parasitic races in terms of reproductive output, and extend this investigation to quantify host specificity of multiple populations, because *O. minor* var. *minor* occurs on numerous other host species besides red clover. Valuable information will be obtained from investigating the differences in solute flux of natural *Orobanche* strains on different hosts, which has to date, only been carried out using weedy species. In conclusion, we propose that physiological shifts in *Orobanche* taxa may underpin the host specificity of different cryptic races, and precede genetic divergence and ultimately speciation in this genus, and that this mode of speciation may be a phenomenon common to parasitic plants.

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