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ARTICLE

How much does the host matter to the parasitoid? Distribution of *Eurytoma* (Hymenoptera, Chalcidoidea) species amongst two locally co-occurring gall-inducing hosts in the genus *Diplolepis* (Hymenoptera, Cynipidae)

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Abstract

Gall wasps in the cynipid genus *Diplolepis* Geoffroy (Hymenoptera: Cynipidae) attack various species of native and introduced roses in Canada. Although gall forms are diverse, gall wasps are parasitised by highly concordant complexes of parasitoids and inquilines. Many species of gall wasps attack the same host plants and develop over the same periods in the season, suggesting that opportunistic parasitoids may be exploiting a range of hosts rather than specialising. We sampled larvae of *Eurytoma* Illiger (Hymenoptera: Cynipidae) from galls of *D. variabilis* (Bassett) and *D. rosaefolii* (Cockerell), gall inducers that develop fairly synchronously late in the growing season on leaves of *Rosa woodsii* Lindl. (Rosaceae) in the Okanagan Valley of central British Columbia, Canada. Galls were sampled at five different sites along a gradient from the north end of the valley to the Canada–United States border, a distance of 100 km. We extracted DNA, then amplified and sequenced the cytochrome b segment for each *Eurytoma* larva. We identified two well-supported clades that were differentiated by neither sampling location nor host. Instead, at least two species of *Eurytoma, E. imminuta* Bugbee and *E. longavena* Bugbee, exist at these localities, and both exploit at least two of the *Diplolepis* hosts found at these sites.

Introduction

One of the biggest challenges to characterising parasitoid communities is the existence of cryptic species. Many congeneric parasitoid species are morphologically similar or identical (Hayward *et al.* 2011; Zhang *et al.* 2014; Hall *et al.* 2017). Consequently, community characterisations are, of necessity, often limited to genus or morphological species-group levels of accuracy. Such lumping can collapse species with different life histories into the same category and confound attempts to elucidate factors that affect community dynamics (Hrček and Godfray 2015). The development of inexpensive and user-friendly techniques for extracting and amplifying DNA and the ready availability of published gene sequences at such sites as the Barcode of Life Data System (Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, Canada) have greatly increased the accuracy and ease of species identification (Hayward *et al.* 2011; Zhang *et al.* 2014; Hrček and Godfray 2015; Hall *et al.* 2017), although care must be taken to ensure the accuracy of the original identification for published barcodes.

Cynipid wasps of the genus *Diplolepis* Geoffroy (Hymenoptera: Cynipidae) induce galls on various tissues of host plants in the genus *Rosa* Linnaeus (Rosaceae) (Shorthouse 2010). In the

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Fig. 1. Galls induced by **A**, *Diplolepis variabilis* and **B**, *D. rosaefolii* on the same leaf of a *Rosa woodsia* ramet.

Okanagan Valley of south–central British Columbia, Canada, *Rosa woodsii* is attacked by seven relatively common *Diplolepis* species (Lalonde and Shorthouse 2000; Shorthouse 2010). Two such species, *D. rosaefolii* Cockerell and *D. variabilis* Bassett, are among the most common and develop at more or less the same time during the late summer and early fall in the area. Both induce galls in leaf tissue and often co-occur on the same plant (Fig. 1). Because of this, both species are available to the same guild of parasitoids at more or less the same time. Although these galls are induced by congenerically close relatives, their shapes are markedly different. *Diplolepis variabilis* induces a highly polymorphic gall composed of soft corky material and ranges from single-inhabitant forms that are the size of a pea to large, amorphous structures formed from the coalesced galls of many individuals (Fig. 1). In contrast, *D. rosaefolii* induces much smaller, single-inhabitant, lenticular galls composed of highly sclerotised walls that have a uniform shape and rarely coalesce (Fig. 1).

Cynipid-induced galls, in general, are host to a large diversity of hymenopterous inquilines – strategists that exploit the gall but not the gall inducer as a food source – and parasitoids (Shorthouse 1973; Stone and Cook 1998). Galls of *Diplolepis* are no exception (Brooks and Shorthouse 1997; Shorthouse 2010; Bannerman *et al.* 2012). All *Diplolepis* species in the region induce galls that support at least one species of parasitoid wasp in the genus *Eurytoma* Illiger (Hymenoptera, Chalcidoidea) (Shorthouse 2010). Parasitoids in the genus *Eurytoma* are fairly flexible in their feeding habits and will feed on larval *Diplolepis*, gall nutritive tissue, and also on other gall inhabitants such as larvae of the inquiline species in the genus *Periclistus* Förster (Hymenoptera: Cynipidae) (Brooks and Shorthouse 1997; Leggo and Shorthouse 2006). Accurate identification of *Eurytoma* species using classical morphological characters is challenging, making it historically difficult to determine the degree to which these wasps specialise on particular hosts (Zhang *et al.* 2014). However, a recent study that combines classical morphology and molecular characterisation has demonstrated that at least four closely related species, *E. calcarea* Bugbee, *E. iniquus* Bugbee, *E. longavena* Bugbee, and *E. imminuta* Bugbee (= *E. spongiosa* 1; Zhang *et al.* 2017), inhabit *D. variabilis* galls in the Okanagan Valley. Two of these, *E. iniquus* and *E. longavena*, also are known to inhabit *D. rosaefolii* galls in Ontario, but the species of *Eurytoma* attacking galls of this species in British Columbia have not been investigated (Zhang *et al.* 2014).

Given the high degree of lifecycle synchrony and spatial concordance between *Diplolepis variabilis* and *D. rosaefolii* in the Okanagan, we hypothesised that these two species will be attacked by the same species of *Eurytoma* at a given locality and that locality will be a better predictor of *Eurytoma* species presence than the species of host. In this study, we therefore sampled

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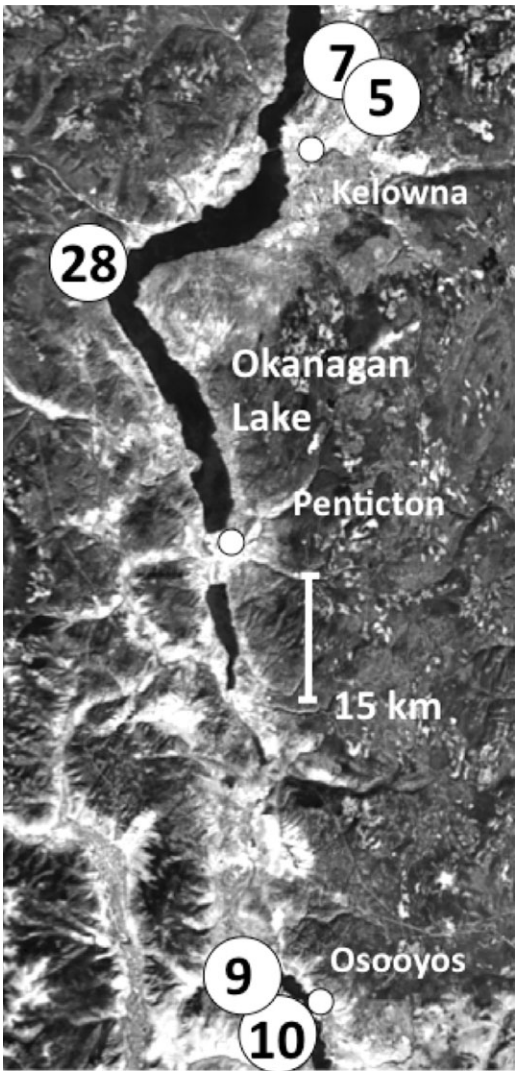

Fig. 2. Map of sampling locations for material used in this study. Galls of both *Diplolepis variabilis* and *D. rosaefolii* were collected at sites near Kelowna (sites 5 and 7), Peachland (site 28), and Osoyoos (sites 9 and 10), British Columbia, Canada.

co-occurring galls of *D. variabilis* and *D. rosaefolii* from a number of sites in the Okanagan Valley, British Columbia and, using molecular tools to identify individuals to species, asked whether or not the assemblage of *Eurytoma* species present in the samples was affected by host species or by geographic location.

Methods

Collections were made during autumn 2009. Each rose bush located within a radius of 10 m represented one sampling site, and the site's position was recorded using a geographic positioning system. Thirty-two sites were sampled for galls of both *D. variabilis* and *D. rosaefolii*. A sample of 15–30 galls of each species was collected at each site and taken back to the lab. Galls were refrigerated and removed only just before dissection. Material from 5 of the 32 sites (sites 5, 7, 9, 10, and 28), spanning a distance of 100 km from near Osoyoos to the Kelowna airport (Fig. 2), was used in

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this study. The sites used were those with recognisable *Eurytoma* larvae in both *D. variabilis* and *D. rosaefolii* galls. *Eurytoma* larvae dissected from galls of *D. rosaefoliii* and *D. variabilis* were first identified using published illustrations (Shorthouse 1973; Leggo and Shorthouse 2006), then reserved for DNA extraction. In total, DNA was extracted from each of 56 samples using a DNeasy® Blood and Tissue kit (Qiagen, Inc., Valencia, California, United States of America).

The extracted DNA was used to amplify a 433-bp segment of the cytochrome b mitochondrial gene, and each clean amplified sample was sequenced. The primers used were *CB1* (5′–3′TATGTACTACCATGAGGACAAATATC) and *CB2* (5′–3′ATTACACCTCCTAATTTATTA GGAAT) (Jermiin and Crozier 1994; Stone and Cook 1998; Hall *et al.* 2017). The Go Taq, Green Master Mix (Promega Corporation, Madison, Wisconson, United States of America) procedure was used for polymerase chain reaction (PCR). The PCR cycle consisted of 3 minutes at 94 °C, followed by 35 cycles of denaturation for 1 minute at 94 °C, annealing for 0.5 minute at 45 °C, and extension for 1 minute at 72 °C. At the end of the 35 cycles, a final 10 minutes at 72 °C occurred, and samples were then held at 4 °C until recovery. Gel electrophoresis was used to confirm amplification. Samples showing clear bands at 433 bp were then sequenced in both forwards and reverse directions. Sanger sequencing was performed by the University of British Columbia's Fragment Analysis and DNA Sequence Service (FADSS; Kelowna, British Columbia, Canada). We then assembled forwards and reverse sequences using Codon Code Aligner (Version 7.0.1; Codon Code Corporation, Centerville, Massachusetts, United States of America) and saved the resulting contigs. Contig sequences that had quality values below 50% were removed from the dataset, and the remaining contigs were aligned using the Muscle alignment option in Seaview 4.7 (Gouy *et al.* 2010). Before tree construction, the Blast function on GenBank was used, and any sequences that did not cluster within the genus *Eurytoma* were discarded. We then downloaded cytochrome b sequences of *E. adleriae* Zerova – a parasitoid of various gall-inducing hosts in Europe and Asia – and *Bruchophagus caucasicus* Zerova – a eurytomid in the same subfamily as the genus *Eurytoma* – from GenBank to use as outgroups. This entire process resulted in 21 high-quality sequences. These and the outgroup sequences were used to construct a maximum likelihood tree using Seaview 4.7 (Gouy *et al.* 2010), per the steps described here.

The cytochrome b sequences were assigned to described species using published cytochrome oxidase I (*COI*) barcodes from Zhang *et al.* (2014). To do this, material sequenced by Earley *et al.* (unpublished) was referenced. Earley *et al.* had extracted DNA from a large collection of Okanagan *Eurytoma* reared from galls of *D. variabilis* and amplified both the cytochrome b and *COI* mitochondrial gene regions for each extracted specimen. Earley *et al.* were thus able to assign their cytochrome b sequences to barcoded species using the associated *COI* sequences. In the current study, a maximum likelihood tree was constructed using Earley *et al.*'s (unpublished) cytochrome b-sequenced material, together with the sequences from the current study, using Seaview 4.7 (Gouy *et al.* 2010). With the exception of one sequence, the current study's material could be assigned to a described and barcoded species with 100% bootstrap confidence.

Results and discussion

Our calibrated cytochrome b tree shows that the valley supports two well-supported clades of *Eurytoma* that parasitise the inhabitants of galls of both *Diplolepis variabilis* and *D. rosaefolii* (Fig. 3), with no evident preference by either parasitoid for a particular host ($X^2 = 1.65$, 1 *df*, $P > 0.05$) or for a particular location (see the second point in the paragraph below).

A number of interesting points emerged from the analysis. Firstly, the *Eurytoma* species that attack *D. rosaefolii* in the Okanagan Valley differ from those that attack this host in Ontario (Zhang *et al.* 2014, 2017). In particular, *E. imminuta* (Zhang *et al.*'s (2014) *E. spongiosa* 1), the most ubiquitous species in the samples collected, was not recorded by Zhang *et al.*

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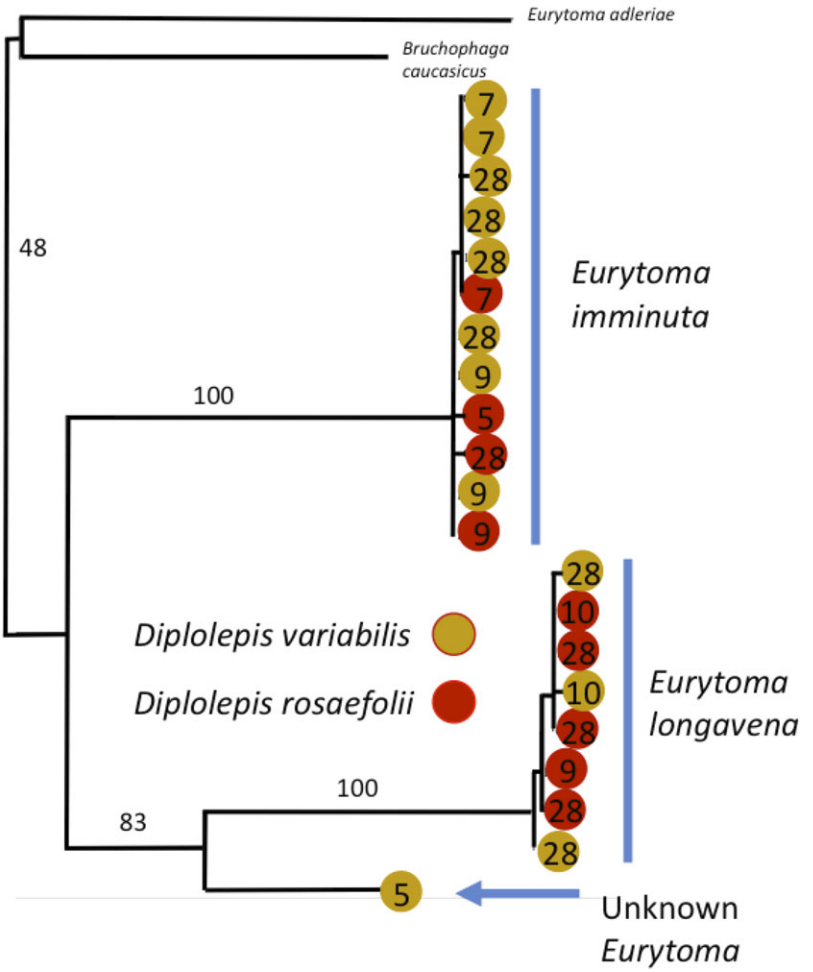

Fig. 3. Maximum likelihood tree of *Eurytoma* samples dissected from galls of *D. variabilis* (light brown circles) and *D. rosaefolii* (red circles). Numbers in circles indicate the collection site, as mapped in Fig. 2. The tree was constructed with a bootstrapped (1000 iterations) maximum likelihood routine on Seaview (Gouy *et al.* 2010) using the cytochrome b sequences obtained from this study's samples and outgroups (*E. adleriae, Bruchophaga caucasicus*) downloaded from GenBank (https://www.ncbi.nlm.nih.gov/nuccore?db=Nucleotide). Values at major nodes indicate percent bootstrap branch support. Species assignments were made by using the published cytochrome oxidase I (*CO1*) barcodes associated with our cytochrome b sequences per Earley *et al.*'s unpublished data.

(2014, 2017) as attacking the inhabitants of galls of *D. rosaefolii*. Secondly, although one of the species, *Eurytoma longavena,* was present only in samples taken from southern and central sites (sites 9, 10, and 28), while the other identified *Eurytoma* species, *E. imminuta,* is distributed amongst the galls of both host species across the entire sampled area in the valley, this apparent local restriction of *E. longavena* does not significantly differ from a random expectation even when we group sites 9 and 10 (southern) and sites 5 and 7 (northern) to reduce the number of categories ($X^2 = 3.333$, 2 *df*, $P > 0.05$). Thirdly, one of our sequences could not be assigned to a species (Fig. 3), suggesting that there is at least one nonbarcoded and possibly undescribed *Eurytoma* species in the valley. Finally, Zhang *et al.* (2014, 2017) report a number of other *Eurytoma* species present within galls of both *D. variabilis* and *D. rosaefolii* that were not found in our samples.

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It should be noted that Zhang *et al.*'s (2014) sample locations for both *D. variabilis* (Kelowna Airport, Kelowna, British Columbia) and *D. rosaefolii* (Ontario) differ from the sample locations used in this study, although their sample of galls of *D. variabilis* did come from a location that was within 500 m of our sites 5 and 7. The above shows that the composition of the *Eurytoma* portion of the parasitoid communities associated with *Diplolepis* gall-formers is diverse and possibly affected by the local pool of available species. No invariant host species-specific assemblage of parasitoids occurs, at least when it comes to *Eurytoma*. This was a result hinted at by Bannerman *et al.* (2012) for the parasitoid assemblage attacking inhabitants of *D. variabilis* galls in the Okanagan and demonstrated by Aebi *et al.* (2006) for invasive populations of the chestnut gall wasp at different global locations. The finding is also consistent with a study in Idaho, United States of America, where the character of the vegetation surrounding a local *Diplolepis* community affected the diversity of the parasitoid community (Looney and Eigenbrode 2011).

We found no apparent subdivision of the *Eurytoma* parasitoid community on the basis of host species in our samples. However, this is only at the level of discrimination afforded by sequencing the cytochrome b region and only across two of the approximately half-dozen galls of species of *Diplolepis* that can be readily found in the Okanagan Valley (Lalonde and Shorthouse 2000). One next step will be to sample *Eurytoma* from galls of other *Diplolepis* species to determine whether subdivision of the parasitoid community occurs across other hosts. In addition, the use of more variable genetic materials, such as single-nucleotide polymorphisms, would help to determine whether subdivision occurs over a shorter timescale than can be demonstrated by the variation present in cytochrome b (Hopper *et al.* 2019).

If further investigation demonstrates that at least some of the species in the *Eurytoma* complex do not discriminate amongst galls induced by *Diplolepis*, the *Diplolepis–Rosa* system could be a useful model for experimentally examining the effects of multiple host–parasitoid dynamics (Holt 1977; Morris *et al.* 2004). In the Okanagan Valley, galls induced by *D. variabilis*, *D. rosaefolii*, and some of the other *Diplolepis* species present in the valley, are convenient subjects for such a study: they are abundant and show high site constancy. Such systems lend themselves to experimentation because of the ease of manipulating and re-visiting individual galls (Fernandes and Price 1992; Price *et al.* 2004). The marked persistence of populations of some *Diplolepis* species, such as *D. variabilis*, and the transience shown by other *Diplolepis* species (Lalonde and Shorthouse 2000) suggest that *Diplolepis–Rosa* systems may be useful models for investigating factors that affect the stability of host–parasitoid systems in general (Holt 1977; Morris *et al.* 2004; Van Veen *et al.* 2006).

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