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Cryptic host-specific diversity among western hemisphere broomrapes (*Orobanche s.l.*, Orobanchaceae)

Adam C. Schneider[1,2,*], Alison E. L. Colwell[2], Gerald M. Schneeweiss[3] and Bruce G. Baldwin[1,2]

[1]*Department of Integrative Biology, 1005 Valley Life Sciences Building, University of California, Berkeley, CA 94720, USA,* [2]*Jepson Herbarium, 1001 Valley Life Sciences Building, University of California, Berkeley, CA 94720, USA and* [3]*Department of Botany and Biodiversity Research, University of Vienna, 1030 Vienna, Austria*
**For correspondence. E-mail acschneider@berkeley.edu*

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• **Background and Aims** The broomrapes, *Orobanche sensu lato* (Orobanchaceae), are common root parasites found across Eurasia, Africa and the Americas. All species native to the western hemisphere, recognized as *Orobanche* sections *Gymnocaulis* and *Nothaphyllon*, form a clade that has a centre of diversity in western North America, but also includes four disjunct species in central and southern South America. The wide ecological distribution coupled with moderate taxonomic diversity make this clade a valuable model system for studying the role, if any, of host-switching in driving the diversification of plant parasites.
• **Methods** Two spacer regions of ribosomal nuclear DNA (ITS + ETS), three plastid regions and one low-copy nuclear gene were sampled from 163 exemplars of *Orobanche* from across the native geographic range in order to infer a detailed phylogeny. Together with comprehensive data on the parasites' native host ranges, associations between phylogenetic lineages and host specificity are tested.
• **Key Results** Within the two currently recognized species of *O*. sect. *Gymnocaulis*, seven strongly supported clades were found. While commonly sympatric, members of these clades each had unique host associations. Strong support for cryptic host-specific diversity was also found in sect. *Nothaphyllon*, while other taxonomic species were well supported. We also find strong evidence for multiple amphitropical dispersals from central North America into South America.
• **Conclusions** Host-switching is an important driver of diversification in western hemisphere broomrapes, where host specificity has been grossly underestimated. More broadly, host specificity and host-switching probably play fundamental roles in the speciation of parasitic plants.

Key words: Amphitropical disjunction, cryptic speciation, holoparasite, host-switching, *Orobanche*, Orobanchaceae, parasite, phylogeny.

INTRODUCTION

Parasitism is a highly successful life strategy that has evolved independently >60 times among animals, at least 12 times among angiosperms, and repeatedly in protozoans and prokaryotes (Poulin and Morand, 2000; Westwood *et al*., 2010). While the evolutionary significance of host–parasite associations has long been recognized (Kellogg, 1913), the main evolutionary mechanisms involved in the generation and maintenance of such ecological and phylogenetic diversity are still poorly understood, especially among parasitic flowering plants (de Vienne *et al*., 2013; Joel *et al*., 2013).

The parasitic broomrapes, *Orobanche sensu lato* (*s.l.*) [alternatively circumscribed as the genera *Aphyllon* and *Myzorrhiza* in the New World, and *Boulardia*, *Orobanche sensu stricto* (*s.s.*) and *Phelipanche* in the Old World: Schneeweiss (2013)], have attracted significant attention as an important system for understanding the evolutionary consequences of parasitism. This attention is in part a result of their extensive worldwide diversity (at least 170 species; Ulrich *et al*., 1995), a detailed and well-supported understanding of their placement within the family Orobanchaceae as well as the relationships among major clades (Schneeweiss *et al*., 2004*a*; Park *et al*., 2008; McNeal *et al*., 2013; Schneeweiss, 2013), and the significant economic damage caused by several Eurasian species to major agricultural systems worldwide (Joel *et al*., 2013).

Despite the interest in this group, relatively little is known about the role of host specificity in broomrape diversification. Understanding host specificity of parasites is predicated on a comprehensive understanding of lineage boundaries in the host (e.g. Labrousse *et al*., 2001; Timko *et al*., 2012) and, more importantly for *Orobanche*, the parasite. That is, failure to recognize evolutionary diversity in the parasite results in an overestimation of host breadth and may limit the ability to understand the evolutionary processes responsible for speciation in plant parasites (Refrégier *et al*., 2008). Therefore, it is important to distinguish true host generalists from taxa that comprise several cryptic lineages artificially united on the basis of superficial similarity but distinguished genetically and ecologically. Host specificity to the family or genus level has been cited as a key factor in the differentiation and genetic isolation of three subspecies of the European *O*. *minor* (Thorogood *et al*., 2008, 2009), but this has not been broadly tested across other *Orobanche* lineages. Several recently described species of

Orobanche sect. *Gymnocaulis*

Orobanche sect. *Nothaphyllon*

nrDNA (ITS + ETS)

O. uniflora subsp. uniflora (Astereae + *Rudbeckia*)

Antennaria + Senecio

Saxifragales

Apioideae

O. uniflora subsp. *occidentalis*

Artemisia

Galium

O. fasciculata

Eriogonum, Eriodictyon, Eriophyllum, + Phacelia

subsp. *valida*

subsp. *howellii*

O. valida (*Garrya*)

O. dugesii

O. cooperi (Heliantheae)

O. bulbosa (*Adenostoma*)

O. parishii subsp. *parishii* (*Artemisia*)

O. ludoviciana (*Artemisia*)

O. corymbosa (*Artemisia*)

O. parishii subsp. *parishii* (Asteroideae)

O. corymbosa (*Artemisia*)

subsp. *grayana* (Astereae)

subsp. *californica* (*Grindelia*)

O. californica

subsp. *parishii*

subsp. *brachyloba*

subsp. *parishii*

subsp. *jepsonii*

subsp. *feudgei* (*Artemisia*)

O. parishii

O. californica

O. vallicola (*Sambucus*)

subsp. *grandis* (*Lessingia*)

subsp. *jepsonii*

subsp. *grandis* (*Lessingia*)

subsp. *condensa* (*Heterotheca*)

Grindelia

Eriophyllum

subsp. *californica*

O. californica

O. californica complex

O. pinorum (*Holodiscus*)

O. tarapacana (*Trixis*)

O. sp. nov (*Zaluzania*)

O. ludoviciana + multiflora (Astereae + *Varilla*)

O. tacnaensis

O. chilensis (*Grindelia*)

O. riparia (Ambrosiinae)

O. arizonica (*Gutierrezia*)

O. ludoviciana complex

Orobanche in North America also have unique host preferences in the Asteraceae: *Orobanche riparia* parasitizes Helianthieae sub-tribe Ambrosiinae and *O. arizonica* parasitizes *Gutierrezia* spp. However, neither these species concepts nor those of the other American *Orobanche* species has ever been tested phylogenetically.

Inclusion of western hemisphere *Orobanche* (sections *Gymnocaulis* and *Nothaphyllon*) in phylogenetic studies has been limited to several exemplars included in larger genus- or family-level analyses. These studies, supported by karyological and morphological evidence, have shown that these two sections are sister groups and together are sister to an Old World clade corresponding to *Orobanche* sect. *Trionychon* (Schneeweiss *et al*., 2004*a*; Park *et al*., 2008), more recently treated as the genus *Phelipanche* (Schneeweiss, 2013). This larger clade is supported by a shared base chromosome base number of $x = 12$ (Heckard and Chuang, 1973; Schneeweiss *et al*., 2004*b*).

Ecologically, *Orobanche* sections *Gymnocaulis* and *Nothaphyllon* parasitize a wide range of eudicot hosts, but most commonly perennial Asteraceae. Taxonomic diversity is concentrated in the California Floristic Province; however, species can be found across the Americas, as far north as the Alaska Peninsula and the Yukon Territory, east to Newfoundland, and south to central Mexico. Four poorly known species are found in South America. Affinities between South American *Orobanche chilensis* and North American *O. ludoviciana* have long been recognized (Beck, 1890), but explicit biogeographic hypotheses for this or other such relationships within the clade have yet to be proposed.

The wide ecological and host diversity among western hemisphere *Orobanche*, as well as its tractable taxonomic diversity, make it a valuable model system for understanding the main ecological and evolutionary processes affecting parasite diversification and speciation. Such investigations, however, are requisite for a robust understanding of evolutionary lineages, their host breadths and their relationships. Specifically our goals were to (1) reconstruct a well-resolved phylogeny of western hemisphere *Orobanche* that could be used to develop a revised, natural classification for the group; (2) evaluate the evolutionary significance of host-switching in *Orobanche* sect. *Gymnocaulis* by comprehensively sampling across the geographic and host ranges of each taxon; (3) test the monophyly of longstanding taxa as well as recently described segregates; and (4) infer biogeographical relationships between North American and South American *Orobanche* spp.

MATERIALS AND METHODS

Taxon and population sampling

163 *Orobanche* populations were sampled either from fresh collected tissue or from herbarium collections: 57 from sect. *Gymnocaulis* and 106 from sect. *Nothaphyllon* (voucher and host information is provided in Supplementary Data Table S1). This data set includes at least one exemplar of all taxa of *Orobanche* recognized within the last 75 years except for *O. weberbaueri*, a poorly known taxon from southern coastal Peru, perhaps known only from the type. Denser population sampling across sect. *Gymnocaulis* enabled more comprehensive geographic and host range sampling in the two currently recognized species of this section, *O. fasciculata* and *O. uniflora* (Fig. 1). Identifying the host breadth for each taxon was challenging, as many collectors note the nearest living plant as the host species without confirming a haustorial connection, resulting in a proliferation of dubious records. Our criteria for accepting a host was that a host taxon must have been independently reported at several populations by more than one collector, or a haustorial connection to an identifiable fragment of host must be present on the herbarium voucher. Host associations for sampled populations are listed in Table S1. For molecular phylogenetic analyses, one individual each of *O. gracilis* and *O. hederae* were used as the outgroup (Park *et al*., 2008; McNeal *et al*., 2013). Sequence data for the *waxy* locus were not available for these outgroups, so instead two more distantly related outgroup taxa were used, *Castilleja ambigua* and *Triphysaria versicolor*.

DNA extraction, amplification and sequencing

DNA was extracted from dried floral tissue using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), or using a CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle, 1987). A total of six regions from the nuclear and plastid genomes were used to estimate the phylogeny: internal and external transcribed spacers of nuclear ribosomal DNA (ITS and ETS, respectively), introns 9–11 of the nuclear low-copy gene *waxy*, as well as the plastid trnL-trnF region (comprising the $trnL_{UAA}$ intron and the $trnL_{UAA}$-$trnF_{GAA}$ intergenic spacer) and the *matK* and *rps*2 genes. ITS, *matK*, and *rps*2 were selected based on their prior use in genus- and family-level phylogenetic studies of *Orobanche* (Schneeweiss *et al*., 2004*a*; McNeal *et al*., 2013), and *waxy* for its use in the related (hemi-)parasitic genus *Castilleja* (Tank and Olmstead, 2008). The remaining two regions, ETS and trnL-trnF, were selected to provide additional rapidly evolving characters from the nuclear and plastid compartments, respectively. Due to difficulty assessing homology within some species of sect. *Nothaphyllon*, the *waxy* locus was mainly used to assess monophyly of sect. *Nothaphyllon* and to infer relationships within sect. *Gymnocaulis*.

Polymerase chain reaction (PCR) amplifications were performed using AccuPower PCR PreMix kits (Bioneer, Alameda, CA, USA) or by generating a master mix of 10 μL of 5× Promega buffer, 4 μL of 25 mM $MgCl_2$, 1·25 μL of 10 mM dNTPs, 1 μL of 20 μM of each primer and 0·25 μL of Go-Taq DNA Polymerase (Promega, Madison, WI, USA) diluted to 50 μL. Complete information about primers, cycling parameters

FIG. 1. Bayesian inference majority-rule consensus tree of 162 *Orobanche* populations inferred from nrDNA (ITS + ETS). Tip labels include the collection number followed by the herbarium accession number, if available. Posterior probabilities >0·9 are shown in bold for nodes with >70 % maximum likelihood bootstrap (BS) support and in italics if BS support is < 70 %. The internal branches leading to section *Gymnocaulis* and section *Nothaphyllon* have been shortened by a factor of 1/2. Host associations are indicated in blue (Asteraceae) or green (other) to the genus or higher taxonomic level. Informally named clades are in purple. Outgroup taxa are not shown. Photographs, from top to bottom: *O. fasciculata* parasitizing *Eriodicyton* sp. (Schneider 606); *O. cooperi* parasitizing *Hymenoclea salsola* (Schneider 415); *O. vallicola* parasitizing *Sambucus mexicana* (Schneider 316); O. *corymbosa* parasitizing *Artemisia tridentata* (Colwell 14–26).

and amplicon sizes are provided in Table 1. PCR products were purified using ExoSAP (USB Products, Cleveland, OH, USA), and both DNA strands were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). GenBank accession numbers can be found in Table S1.

Sequence alignment and phylogenetic reconstruction

Sequences were checked for base-calling errors and assembled into contigs using Geneious v.6.1.7 (Biomatters, Auckland, New Zealand). Sequence alignments were generated using the MUSCLE plug-in with default settings. Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted separately on the concatenated chloroplast DNA matrix (cpDNA), the concatenated ribosomal spacers (nrDNA) and the *waxy* locus using the CIPRES Science Gateway (Miller *et al*., 2010). The ML analyses were performed with RAxML-HPC2 v.8.2.6 (Stamatakis, 2014) using the GTRCAT model with 25 rate categories and 1000 rapid bootstrap (BS) replicates. The BI analyses were performed using MrBayes v.3.2.6 (Ronquist *et al*., 2012). We used an AIC (Akaike information criterion) comparison implemented in jmodeltest2 (Darriba *et al*., 2012) to select a GTR + Γ substitution model (approximated using four rate categories). The estimated substitution rates for the nrDNA, cpDNA and *waxy* alignments were then used as priors in the MrBayes analysis. Default settings were used for other priors. Three independent runs of four chains each (one cold, three heated) were sampled every 1000 generations for 2 500 000 generations. The first 20 % of samples were discarded as burn-in. Convergence was assessed in several ways: the average standard deviation of split frequencies was <0·01, the potential scale reduction factor was close to 1·00 for all parameters, and the effective sample sizes (ESS) were >800.

RESULTS

nrDNA

Strongly supported clades in the Bayesian ITS/ETS analysis (Fig. 1) were consistent with those identified by ML (data not shown). *Orobanche* sect. *Gymnocaulis* and sect. *Nothaphyllon* were both resolved as monophyletic [posterior probability (PP) = 1·0, BS = 100] and sister to each other. Within sect. *Gymnocaulis*, seven major clades were resolved (PP = 1·0, BS ≥ 80). Under the current classification, three of these together correspond to a paraphyletic *O. fasciculata*. Plants from each of these clades showed unique host preferences: plants in two of these groups parasitize hosts of single genera, *Artemisia* (Asteraceae) and *Galium* (Rubiaceae). The third group of plants form a clade of generalists that parasitize numerous species within *Eriogonum* (Polygonaceae), *Eriophyllum* (Asteraceae), and *Eriodictyon* and *Phacelia* (Hydrophyllaceae). The remaining four clades constituted a monophyletic *O. uniflora* (PP = 1·0, BS = 100). Three of these clades include parasites specific to hosts in the genera *Antennaria* and *Senecio* (Asteraceae), on members of Saxifragaceae and Crassulaceae (Saxifragales *s.s.*) and on Apioideae (Apiaceae), respectively. These clades together are currently recognized as *O. uniflora* subsp. *occidentalis* and were resolved sister to the fourth clade corresponding to subsp. *uniflora*. Members of this clade parasitize *Rudbeckia* and several genera of Astereae in the Asteroideae.

Populations of the remaining American *Orobanche* species, representing sect. *Nothaphyllon* were generally resolved in one of eight major clades (PP > 0·95, BS > 90): (1) a clade of populations from the western USA parasitic on *Artemisia* previously determined as one of three taxa: *O. parishii* subsp. *parishii*, *O. ludoviciana* or *O. corymbosa*; (2) a taxonomically and ecologically diverse clade, the *O. californica* complex, which included *O. californica* and *O. vallicola*, as well as the remainder of *O. parishii* and *O. corymbosa* populations; (3) *O. pinorum*; (4) *O. tarapacana*; (5) the *O. ludoviciana* complex, including *O. multiflora*, *O riparia*, *O. chilensis*, *O. tacnaensis*, *O arizonica*, the remainder of *O. ludoviciana* and a collection from Hidalgo, Mexico (Yatskievych 85-215) that does not match the morphology of any described species; (6) *O. valida*; (7) *O. cooperi* and *O. dugesii*; and (8) *O. bulbosa*. Clades 6–8, found predominantly in south-western North America, constituted a monophyletic group (PP = 0·95, BS = 77) that was sister to the rest of the section (clades 1–5). Resolution at the subspecific level of the paraphyletic *O. californica* was variable. For example, populations of subsp. *californica* along the central California coast parasitizing *Grindelia stricta* and those in far northern California and Washington parasitizing *Grindelia integrifolia* were resolved in separate strongly supported subclades within the *O. californica* complex (clade 2, above). Other subspecies, such as subsp. *grandis* and subsp. *condensa*, formed a polytomy. The polyploid *O. parishii* subsp. *brachyloba* was nested within one of three separate clades of *O. parishii* subsp. *parishii*.

cpDNA

Strongly supported clades from the Bayesian analysis of three plastid regions (Fig. 2) were consistent with those identified by ML (data not shown). *Orobanche* sect. *Gymnocaulis* was resolved as monophyletic (PP = 1·0, BS = 100). Within sect. *Gymnocaulis*, six host-specific clades were resolved, congruent with the nrDNA results. Three of these were sub-clades of the monophyletic *O. uniflora* (PP = 0·99, BS = 97): a clade of plants parasitizing *Antennaria* and *Senecio* (PP = 1·0, BS = 93) and a less supported clade of plants parasitizing Apioideae (Apiaceae), Saxifragaceae and Crassulaceae (PP = 0·71, BS = 88), together corresponding to subsp. *occidentalis* (PP = 1·0, BS = 100) and sister to a clade of plants that parasitize several genera of Asteroideae corresponding to subsp. *uniflora* (PP = 1, BS = 100). *Orobanche fasciculata* was found to be paraphyletic: a strongly supported clade parasitizing *Artemisia* (PP = 1·0, BS = 100) was resolved sister to *O. uniflora*. The remaining two clades of *O. fasciculata* were resolved as sister groups, one strongly supported and parasitizing *Galium* spp. in California and Oregon (PP = 1·0, BS = 100), and the other weakly supported and parasitizing a variety of distantly related core eudicot genera (PP = 0·65, BS < 50).

Deep relationships within *Orobanche* sect. *Nothaphyllon* were generally well resolved, albeit with variable support at the species and subspecies level. Populations of *O. bulbosa* formed a clade (PP = 1·0, BS = 96) that was sister to the remainder of the section, which in turn comprised two well-

TABLE 1. *Molecular regions used in the phylogenetic analyses of* Orobanche *sections* Gymnocaulis *and* Nothaphyllon*, approximate lengths of complete ingroup sequences, PCR primers (5′–3′) and thermocycling parameters*

Gene region	Approximate amplicon length	Primer sequences	Reference	Thermocycling parameters
ITS	590 bp	AB_101: TGG TCC CGT GAA GTG TTC G	Schneeweiss *et al*. (2004a)	94 °C, 4 min; 35 × (95 °C, 1 min; 48 °C, 1 min; 72 °C, 1 min); 72 °C, 10 min.
		AB_102: CCG GTT CGC TG CCG TAA C	Schneeweiss *et al*. (2004a)	
ETS	430 bp	ETS_B: ATA GAG CGC GTG AGT GGT G	Beardsley and Olmstead, 2002)	96 °C, 2 min; 35 × (94 °C, 30 s; 56 °C, 30 s; 72 °C, 45 s); 72 °C, 3 min.
		ETS_seq: (C) TGG CAG GAT CAA CCA GGT A	This study	
waxy (introns 9–11)	585–630 bp	waxy_9F-ORO: GAT GCT AAG CCW TTG TTG A	This study	92 °C, 5 min; 40 × (94 °C, 45 s; 53·5 °C, 45 s; 72 °C, 1 min); 72 °C, 5 min.
		waxy_11R: CCA TRT GGA ASC CAG TRT A	Tank and Olmstead (2009)	
matK 3′ intron	680–760 bp	matK 8: CTT CGA CTT TCT TGT GCT	Steele and Vilgalys (1994)	94 °C, 5 min; 40 × (92 °C, 1 min; 51 °C, 40 s; 72 °C, 1 min); 72 °C, 10 min.
		matK_psbA5′R: AAC CAT CCA ATG TAA AGA CGG TTT	Shaw *et al*. (2005)	
rps2	675 bp	rps2_2F: AAA TGG AAT CCT AAA ATG GC	This study	94 °C, 2 min 30 s; 35 × (94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min); 72 °C, 7 min.
		rps2_18F: GGR KAR AAA TGA CAA GAA GAT ATT GG	dePamphilis *et al*. (1997)	
		rps2_661R: ACC CTC ACA AAT GCG AAT ACC AA	dePamphilis *et al*. (1997)	
trnL-trnF spacer	710–810 bp	trL ‘c’: CGA AAT CGG TAG ACG CTA CG	Taberlet *et al*. (1991)	94 °C, 5 min; 40 × (92 °C, 1 min; 51·5 °C, 1 min; 72 °C, 1 min); 72 °C, 5 min.
		trnF ‘f’: ATT TGA ACT GGT GAC ACG AG	Taberlet *et al*. (1991)	

Two different forward primers for *rps*2 were used.

supported sub-clades (PP = 1·0, BS > 95). The first included strongly supported clades corresponding to single taxa that diverged from the remainder of the sub-clade in succession: *O. valida* (PP = 1·0, BS = 100), *O. parishii* (PP = 1·0, BS = 100) and finally *O. tarapacana* (PP = 0·94, BS = 72), which was sister to a clade of *O. cooperi*, *O. dugesii* and one accession of *O. corymbosa* (PP = 0·98, BS = 68). The second well-supported sub-clade included the only sampled population of *O. pinorum* sister to the *O. californica* and *O. ludoviciana* complexes. Relationships within this sub-clade were poorly resolved, except for strong support of *O. riparia* + *O. arizonica*, *O. vallicola*, a clade of *O. californica* subsp. *californica* parasitic on *Eriophyllum staechadifolium*, and *O. chilensis* + several populations from central North America (PP = 1·0, BS > 97).

waxy

Orobanche sect. *Gymnocaulis* and sect. *Nothaphyllon* were each resolved as monophyletic (PP = 0·99, BS > 75). Within sect. *Gymnocaulis*, five host-specific clades were resolved with strong support (PP > 0·92, BS > 73), congruent with both nrDNA and cpDNA results. These included a clade of plants parasitizing several genera in the Asteroideae corresponding to *O. uniflora* subsp. *uniflora*, as well as two clades together corresponding to *O. uniflora* subsp. *occidentalis* – the first, which was comprised of plants parasitizing Saxifragaceae and Crassulaceae (Saxifragales *s.s*), and another that included a sub-clade of parasites on *Antennaria* and *Senecio* (Asteraceae) united in a moderately supported polytomy with several populations that parasitize Apioideae (PP = 0·89, BS = 0·74). The remaining two strongly supported clades include plants currently recognized as *O. fasciculata*: one was sister to *O. uniflora* and parasitizes *Artemisia*; the other parasitizes *Galium* and was sister to the remaining populations of *O. fasciculata*, which formed a third, weakly supported clade (PP = 0·74, BS = 67) including parasites on a variety of core eudicot hosts. In contrast to *Orobanche* sect. *Gymnocaulis*, infraspecific sampling density and phylogenetic resolution within *O.* sect. *Nothaphyllon* was limited, although conspecific populations of *O. valida*, *O. californica* subsp. *californica* and *O. cooperi*, as well as *O. chilensis* + *O. multiflora* were each resolved as monophyletic (PP > 0·94, BS > 90). Tree files were uploaded to Open Tree of Life (http://www.opentreeoflife.org), study ID ot_732.

DISCUSSION

Host specificity and speciation

Among extant western hemisphere *Orobanche*, we report many previously unrecognized, host-specific lineages in both sect. *Gymnocaulis* and sect. *Nothaphyllon* that are strongly supported by both plastid and nuclear DNA sequences (Figs 1–3). This cryptic diversity has two complementary implications – one evolutionary, the other ecological. First, biodiversity within

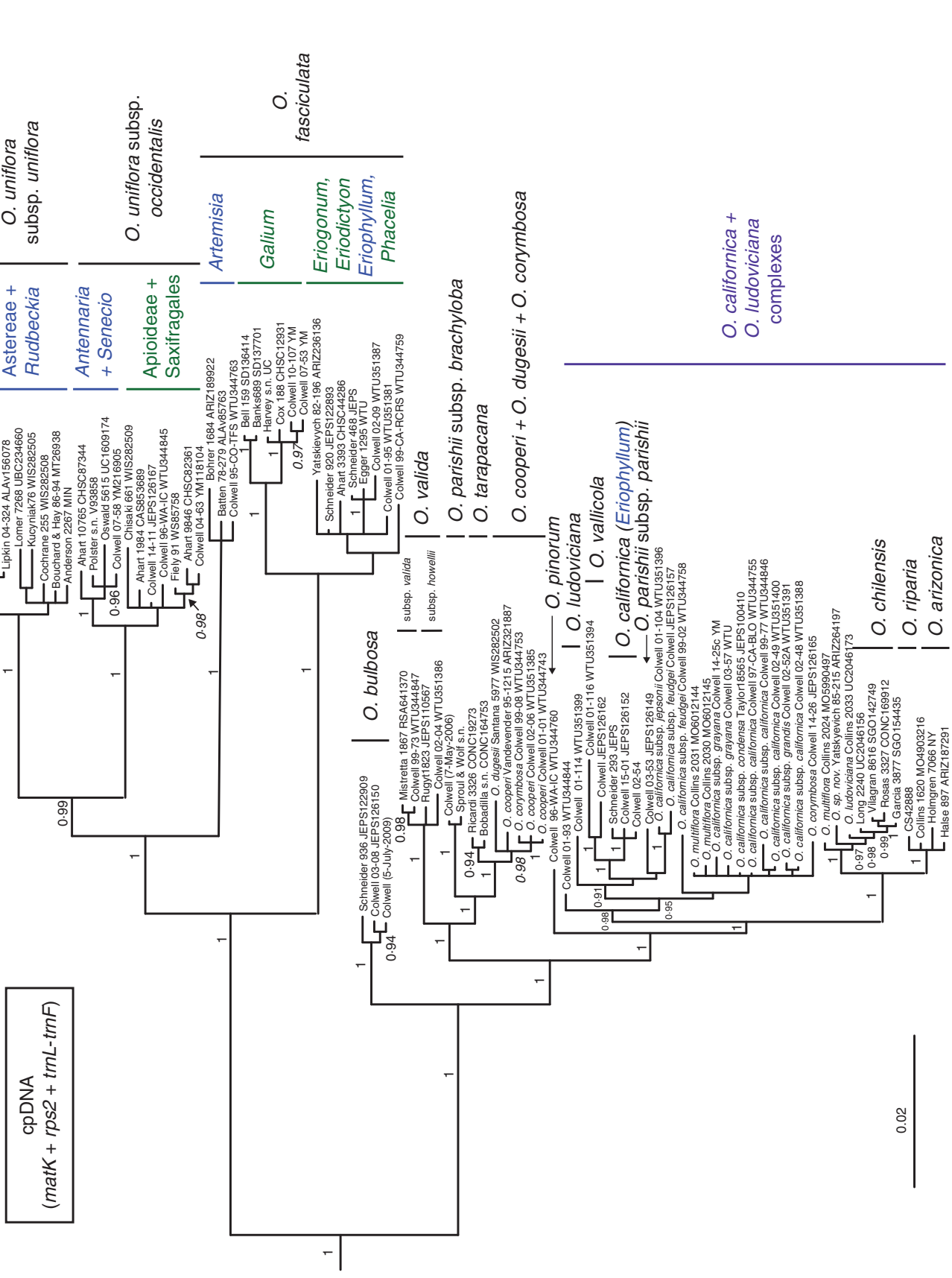

FIG. 2. Bayesian inference majority-rule consensus tree of 86 *Orobanche* populations inferred from three concatenated cpDNA regions (*matK*, *rps2* and trnL-trnF). Tip labels consist of the taxon name (if not included in a sidebar), the collection number and the herbarium accession numbers if available. Posterior probabilities >0·9 are shown in bold for nodes with >70 % maximum likelihood bootstrap (BS) support and in italics if BS support is < 70 %. Some host associations are indicated in blue (Asteraceae) or green (other) to the genus or higher taxonomic level; for others, see Fig. 1. Informally named groups are given in purple. Outgroups are not shown.

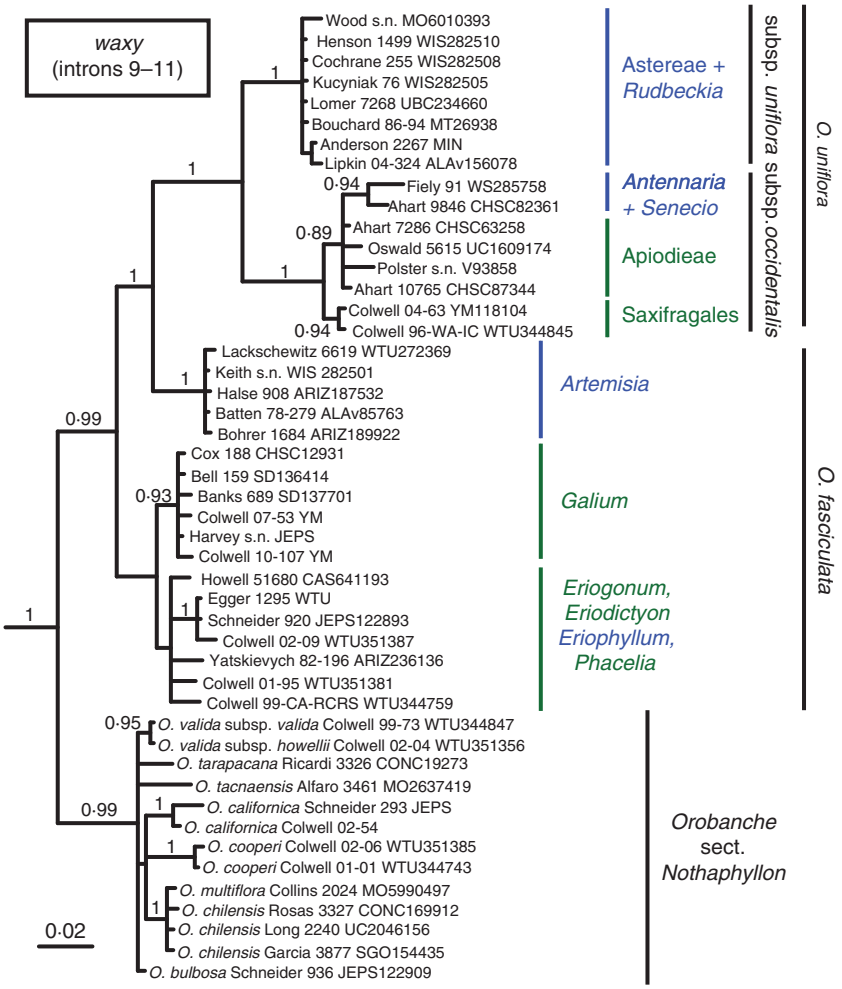

FIG. 3. Bayesian inference majority-rule consensus tree of 47 *Orobanche* populations inferred from the *waxy* locus (introns 9–11). Tip labels consist of the taxon name (if not included in a sidebar), the collection number and the herbarium accession numbers if available. Posterior probabilities >0·8 are shown. All labelled nodes have maximum likelihood bootstrap scores ≥74 %. Host associations for sect. *Gymnocaulis* are indicated in blue (Asteraceae) or green (other) to the genus or higher taxonomic level; for sect. *Nothaphyllon* see Fig. 1 or Table S1. Outgroups are not shown.

western hemisphere *Orobanche* is substantially richer than recognized by current taxonomy, perhaps because extensive reduction of structural characters in these parasites has limited the potential for morphological diagnosis of recently diverged evolutionary lineages. Secondly, the host breadth of each evolutionary lineage is narrower than previously assumed, although some lineages with wide host ranges are still present (e.g. *O. fasciculata* p.p.). Host specificity in plant parasites has been correlated to various life history and other host traits such as weediness or perenniality (Schneweeis, 2007). Host-switching has been cited as a driver of speciation of numerous parasites across the tree of life (Ricklefs *et al*., 2004; de Vienne *et al*., 2013), including other lineages of parasitic plants (Norton and Carpenter, 1998; Norton and Lange, 1999; Bolin *et al*., 2011), as well as within the genus *Orobanche* (Thorogood *et al*., 2009). Our evidence strongly supports this hypothesis. The abundance of host-specific clades found here suggests that host-switching may be an even more important driver of evolutionary divergence in parasitic plants than previously recognized.

Although some *Orobanche* taxa are specific to a single host species, most parasitize several closely related species that are unique and sometimes phylogenetically distant from the hosts of their nearest relatives. In many ways, *Orobanche* spp. occupy an ecological middle-ground between species such as *Epifagus virginiana* (Orobanchaceae), which can only grow on *Fagus grandifolia*, and true generalists such as dodders (*Cuscuta* spp., Convolvulaceae) in which a single individual may parasitize numerous distantly related hosts (Press and Graves, 1995). Therefore, it is unlikely that host–parasite co-speciation plays an appreciable role in driving diversification in western hemisphere *Orobanche* in contrast to some plant–animal, animal–animal or prokaryote–animal host–parasite systems (de Vienne *et al*., 2013). Instead, we argue that the more common mode – host-switching followed by physiological specialization and divergence – is dominant in this system.

Specialization and evolutionary divergence (cladogenesis) following host-switching is an expected outcome given the complex challenges of host detection, host invasion and evasion or

neutralization of host defences, which may occur pre- or post-attachment. Pre-attachment host defences may include reduced germination stimulants (i.e. strigolactones, Cameron *et al*., 2006; Xie *et al*., 2010), increased germination inhibitory compounds (Fernández-Aparicio *et al*., 2011), chemical inhibition of haustorial development (Pérez-de-Luque *et al*., 2005*a*, *b*) or structural fortifications to serve as a mechanical barrier to invasion. Potential hosts can repel parasitic plants following attachment using a variety of mechanisms that disrupt the flow of nutrients or block vessel elements (Goldwasser *et al*., 1999, 2000; Pérez-de-Luque *et al*., 2005*a*), initiate programmed cell death (Gurney *et al*., 2006), increase lignification and suberization of cell walls (Labrousse *et al*., 2001; Pérez-de-Luque *et al*., 2008) or elicit chemical defence through increased peroxidases or the transfer of toxins from the host to the parasite (Gurney *et al*., 2003). These multiple layers of incompatibility must be overcome for a successful invasion of the host, and provide the physiological basis for host specificity in parasitic Orobanchaceae (Yoder, 1997; Yoshida and Shirasu, 2009; Thorogood and Hiscock, 2010). Consequently, distantly related hosts with more divergent physiologies probably require different invasion strategies. Various suites of host-specific traits may therefore represent different adaptive peaks for an *Orobanche* lineage.

Drès and Mallet (2002) cite a number of insect–plant systems to show how the formation of host-specific races may eventually lead to sympatric speciation of parasites through outbreeding depression, even in the presence of gene flow. The generalist clade of *O*. *fasciculata* shows poorly supported phylogenetic substructure and may provide the opportunity to explore this hypothesis in a plant–parasite system. Among the other host-specific clades of *O*. sect. *Gymnocaulis*, sympatric speciation following this model may already have occurred. The strong support for these clades by all three loci (nrDNA, cpDNA and *waxy*) suggest minimal, if any, continued gene flow among these lineages, even between geographically neighbouring populations. Isolation by host may also be reinforced by autogamy or apomixis, which is common in New World *Orobanche* species in contrast to more variable mating systems among species of Eurasian *Orobanche* and predominance of outcrossing among other lineages of parasitic angiosperms (Musselman *et al*., 1982; Jones, 1989; Bellot and Renner, 2013). Autogamy has been identified as the predominant mating system in *O*. *pinorum*, with occasional outcrossing by bees (Ellis *et al*., 1999), is common among *O*. *fasciculata* parasitizing *Artemisia* (Reuter, 1986), and has been anecdotally reported in *O*. *uniflora* subsp. *occidentalis* and *Orobanche bulbosa* (K. L. Chambers 2952, OSC198410; Butterwick 5434 & Parfitt, ASU, JEPS; Schneider 1032, JEPS (Parfitt and Butterwick, 1981)). Some populations of *Orobanche uniflora* subsp. *uniflora* are obligatorily parthenogenic, while other populations show a 'wholly different. . .reproductive process' (Jenson, 1951). As discussed previously, gene flow between different host races is expected to be detrimental if parent taxa are adapted to separate hosts, since a hybrid may be adapted to neither of them.

Geographic differentiation may play a subordinate role in lineage diversification, and may be restricted to cases where sister clades parasitize closely related hosts, such as between the subspecies of *O*. *valida*, which both parasitize *Garrya*. Much more commonly, ranges are at least partially overlapping, and closely related parasite lineages differing in their hosts can co-occur on a regional or even a local scale. This is particularly well pronounced in sect. *Gymnocaulis*, discussed in detail below.

Cryptic diversity in section Gymnocaulis

Cryptic lineages are found in both sections of New World *Orobanche* [e.g. a polyphyletic *O*. *parishii* subsp. *parishii* (Fig. 1)], but most extensively in *O*. sect. *Gymnocaulis*, in which we identified over twice as many host-specific clades as exist commonly recognized taxa. Moreover, these clades are often subtended by long stem branches relative to clades that represent different recognized species in sect. *Nothaphyllon*. This disparity, which is robust to the gene region(s) used (Figs 1–3), may be due to more extensive reduction of morphological and thus diagnostic features in sect. *Gymnocaulis*, as well as more limited systematic and taxonomic study of this section (Achey, 1933; Watson, 1975) relative to sect. *Nothaphyllon* (Munz, 1930; Collins, 1973; Heckard, 1973; Heckard and Chuang, 1975; Collins and Yatskievich, 2015). Similar levels of cryptic diversity may be found in other holoparasitic lineages, particular endoparasites such as *Cytinus* (Cytinaceae) that show even more extensive morphological reduction than *Orobanche* and a more intimate host–parasite relationship (De Vega *et al*., 2008).

Each clade of *Orobanche* sect. *Gymnocaulis* shows at least partial range overlap with its sister group, with generally increasing overlap with decreasing phylogenetic distance (Fig. 4). The clade of *O*. *fasciculata* parasitic on *Galium* is entirely included within the range of its sister group, which is a generalist clade parasitic on various eudicot hosts. The clade of *O*. *fasciculata* parasitic on *Artemisia* grows coarsely sympatrically (i.e. sympatric at regional scales) with both subspecies of its sister group, *O*. *uniflora*. These subspecies, *O*. subsp. *uniflora* and *O*. subsp. *occidentalis*, once thought to be allopatric, are now known to co-occur based on a recent floristic discovery in southern British Columbia and subsequent reinterpretation of historic herbarium records (A. C. Schneider, unpubl. data). Most strikingly, the three closely related clades resolved within *O*. *uniflora* subsp. *occidentalis*, which parasitize species in the Asteraceae, Apiaceae, and Saxifragaceae plus Crassulaceae, respectively, share nearly entirely overlapping ranges at both coarse continental and local scales. For example, populations of all three clades can be found in Yosemite National Park and the adjacent Sierra National Forest.

Relationships in section Nothaphyllon

In sect. *Nothaphyllon*, we also find strong support for host-specific species, including the recently described *O*. *arizonica*, *O*. *riparia* and a clade currently recognized as *O*. *californica* subsp. *californica* that parasitzes *Eriophyllum stachaedifolium* on the central California coast, which is currently being described by the second author and George Yatskievych. Most other clades have distinct host associations, generally with perennial Asteraceae, but usually not specific to the species level (Fig. 1).

Most of the taxonomic diversity in *O*. sect. *Nothaphyllon* is concentrated in a large clade supported by nrDNA and cpDNA, which is comprised of two sub-clades supported by nrDNA (Fig. 1) and morphological analysis (Collins 1973;

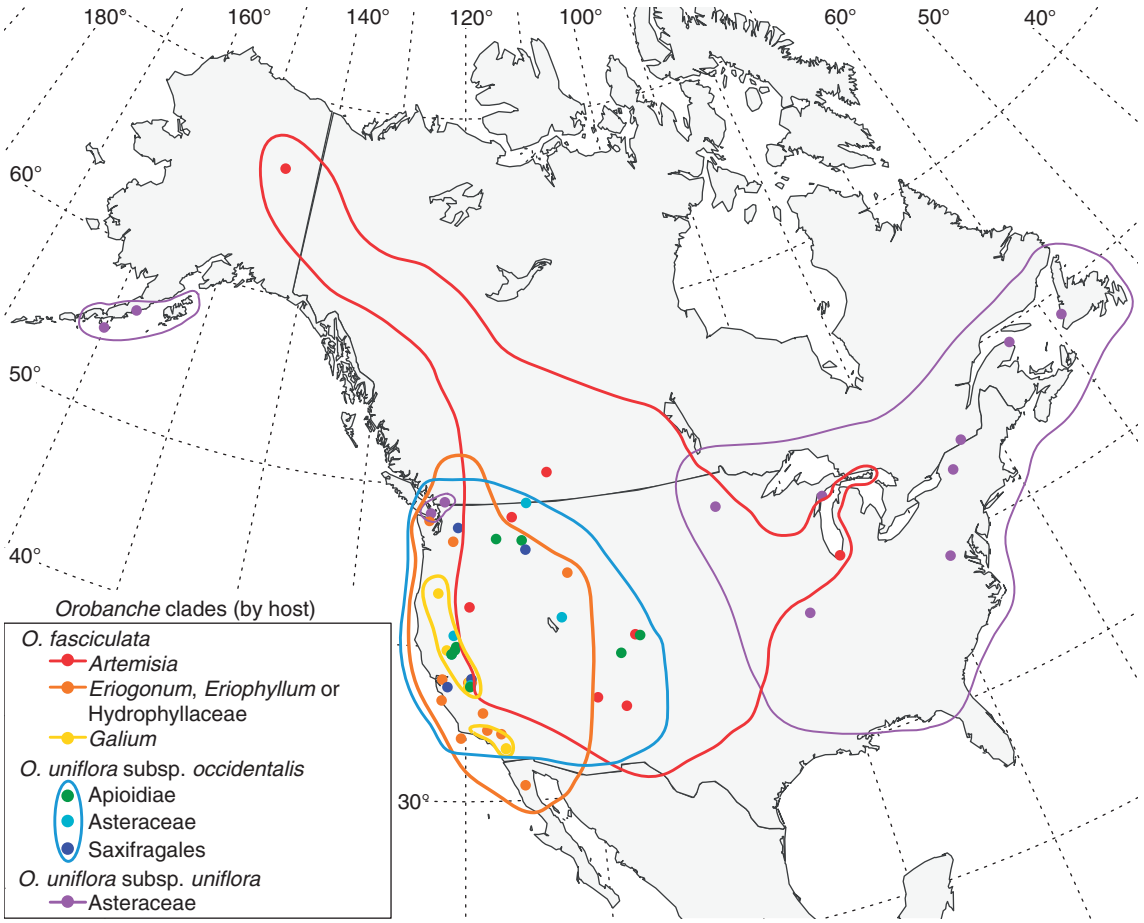

FIG. 4. Range map of host-specific clades of *Orobanche* sect. *Gymnocaulis*. Coloured circles represent individuals sampled in the phylogeny (Figs 1–3). Coloured lines show the approximate range of each clade. Further study is needed to determine the range of each of the three host-specific lineages of *O. uniflora* subsp. *occidentalis*, which in this figure are treated as one unit. Range maps should be considered tentative, particularly in northern Canada and west-central USA, pending a thorough taxonomic and phytogeographical study.

Heckard, 1973). The first sub-clade corresponds to the *O. californica* complex, which includes *O. californica* and its subspecies, *O. parishii*, *O. corymbosa* and *O. vallicola*. The second clade represents the *O. ludoviciana* complex, which includes *O. ludoviciana* (except for populations parasitizing *Artemisia*), *O. multiflora*, the recently described *O. arizonica*, *O. riparia*, the disjunct South American species *O. chilensis* and *O. tacnaensis*, and a collection from Hidalgo, Mexico that does not fit the description of any described taxon (Yatskievych 85-215; ARIZ).

Several earlier diverging lineages native to western North America are also strongly supported as monophyletic by both nrDNA and cpDNA, including *O. valida*, *O. bulbosa* and the recently revised *O. cooperi* + *O. dugesii* complex (Figs 1 and 2; Collins and Yatskievych, 2015). However, relationships among these lineages are unclear: *O. bulbosa* is resolved either as sister to the rest of the section (nrDNA, Fig. 1) or as a grade with *O. bulbosa* diverging earliest (cpDNA, Fig. 2). The conflict among gene partitions is in most cases probably explained by incomplete lineage sorting, but in other cases may be a result of reticulate evolution. For example, based on its phylogenetic placement in two separate clades (Fig. 1), and morphological and host affinities (*Artemisia*, especially *A. tridentata*), *O. corymbosa* may represent a hybrid between *O. californica* and *O. ludoviciana*, both of which in part also parasitize *Artemisia*. In certain other cases, polyploidy may be a driver of speciation. Heckard and Chuang (1975) published detailed chromosome counts for most species of North American *Orobanche*. The octoploid *O. parishii* subsp. *brachyloba* forms a clade nested within *O. parishii* subsp. *parishii* (Fig. 1), its likely tetraploid progenitor (ploidy assignment based on a chromosome base number of $x = 12$; for a more detailed discussion, see Schneeweiss *et al.*, 2004*b*), or, if an allopolyploid, one of two parental lineages. Octoploid lineages have also been reported in *O. cooperi* and *O. corymbosa* subsp. *corymbosa* (but not *O. ludoviciana*). A full discussion of the systematics and taxonomy of these and other individual species is needed, but is beyond the scope of this paper.

Repeated dispersal to South America

Based on the nrDNA phylogeny, we find support for the longstanding hypothesis that *O. chilensis* is closely related to *O.*

ludoviciana and *O. multiflora* (Beck, 1890), thereby contributing to the broadly recognized pattern of amphitropical disjunction between the Great Plains of North America and northern Chile/southern Peru (Wen and Ickert-Bond, 2009). Of the two other sampled *Orobanche* species from South America, *O. tacnaensis*, was resolved with *O. chilensis*, but the two samples of *O. tarapacana* Phil. formed a separate, earlier diverging lineage resulting from north to south dispersal. Phylogenetic placement of *O. tarapacana* is uncertain due to conflict between the nrDNA and cpDNA trees; *O. tarapacana* is sister to either the *O. ludoviciana* complex, the *O. cooperi* complex or perhaps a hybrid between the two (Figs 1 and 2).

Conclusions and future directions

Parasitic Orobanchaceae is becoming a model system for understanding plant parasitism at various levels of biological organization and scale (Joel *et al*., 2013; McNeal *et al*., 2013; Wicke *et al*., 2013; Yang *et al*., 2015). Our results emphasize the importance of host specificity and host-switching as a driver of evolutionary divergence in obligate plant parasites. We find evidence for twice as many host-specific lineages in *O*. sect. *Gymnocaulis* as recognized taxa, and denser sampling in other clades such as *O*. sect. *Nothaphyllon* is likely to uncover more. This robust understanding of fine-scale evolutionary relationships provides the necessary phylogenetic framework to develop a more natural classification for this group, and understand genetic, ecological, functional and life-history consequences of host–parasite associations more broadly.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: vouchers, host associations and GenBank accession numbers for 163 *Orobanche* populations and outgroup taxa.

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