Testing species limits of Eurytomidae (Hymenoptera) associated with galls induced by *Diplolepis* (Hymenoptera: Cynipidae) in Canada using an integrative approach

Y. Miles Zhang,[1] Michael W. Gates, Joseph D. Shorthouse

Abstract—Studies of insect host-parasitoid relationships are often confounded by the difficulties associated with species delimitation in taxonomically challenging groups. Eurytomidae (Hymenoptera) are common parasitoids associated with galls induced by Cynipidae (Hymenoptera) and are difficult to identify due to their small size, morphological conservatism, and unreliable published host records. This study tests the species limits of eurytomids associated with galls induced by *Diplolepis* Geoffroy (Hymenoptera: Cynipidae) in Canada using an integrative taxonomy approach including adult morphology, the mitochondrial gene *cytochrome c oxidase I*, host records, and geographical range. Incongruences between morphological and molecular data were found within the *Eurytoma discordans* Bugbee complex, as *Eurytoma discordans*, *Eurytoma acuta* Bugbee, and *Eurytoma calcarea* Bugbee were shown to be **new synonyms**. The results also revealed the presence of cryptic species within *Eurytoma spongiosa* Bugbee. Furthermore, issues that have impeded ecological and biological studies of eurytomids associated with rose galls such as host specificity and sex association were resolved using DNA barcodes, providing new insights into the evolutionary history of this difficult group.

Résumé—Les études sur les interactions hôtes-parasitoïdes chez les insectes sont souvent limitées par les difficultés associées à l'identification des espèces, surtout lorsqu'elles proviennent de groupes taxonomiques complexes. Les Eurytomidae (Hymenoptera) sont des parasitoïdes communs associés aux galles formées par les Cynipidae (Hymenoptera), et leur identification peut s'avérer laborieuse en raison de leur taille minuscule, leur similitude morphologique et un recueil peu fiable des espèces d'hôtes connues. Le but de cette étude est de redéfinir les limites taxonomiques des eurytomides associés aux galles formées par *Diplolepis* Geoffroy (Hymenoptera: Cynipidae) au Canada en utilisant une méthodologie taxonomique intégrée basée sur la morphologie des adultes, le gène mitochondrial *cytochrome c oxidase I*, les hôtes exploités et l'étendue géographique des espèces. Nous avons noté certaines incompatibilités entre les données morphologiques et moléculaires dans le complexe de *Eurytoma discordans* Bugbee, trois espèces s'avérant analogues. Nos résultats révèlent également la présence d'espèces cryptiques au sein d'*Eurytoma spongiosa* Bugbee. Cette étude démontre donc que les difficultés rencontrées lors de recherches écologiques et biologiques sur les eurytomides associés aux galles des rosiers, comme par exemple leur spécifité d'hôtes et l'identification des sexes, peuvent être contournées par l'utilisation de marqueurs génétiques. Cette méthodologie permettra ainsi d'approfondir nos connaissances de l'histoire évolutive de ce groupe taxonomique complexe.

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Y.M. Zhang,[1] J.D. Shorthouse, Department of Biology, Laurentian University, Sudbury, Ontario, Canada P3E 2C6

M.W. Gates, United States Department of Agriculture, Systematic Entomology Laboratory, Agriculture Research Service, c/o National Museum of Natural History, Washington, DC 20013-7012, United States of America

[1]Present address: Department of Entomology, University of Manitoba, Winnipeg, Manitoba, Canada R3 T 2N2

[1]Corresponding author (e-mail: yuanmeng.zhang@gmail.com).

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Introduction

Communities of insects associated with plant galls are useful model systems for studying trophic interactions among insects (Csóka *et al.* 2005), and species level identification of the inhabitants is necessary to analyse food webs (Gómez *et al.* 2011). Besides the inducers, gall communities are commonly comprised of parasitoids that feed on inducers or other inhabitants, and inquilines that feed on galls tissues (Shorthouse 2010). One of the driving forces behind the speciation of gall parasitoids is the phenological patterns of their hosts, as parasitoids can only reach their hosts by oviposition through gall tissues, which proliferate and enlarge as the galls develop (reviewed in Csóka *et al.* 2005). Several studies have also shown that the host plant, host organ, and the time required for gall development all affect richness of parasitoid species (*e.g.*, Schönrogge *et al.* 1995, 1996; Plantard *et al.* 1996; Plantard *et al.* 1999); however, the reason why many parasitoids are highly host specific, despite the presence of other potential hosts on the same host plant, are largely unknown (Csóka *et al.* 2005). All known parasitoids that attack cynipid hosts are wasps within the superfamilies Chalcidoidea and Ichneumonoidea (Shorthouse 2010).

The superfamily Chalcidoidea contains an estimated 500 000 species, making it one of the most biologically and morphologically diverse groups of parasitic wasps (Gibson *et al.* 1997, 1999; Munro *et al.* 2011). While some chalcidoids are phytophagous, the majority are entomophagous and their hosts include all life-history stages of 13 orders of insects, two orders of arachnids, and one family of nematodes (Gibson *et al.* 1997, 1999). Chalcids within the family Eurytomidae have over 1400 nominal species in 84 genera and are found in most zoogeographical regions (Lotfalizadeh *et al.* 2007b; Gates 2008; Noyes 2012). Eurytomids are largely endophytic as seed feeders, gall inducers or parasitoids of phytophagous insects (Lotfalizadeh *et al.* 2007b). The accurate identification of eurytomids, in particular the genus *Eurytoma* Illiger, which includes more than 700 described species, has proven difficult using existing morphological keys due to overlapping diagnostic characters and lack of illustrations (*e.g.*, Bugbee 1967). As a result, phylogenetic, ecological, and evolutionary studies of eurytomids have been impeded. The degree of morphological conservatism is particularly prominent in members of the *Eurytoma rosae* Nees species group, which parasitises various gall-inducing cynipids, Tephritidae (Diptera), and Curculionidae (Coleoptera) (Lotfalizadeh *et al.* 2007b). Members of the *E. rosae* group are often morphologically similar and impossible to segregate into morphospecies, despite being ecologically and genetically distinct (Ács *et al.* 2002; Lotfalizadeh *et al.* 2007a; Gómez *et al.* 2011).

A total of 14 native species of cynipid wasps of the genus *Diplolepis* Geoffroy (Hymenoptera: Cynipidae) have been recorded in Canada, all of which induce structurally distinct galls on *Rosa* Linnaeus (Rosaceae) (Shorthouse 2010). These rose galls are heavily attacked by chalcid parasitoids, of which eurytomids are the most abundant (Shorthouse *et al.* 2005; Shorthouse 2010). Ten species of eurytomids are known to be associated with galls of *Diplolepis* in Canada feeding as koinobiont ectoparasitoids of either the inducers or cynipid inquilines of the genus *Periclistus* Förster (Noyes 2012). While most eurytomids are univoltine and overwinter within galls before exiting the following spring, cases of fall emergence have been recorded where mature eurytomids pupate and exit the gall in the fall of the year of gall initiation (Shorthouse 1973, 2010; Brooks and Shorthouse 1997). Several studies have been conducted on both adult (Lotfalizadeh *et al.* 2007a, 2007b) and larval (Gómez *et al.* 2011) eurytomids associated with rose gall communities in the Palearctic region, whereas the Nearctic species have received little taxonomic attention. Bugbee (1951a, 1951b, 1973) described the majority of the Nearctic species; however, many of the original species descriptions are brief, and were often based on a limited number of specimens collected from a single locality. Further, morphological variations from the type specimens were assigned as subspecies, resulting in even more indistinguishable taxa. While identification keys were provided for *Eurytoma* by Bugbee (1951b, 1967), they were based only on females and distinguishing characters used were often ambiguous and generally lacking illustrations. These impediments confound studies

Fig. 1. Map of Canada indicating the sampling locations of Eurytomidae used in this study.

on host-parasitoid relationships within galls induced by *Diplolepis* (*e.g.*, Shorthouse *et al.* 2005; Leggo and Shorthouse 2006; Shorthouse 2010), and thus a novel approach is needed to delimit these morphologically similar species.

With advances in molecular biology, the use of molecular markers has proven essential for delimiting closely related species among Hymenoptera parasitoids (Heraty 2009; Santos *et al.* 2011). The mitochondrial genome in particular serves as a good model for the study of molecular evolution and population genetics, with high rates of evolution and genome reorganisation observed in known chalcid wasp genomes (Dowton and Austin 1995; Oliveira *et al.* 2008). A short fragment of the mitochondrial cytochrome *c* oxidase I (COI) gene is the core DNA barcoding animal gene and has proven successful in species identification, and distinct molecular clades or haplogroups have been used in the identification of morphologically cryptic taxa (*e.g.*, Hebert *et al.* 2004; Smith *et al.* 2006, 2007). However, controversy exists on the exclusive reliance of mitochondrial DNA in species delimitation without the inclusion of morphological or ecological datasets (*e.g.*, Cognato 2006; Meier *et al.* 2006). Thus, an integrative taxonomy approach is preferred using multiple independent character data sources and avoiding reliance on key characters alone in testing species hypotheses for problematic groups (Dayrat 2005; Will *et al.* 2005). Cytochrome *c* oxidase I has been shown to be a valuable tool in identifying cryptic taxa, in combination with morphological and ecological data, for testing host-specificity and geographical variability for Hymenoptera (*e.g.*, Smith *et al.* 2008; Sheffield *et al.* 2009; Ács *et al.* 2010; Kaartinen *et al.* 2010; Sun *et al.* 2011; Gebiola *et al.* 2012) including members of Eurytomidae (Lotfalizadeh *et al.* 2007a; Li *et al.* 2010).

The purpose this study was to use an integrative taxonomy approach to delimit eurytomids associated with galls of 14 species of *Diplolepis* from Canada by testing congruency of genetic variation, morphological differences, host specificity, and geographical distribution between different populations. Additionally the validity of species limits proposed by Bugbee (1967) was tested using COI sequences.

Materials and methods

Sample collection

Maturing or mature galls were collected from various sites in Canada from 1998 to 2011 (Fig. 1), either in the spring after snow melt for

galls induced the previous year, or in the fall after galls had matured. Galls from the previous year were stored in jars at room temperature allowing the inhabitants to exit the galls. Galls collected in the fall were subjected to −5 °C for three to four months to break diapause. All inhabitants were either aspirated or removed with a paint brush, and then stored in 100% ethanol. The specimens used for this study were limited to those with sufficient ecological and geographical data to unambiguously identify host galls ($n = 423$). Eurytomids were selected from pinned specimens and bulk samples stored in 100% ethanol and identified to the species level based on dichotomous morphological keys by Bugbee (1951a, 1967) in combination with host records whenever possible. Specimens that could not be confidently identified were separated into morphospecies. Localities of the eurytomids used in the study are shown in Figure 1. This map was generated using Simplemappr (Shorthouse 2012).

DNA extraction and PCR amplification of COI barcoding region

DNA extractions were performed at the Canadian Centre for DNA Barcoding (CCDB) in Guelph, Ontario, Canada using a silica-based 96-well automated extraction according to the protocol described by Ivanova *et al.* (2006, 2007) in combination with the nondestructive voucher retrieval method described in Porco *et al.* (2010). A series of primers were used listed in Table 1. PCR amplification and sequencing were performed according to the standard protocol used by CCDB (Ivanova and Grainger 2007a, 2007b).

Phylogenetic inference

Contigs were assembled using Sequencher version 4.5 and aligned by CLUSTALX in MEGA version 5.05 (Tamura *et al.* 2011) then manually checked by eye. Genetic distances were calculated in the Barcode of Life Data System (BOLD) using Kimura-2-Parameter (K2P) (Ratnasingham and Hebert 2007). Sequences of eurytomids with >350 base pairs were used in all analyses, with the sequences of *Orthopelma mediator* Thunberg (Hymenoptera: Ichneumonidae), *Ormyrus rosae* Ashmead (Hymenoptera: Ormyridae), and *Torymus bedeguaris* (Linnaeus) (Hymenoptera: Torymidae) as outgroups.

Maximum likelihood analyses were performed using the K2P distance model (Kimura 1980) in MEGA 5.05 and visualised as a phylogenetic tree. Branch support was assessed with 1000 bootstrap pseudoreplicates and was considered as supported when bootstrap value was >70%. Similarly, Bayesian inference using gamma-distributed rate variation across sites and a proportion of invariable sites with HKY + I + G model, as selected by JModeltest version 0.1.1 (Posada 2008) was performed using MrBayes 3.2 (Ronquist *et al.* 2012). Two parallel runs of four simultaneous Monte Carlo Markov chains (three heated and one cold) were run for four million generations, and trees sampled every 1000 generations. The burn-in value was set at 25% of the total sampled topologies, with the phylogeny estimated from a majority-rule consensus of the remaining trees at the threshold for clade acceptance set at 0.95. The trace files sequences and specimen

Table 1. Primers used for PCR and sequencing.

Primer name	Direction	Primer sequence (5′–3′)	Primer source
LepF1	Forward	ATTCAACCAATCATAAAGATATTGG	Hebert *et al.* (2004)
FWPTF1	Forward	CCTGGTTCTTTRATTGGTAATGATC	Li *et al.* (2010)
RonMWASPdeg_t1	Forward	TGTAAAACGACGGCCAGTGGWTCW CCWGATATAKCWTTTCC	Smith *et al.* (2008)
UEA3	Forward	TATAGCATTCCCACGAATAAATAA	Lunt *et al.* (1996)
TL2-N-3014	Reverse	TCCATTGCACTAATCTGCCATATTA	Simon *et al.* (1994)
C_ANTMR1D-RonIIdeg_R	Reverse	GGRGGRTARAYAGTTCATCCWGTWCC	Modified from Simon *et al.* (1994)
C_ANTMR1D-AMR1deg_R	Reverse	CAWCCWGTWCCKRMNCCWKCAT	Smith *et al.* (2005)
LepR1	Reverse	TAAACTTCTGGATGTCCAAAAATCA	Hebert *et al.* (2004)

information are deposited in the project Eurytomidae associated with galls of *Diplolepis* in Canada (project code MZEDO) on BOLD (www.boldsystems.org), and all sequences have been deposited in GenBank under accession numbers KC685087–KC685296.

Morphological study

Morphospecies were compared with the voucher specimens used for molecular study *a posteriori*, and sorted according to haplogroups. These specimens were chemically dried using Hexamethyldisilazane (Heraty and Hawks 1998) before being point or card mounted. Scanning electronic microscopy or stereomicroscope photographs were taken using methods described by Gates and Pérez-Lachaud (2012). The vouchers were also compared with type specimens located in the National Museum of Natural History (USNM) in Washington, DC, United States of America or the Canadian National Collection of Insects (CNCI) in Ottawa, Ontario, Canada. The DNA extracts are stored at the Biodiversity Institute of Ontario (Guelph, Ontario, Canada), while the specimens are deposited at USNM and CNCI.

Results

Cytochrome *c* oxidase I species delimitation

A variety of primers were used due to the difficulty in amplifying the COI region of chalcids as a result of the poly-T runs in priming region. This is also the likely cause of the low success rate, as COI sequences were obtained only from 220 of 423 specimens. Sequence lengths ranged from 223 base pairs to 632 base pairs, and show a strong A + T nucleotide bias (mean = 0.752) in comparison to C + G (mean = 0.248). Phylogenetic analyses identified eight haplogroups of eurytomids, seven of which have successfully matched identified females with male conspecifics that were morphologically unidentifiable. All haplogroups were well supported by maximum likelihood bootstrap and Bayesian posterior probabilities (Fig. 2). Both the subfamily Eurytominae (*Tenuipetiolus* + *Eurytoma*) and the genus *Eurytoma* were recovered as monophyletic, and *Eurytoma iniquus* Bugbee, *Eurytoma longavena* Bugbee, and *Tenuipetiolus ruber* Bugbee were recovered as distinct clades (Table 2). Deeply divergent lineages were revealed in *Eurytoma spongiosa* Bugbee, including an additional clade "*E. spongiosa* 2". *Eurytoma discordans* Bugbee, *Eurytoma acuta* Bugbee, and *Eurytoma calcarea* Bugbee were grouped together into one genetically variable clade (Fig. 3). In addition, two rare haplogroups were found among unidentified species, *Eurytoma* species 1 with four specimens, and a single male specimen as *Eurytoma* species 2 (Fig. 2; Table 2). The intra-specific variation ranged from 0.2% to 3.8%, whereas the inter-specific divergence was 5.7–20.2% (Table 3).

Morphological study

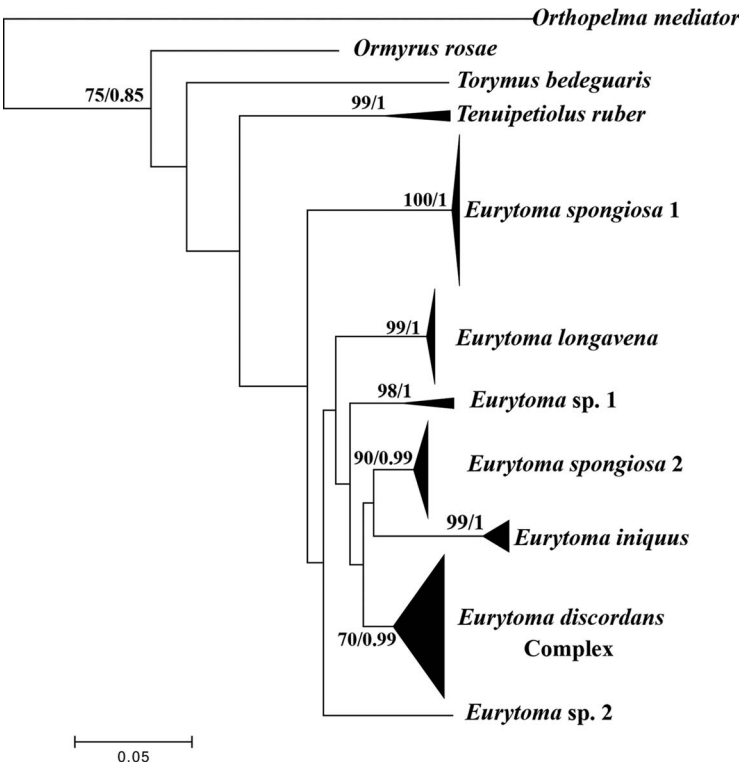
In total, eight morphospecies of eurytomids were found associated with galls induced by *Diplolepis* from the collection sites, including five of the 10 species previously known from Canada (Noyes 2012). *Eurytoma obtusilobae* Ashmead was only observed in a single collection of galls of *Diplolepis radicum* (Osten Sacken) found near Kelowna, British Columbia, Canada in 1999. These specimens failed to generate sequences and were not used for this study. With the exception of *T. ruber* Bugbee, seven other morphospecies belong to the genus *Eurytoma* within the *E. rosae* species group, characterised by the presence of postgenal depressions and the raised adscrobal carina which forms the precoxal tooth in front of the mesocoxal cavities in lateral view (Lotfalizadeh *et al.* 2007a).

Key morphological characteristics traditionally used to distinguish eurytomids were found to be ambiguous, with morphological variations often correlated with size, and the absence or atrophy of key characters in the smaller specimens. Other variation included colour of the scape, forelegs and midlegs (yellow to black) and the ratio of wing vein length. New characters such as the number and arrangement of multiporous plate sensilla, and sculpturing on the petiole were useful in distinguishing male specimens, many of which were previously unidentifiable if encountered singly.

Host and geographical records

Ten new provincial records were established as a result of this study (Table 2), including a new Canadian record for *E. iniquus*. In addition, 18 new host associations were discovered,

Fig. 2. Phylogenetic tree for species of Eurytomidae associated with rose galls induced by *Diplolepis* in Canada based on cytochrome *c* oxidase I (COI) data. Maximum likelihood bootstrap support (first value) and Bayesian posterior probabilities (second value) are shown at each node. The scale bar represents the number of nucleotide substitutions per site. *Orthopelma mediator* (Hymenoptera: Ichneumonidae), *Ormyrus rosae* (Hymenoptera: Ormyridae), and *Torymus bedeguaris* (Hymenoptera: Torymidae) are used as outgroups.



greatly expanding the known host records in North America. With the exception of *Eurytoma* species 2, which has only been observed in association with galls of *Diplolepis ignota* (Osten Sacken), all other haplogroups are associated with two to six different hosts (Table 4). In addition, two generations of *E. longavena* and *E. spongiosa* 2 were collected, from both spring (*e.g.*, *Diplolepis polita* (Ashmead)) and fall initiated galls (*e.g.*, *Diplolepis nebulosa* (Bassett)).

Discussion

Testing species limits using COI

Accelerated rates of evolution of the chalcid mitochondrial genome have been correlated with parasitic lifestyles (Xiao *et al.* 2011); however, testing the species limits of recently diverged lineages is difficult because the organisms often had insufficient time for the evolution of diagnostic characters or complete reproductive isolation (Xiao *et al.* 2011; Gebiola *et al.* 2012). Independent lines of evidence were used in the testing of species limits in these studies, thus avoiding the reliance of one particular dataset.

The COI sequences found in present study resolved closely related species of eurytomids that are difficult or impossible to distinguish morphologically. This first "screening" of morphospecies using COI drew attention to problematic clades that required further investigation (Li *et al.* 2010). An additional benefit of molecular analyses is the ability to associate sexually dimorphic eurytomids in a simple and precise way, where males were previously unknown or indistinguishable due to the lack of detailed species descriptions. The results of this study show the COI sequences are taxonomically informative in identifying eurytomid species and species boundaries defined by deep COI

Table 2. Collection locality and host information for eurytomid morphospecies and haplogroups.

Morphospecies	Haplogroups	Locality	Host gall	Host plant	Sample size
Eurytoma acuta	*Eurytoma discordans*	Alberta	*D. nodulosa* (Beutenmueller)	*R. woodsii* Lindley	4
		Alberta	*D. tumida* (Bassett)	*R. woodsii*	7
Eurytoma calcarea		Alberta	*D. bicolor* (Harris)	*R. woodsii*	10
		British Columbia	*D. variabilis* (Bassett)	*R. woodsii*	3
Eurytoma discordans		Ontario	*D. nodulosa*	*R. blanda* Gray	4
		Ontario	***D. radicum*** (Osten Sacken)	*R. acicularis* Lindley	6
		Ontario	*D. spinosa* (Ashmead)	*R. blanda*	20
Eurytoma iniquus	***Eurytoma iniquus***	**Alberta**	***D. bicolor***	*R. woodsii*	2
		British Columbia	***D. variabilis***	*R. woodsii*	1
		Ontario	***D. bicolor***	*R. blanda*	4
		Ontario	***D. nodulosa***	*R. blanda*	3
		Ontario	***D. rosaefolii*** (Cockerell)	*R. acicularis*	2
Eurytoma longavena	*Eurytoma longavena*	Alberta	***D. nebulosa*** (Bassett)	*R. woodsii*	2
		British Columbia	***D. variabilis***	*R. woodsii*	1
		Quebec	*D. polita* (Ashmead)	*R. acicularis*	6
		Ontario	*D. nebulosa*	*R. blanda*	6
		Ontario	***D. fusiformans*** (Ashmead)	*R. acicularis*	2
		Ontario	*D. polita*	*R. acicularis*	4
		Ontario	***D. rosaefolii***	*R. acicularis*	15
Eurytoma spongiosa	*Eurytoma spongiosa* 1	**Alberta**	***D. ignota*** (Osten Sacken)	*R. woodsii*	2
		Alberta	*D. tumida*	*R. woodsii*	5
		British Columbia	***D. variabilis***	*R. woodsii*	4
		Ontario	*D. spinosa*	*R. blanda*	15
		Ontario	*D. spinosa*	*R. rugosa* Thunberg	7
		Ontario	*D. triforma* Shorthouse and Ritchie	*R. canina* L.	24
	Eurytoma spongiosa 2	Alberta	*D. ignota*	*R. arkansana* Porter	5
		Ontario	***D. nebulosa***	*R. blanda*	1
		Ontario	*D. triforma*	*R. acicularis*	7
		Ontario	*D. triforma*	*R. canina*	19
		Quebec	***D. polita***	*R. acicularis*	4
Eurytoma species	***Eurytoma* sp 1**	**Ontario**	***D. nodulosa***	*R. blanda*	1
		Ontario	***D. rosaefolii***	*R. acicularis*	3
	***Eurytoma* sp 2**	**Alberta**	***D. ignota***	*R. arkasana*	1
Tenuipetiolus ruber	*Tenuipetiolus ruber*	Ontario	***D. triforma***	*R. acicularis*	2
		Quebec	***D. polita***	*R. acicularis*	2

Note: New records are indicated in bold.

Fig. 3. Expanded phylogenetic tree for the *Eurytoma discordans* complex. Codes after species identification indicates location of host galls on plant organ (LE, leaf; RO, root; ST, stem). The codes after the underscore are the collection location in Canada (AB, Alberta; BC, British Columbia; ON, Ontario). Maximum likelihood bootstrap support (first value) and Bayesian posterior probabilities (second value) are shown at each node.

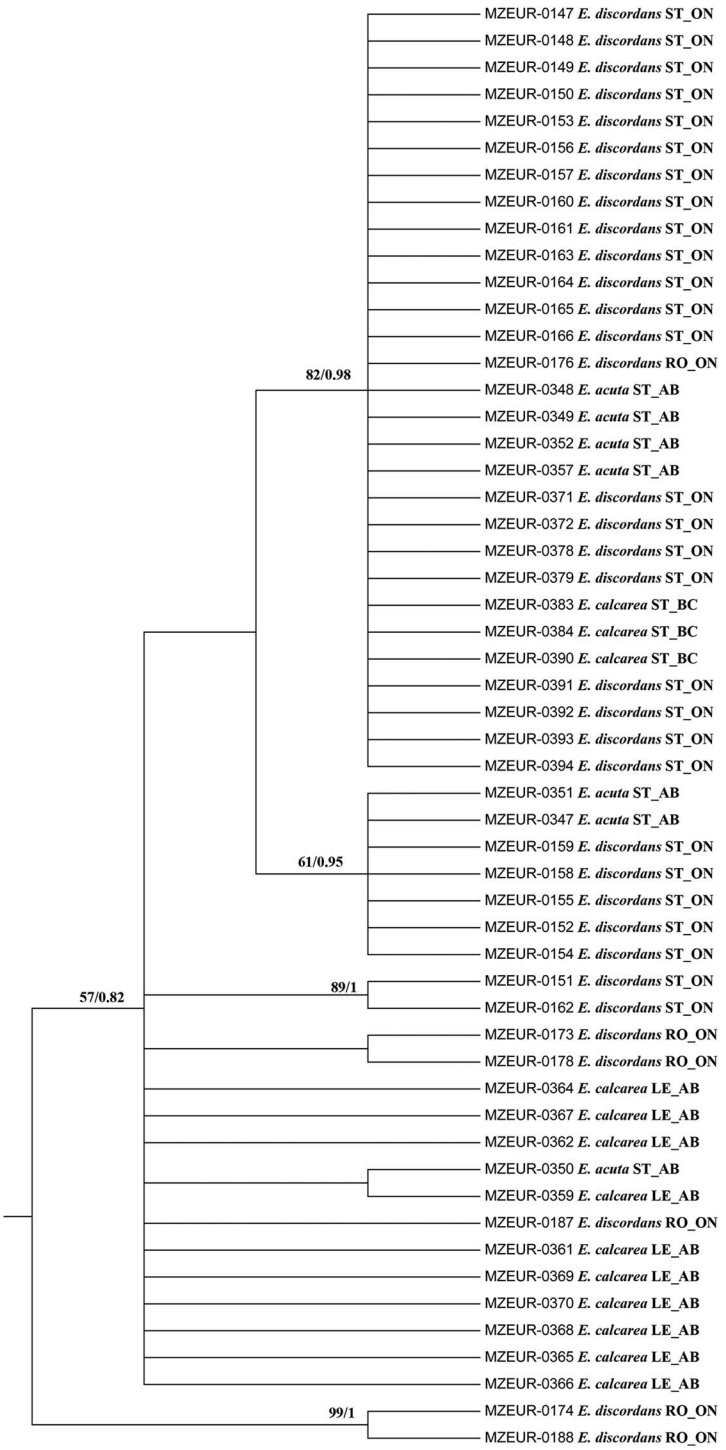


Table 3. Intra-specific and inter-specific divergence for all haplogroups. Standard errors are shown in reverse of the matrix for interspecific divergence

Interspecific divergence	*Eurytoma discordans*	*Eurytoma iniquus*	*Eurytoma longavena*	*Eurytoma* sp 1	*Eurytoma* sp 2	*Eurytoma spongiosa* 1	*Eurytoma spongiosa* 2	*Tenuipetiolus ruber*	Intraspecific divergence	SE
Eurytoma discordans		0.018	0.017	0.016	0.021	0.023	0.014	0.025	0.014	0.005
Eurytoma iniquus	0.092		0.024	0.021	0.023	0.024	0.019	0.028	0.012	0.004
Eurytoma longavena	0.079	0.126		0.018	0.022	0.023	0.018	0.027	0.002	0.002
Eurytoma sp 1	0.076	0.117	0.090		0.021	0.022	0.018	0.028	0.028	0.008
Eurytoma sp 2	0.097	0.132	0.103	0.112		0.023	0.021	0.029	N/A	N/A
Eurytoma spongiosa 1	0.128	0.134	0.118	0.125	0.120		0.021	0.029	0.004	0.002
Eurytoma spongiosa 2	0.057	0.088	0.076	0.089	0.097	0.103		0.026	0.005	0.002
Tenuipetiolus ruber	0.161	0.197	0.175	0.194	0.202	0.198	0.170		0.038	0.011

Table 4. Eurytomid haplogroups and associated rose galls.

Species	Plant organ	*Eurytoma discordans*	*Eurytoma iniquus*	*Eurytoma longavena*	*Eurytoma spongiosa* 1	*Eurytoma spongiosa* 2	*Eurytoma* sp 1	*Eurytoma* sp 2	*Tenuipetiolus ruber*
D. bicolor	Leaf	**X**	**X**						
D. ignota	Leaf				**X**	**X**		**X**	
D. nebulosa	Leaf			**X**		**X**			
D. polita	Leaf			**X**		**X**			**X**
D. rosaefolii	Leaf		**X**	**X**			**X**		
D. variabilis	Leaf	**X**	**X**	**X**	**X**				
D. fusiformans	Stem			**X**					
D. nodulosa	Stem	**X**	**X**				**X**		
D. spinosa	Stem	**X**			**X**				
D. triforma	Stem				**X**	**X**			**X**
D. tumida	Stem	**X**			**X**				
D. radicum	Root	**X**							

divergences are incongruent with morphological studies by Bugbee (1951a, 1951b, 1967, 1973). While this study did not include additional genes, similar studies on eurytomids have shown that mitochondrial and nuclear genes corroborated each other (Lotfalizadeh *et al.* 2007a; Li *et al.* 2010), therefore the results of the current study based only on mitochondrial genes is likely robust when combined with ecological and host records. The species limits of three of the eight haplogroups were resolved by COI sequences although the other five haplogroups showed conflicting results with existing morphological data (Bugbee 1951b, 1967). The intraspecific divergence is $<2\%$, which is consistent with other published studies on Hymenoptera (*e.g.*, Sheffield *et al.* 2009; Li *et al.* 2010). Deep phylogenetic divergences within the COI data support the existence of cryptic genetic species in *Eurytoma*, consistent with previous studies of eurytomids of Palearctic gall communities (Ács *et al.* 2002; Lotfalizadeh *et al.* 2007a; Gómez *et al.* 2011). The presence of cryptic species within specimens identified as *E. spongiosa* was expected, as the species was originally described as a morphologically variable generalist that attacked a wide variety of hosts (Bugbee 1951a). A possible hypothesis for the lack of consistent morphological differences between *E. spongiosa* 1 and *E. spongiosa* 2 despite host differences could be related to the presence of *Wolbachia* Hertig (Rickettsiaceae), a common and widespread group of intracelluar bacteria found in the reproductive organs of arthropods that can cause cytoplasmic incompatibility (reviewed in Werren *et al.* 2008). The presence of *Wolbachia* is much higher within Hymenoptera in comparison to other groups of arthropods, which has been hypothesised to be the cause of host speciation (Bordenstein *et al.* 2001; Sun *et al.* 2011; Smith *et al.* 2012). As the presence of other cryptic chalcid parasitoids has been reported in association with cynipid galls (Lotfalizadeh *et al.* 2007a; Nicholls *et al.* 2010), further investigation on screens for *Wolbachia* may help to delimit the two *E. spongiosa* species.

Eurytoma acuta, *E. calcarea*, and *E. discordans* were described as morphologically distinct from each other based on the colour of the scape and shape of the stigmal club and marginal vein (Bugbee 1951b). Based on specimens examined in this study, these characters were shown to be extremes of a continuum rather than stable characters and are thus unreliable. The three species were also previously distinguished by their range and host, which has been expanded and now overlap as a result of additional data presented in this study. The high rate of intraspecific divergence of this clade suggests the presence of a species complex, where retention of ancestral polymorphism and hybridisation may have resulted in the failure of molecular tracing of species boundaries (Li *et al.* 2010). Hence, *E. acuta* and *E. calcarea* should be synonymised, under the more senior name *E. discordans*, **new synonyms**.

Host specificity and the evolution of eurytomids on roses

The new distribution and host records suggest that eurytomids exhibit a much wider host range than previously reported (Noyes 2012), as the majority of species are either oligophagous or polyphagous and were found wherever their hosts occur. Thus, using host records and range as key characteristics (Bugbee 1951b, 1967, 1973) in species delimitation is likely prone to error. The presence of fall emergents in *E. longavena* and *E. spongiosa* 2 in both spring-initiated and late summer-initiated galls suggests that these species are bivoltine, where the first generation emerges in the spring and attacks freshly initiated galls, while the second generation develops and exits from spring galls in the late summer to attack the galls of other species of *Diplolepis* that are maturing at this time (Shorthouse 1973).

The radiation of *Diplolepis* species onto novel host plants and organs was likely in response to selection for exclusion of natural enemies (enemy-free space) such as eurytomids (Stille 1984; Price *et al.* 1987; Stone *et al.* 2002). Most eurytomids are found across a wide geographical range within galls found on multiple species of wild roses; thus, their natural range likely mirrors their hosts. For instance, in cases where *Diplolepis spinosa* (Ashmead) shifted hosts from *Rosa blanda* Aiton to the domestic rose *Rosa rugosa* Thunberg (Shorthouse 1988), the species of eurytomid parasitoids that are normally associated with *D. spinosa* are also

found attacking galls on the new host plant (Table 2). In a study by Nicholls *et al.* (2010), evidence was provided for parasitoids of oak galls that have tracked their hosts through space and time, showing radiation into cryptic species together with host radiations at multiple trophic levels. It is likely that eurytomids associated with rose galls also have stable, long-term co-evolutionary interactions with other species in the cynipid community, responding as a single unit to environmental perturbations (Nicholls *et al.* 2010).

Several species of eurytomids examined in this study showed a close evolutionary relationship with their hosts, often only attacking hosts inducing galls on a specific plant organ (Table 4). *Eurytoma longavena* was observed almost exclusively in single-chambered galls such as those induced on leaves (*e.g.*, *D. polita*). The only exception was galls induced by *Diplolepis fusiformans* (Ashmead), a small, single-chambered stem gall that is closely related to the other basal lineages of leaf-gall inducing species (Plantard *et al.* 1998). Likewise *E. discordans* was found in multi-chambered stem galls where it sometimes consumes several hosts by tunnelling from one larval chamber to another (Brooks and Shorthouse 1997). *Tenuipetiolus ruber* was rarely found in galls of *D. polita* and *Diplolepis triforma* Shorthouse and Ritchie, and the intraspecific divergence of specimens identified as *T. ruber* (3.8%) suggests the presence of cryptic species. In addition to *Diplolepis*, this species has also been found in association with cynipid galls on blackberry induced by *Diastrophus* Hartig (Hymenoptera: Cynipidae) (Bugbee 1951a). Additional specimens from other hosts to determine the species limit of *T. ruber*. The two unidentified species of *Eurytoma* and *E. iniquus* were collected from galls with high levels of attack by inquilines of the genus *Periclistus* Förster (Hymenoptera: Cynipidae) (Table 4). *Eurytoma nigricoxa* Provancher is the only species in Canada that has been recorded in association with *Periclistus*-modified galls (Bugbee 1967); however, none of the three species matches *E. nigricoxa* upon comparison with the holotype. It is likely these three *Eurytoma* species are parasitoids of *Periclistus*; although more specimens are needed to further investigate these host relationships.

This study has established a DNA barcode reference library for eurytomids, particularly *Eurytoma* associated with galls of *Diplolepis* in Canada. This is the first phylogenetic study of Nearctic *Eurytoma* and suggests that many eurytomid species associated with rose galls (Bugbee 1951a, 1951b, 1967, 1973) require further investigation. Detailed studies of *E. spongiosa* 1, *E. spongiosa* 2, and the *E. discordans* species complex will undoubtedly aid in the identification of species. In addition, the larval forms of the eurytomids included in this study have not been described, therefore matching larvae with their corresponding adults using COI could provide valuable useful information on species delineation. Such studies of other eurytomid larvae have been morphologically informative when the adults were difficult to identify (Claridge and Askew 1960; Henneicke *et al.* 1992; Gómez *et al.* 2011).

The presence of synonymous and cryptic species likely occurs in other eurytomid species treated by Bugbee and are in need of taxonomic revision as many morphological characters used to distinguish Nearctic eurytomids are highly variable. Issues that have impeded the identification of eurytomids associated with cynipid rose galls such as host specificity and sex association were resolved using DNA barcoding, providing new insights into the evolutionary history of this taxonomically difficult group.

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References

Ács, Z., Challis, R., Bihari, P., Blaxter, M., Hayward, A., Melika, G., *et al*. 2010. Phylogeny and DNA barcoding of inquiline oak gallwasps (Hymenoptera: Cynipidae) of the western Palearctic. Molecular Phylogenetics and Evolution, **55**: 210–225.

Ács, Z., Melika, G., Kalo, P., and Kiss, G.B. 2002. Molecular analysis in *Eurytoma rosae* species-group (Chalcidoidea: Eurytomidae). *In* Parasitic wasps: evolution, systematics, biodiversity and biological control. *Edited by* G. Melika and C. Thuróczy. Agroinform, Budapest, Hungary. Pp. 234–240.

Bordenstein, S.R., O'Hara, F.P., and Werren, J.H. 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. Nature, **409**: 707–710.

Brooks, S.E. and Shorthouse, J.D. 1997. Biology of the rose stem galler *Diplolepis nodulosa* (Hymenoptera: Cynipidae) and its associated component community in central Ontario. The Canadian Entomologist, **129**: 1121–1140.

Bugbee, R.E. 1951a. A new genus of two previously described and two new species of the Family Eurytomidae bred from cynipid and dipterous hosts. Journal of the Kansas Entomological Society, **24**: 37–45.

Bugbee, R.E. 1951b. New and described parasites of the genus *Eurytoma* Illiger from rose galls caused by species of the cynipid genus *Diplolepis* Geoffrey. Annals of the Entomological Society of America, **44**: 213–261.

Bugbee, R.E. 1967. Revision of chalcid wasps of genus *Eurytoma* in America north of Mexico. Proceedings of the United States National Museum, **118**: 433–552.

Bugbee, R.E. 1973. New species of the genus *Eurytoma* from the United States and Canada (Hymenoptera: Eurytomidae). Journal of the Georgia Entomological Society, **8**: 11–15.

Claridge, M.F. and Askew, R.R. 1960. Sibling species in the *Eurytoma rosae* group (Hym: Eurytomidae). Entomophaga, **5**: 141–153.

Cognato, A.I. 2006. Standard percent DNA sequence difference for insects does not predict species boundaries. Journal of Economic Entomology, **99**: 1037–1045.

Csóka, G., Stone, G.N., and Melika, G. 2005. Biology, ecology and evolution of gall-inducing Cynipidae. *In* Biology, ecology, and evolution of gall-inducing arthropods, volume 2. *Edited by* A, Raman, C.W. Schaefer, and T.M. Withers. Science Publishers, Enfield, New Hampshire, United States of America. Pp. 573–642.

Dayrat, B. 2005. Towards integrative taxonomy. Biological Journal of the Linnean Society, **85**: 407–415.

Dowton, M. and Austin, A.D. 1995. Increased genetic diversity in mitochondrial genes is correlated with the evolution of parasitism in the Hymenoptera. Journal of Molecular Evolution, **41**: 958–965.

Gates, M. 2008. Species revision and generic systematics of world Rileyinae. Vol. 127. University of California Press Publications in Entomology, Berkeley, California, United States of America. 332 pp.

Gates, M.W. and Pérez-Lachaud, G. 2012. Description of *Camponotophilus delvarei*, gen. n. and sp. n. (Hymenoptera: Chalcidoidea: Eurytomidae), with discussion of diagnostic characters. Proceedings of the Entomological Society of Washington, **114**: 111–124.

Gebiola, M., Goméz-Zurita, J., Monti, M.M., Navones, P., and Bernardo, U. 2012. Integration of molecular, ecological, morphological and endosymbiont data for species delimitation within the *Pnigalio soemius* complex (Hymenoptera: Eulophidae). Molecular Ecology, **21**: 1190–2108.

Gibson, G.A.P., Heraty, J.M., and Woolley, J.B. 1999. Phylogenetics and classification of Chalcidoidea and Mymarommatoidea – a review of current concepts (Hymenoptera, Apocrita). Zoologica Scripta, **28**: 87–124.

Gibson, G.A.P., Huber, J.T., and Woolley, J.B. 1997. Annotated keys to the genera of Nearctic Chalcidoidea (Hymenoptera). National Research Council Research Press, Ottawa, Ontario, Canada.

Gómez, J.F., Nieves-Aldrey, J.L., Hernández Nieves, M., and Stone, G.N. 2011. Comparative morphology and biology of terminal-instar larvae of some *Eurytoma* (Hym. Eurytomidae) species parasitoids of gall wasps (Hym. Cynipidae) in western-Europe. Zoosystema, **33**: 287–323.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H., and Hallwachs, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the United States of America, **101**: 14812–14817.

Henneicke, K., Dawah, H.A., and Jervis, M.A. 1992. Taxonomy and biology of the final-instar larvae of some Eurytomidae (Hymenoptera: Chalcidoidea) associated with grasses in the UK. Journal of Natural History, **26**: 1047–1087.

Heraty, J. 2009. Parasitoid biodiversity and insect pest management. *In* Insect biodiversity: science and society. *Edited by* R.G. Foottit and P.H. Adler. Wiley-Blackwell, Chichester, United Kingdom. Pp. 445–462.

Heraty, J. and Hawks, D. 1998. Hexamethyldisilazane – a chemical alternative for drying insects. Entomological News, **109**: 369–374.

Ivanova, N.V., deWaard, J.R., and Hebert, P.D.N. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Molecular Ecology Notes, **6**: 998–1002.

Ivanova, N.V., deWaard, J.R., and Hebert, P.D.N. 2007. CCDB protocols, glass fiber plate DNA extraction [online]. Available from http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_DNA_Extraction.pdf [accessed 15 April 2012].

Ivanova, N.V. and Grainger, C.M. 2007a. CCDB protocols, COI amplification [online]. Available from http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Amplification.pdf [accessed 15 April 2012].

Ivanova, N.V. and Grainger, C.M. 2007b. CCDB protocols, sequencing. Available from http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Sequencing.pdf [accessed 15 April 2012].

Kaartinen, R., Stone, G.N., Hearn, J., Lohse, K., and Roslin, T. 2010. Revealing secret liaisons: DNA barcoding changes our understanding of food webs. Ecological Entomology, **35**: 623–638.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, **16**: 111–120.

Leggo, J.J. and Shorthouse, J.D. 2006. Modification of galls of *Diplolepis triforma* (Hymenoptera: Cynipidae) by the parasitoids *Eurytoma spongiosa* (Hymenoptera: Eurytomidae) and *Glyphomerus stigma* (Hymenoptera: Torymidae). The Canadian Entomologist, **138**: 681–696.

Li, Y.W., Zhou, X., Feng, G., Hu, H., Niu, L., Hebert, P.D.N., *et al*. 2010. COI and ITS2 sequences delimit species, reveal cryptic taxa and host specificity of fig-associated *Sycophila* (Hymenoptera, Eurytomidae). Molecular Ecology Resources, **10**: 31–40.

Lotfalizadeh, H., Delvare, G., and Rasplus, J.-Y. 2007a. *Eurytoma caninae* sp. n. (Hymenoptera, Eurytomidae), a common species previously overlooked with *E. rosae*. Zootaxa, **1640**: 55–68.

Lotfalizadeh, H., Delvare, G., and Rasplus, J.-Y. 2007b. Phylogenetic analysis of Eurytominae (Chalcidoidea: Eurytomidae) based on morphological characters. Zoological Journal of the Linnean Society, **151**: 441–510.

Lunt, D., Zhang, D.-X., Szymura, J., and Hewitt, G.M. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Molecular Biology, **5**: 153–165.

Meier, R., Shiyang, K., Vaidya, G., and Ng, P. 2006. DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Systematic Biology, **55**: 715–728.

Munro, J.B., Heraty, J.M., Burks, R.A., Hawks, D., Mottern, J., Cruaud, A., *et al*. 2011. A molecular phylogeny of the Chalcidoidea (Hymenoptera). Public Library of Science One, **6**: e27023.

Nicholls, J.A., Preuss, S., Hayward, A., Melika, G., Csóka, G., Nieves-Aldrey, J.-L., *et al*. 2010. Concordant phylogeography and cryptic speciation in two western Palearctic oak gall parasitoid species complexes. Molecular Ecology, **19**: 592–609.

Noyes, J.S. 2012. Universal Chalcidoidea database [online]. Available from http://www.nhm.ac.uk/chalcidoids/ [accessed 28 April 2012].

Oliveira, D.C.S.G., Raychoudhury, R., Lavrov, D.V., and Werren, J.H. 2008. Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp *Nasonia* (Hymenoptera: Pteromalidae). Molecular Biology and Evolution, **25**: 2167–2180.

Plantard, O., Rasplus, J.-Y., and Hochberg, M.E. 1996. Resource partitioning in the parasitoid assemblage of the oak galler *Neuroterus quercusbaccarum* L. (Hymenoptera: Cynipidae). Acta Oecologica, **17**: 1–15.

Plantard, O., Rasplus, J.-Y., Mondor, G., Le Clainche, I., and Solignac, M. 1999. Distribution and phylogeny of *Wolbachia*-inducing thelytoky in Rhoditini and 'Aylacini' (Hymenoptera: Cynipidae). Insect Molecular Biology, **8**: 185–191.

Plantard, O., Shorthouse, J.D., and Rasplus, J.-Y. 1998. Molecular phylogeny of the genus *Diplolepis* (Hymenoptera: Cynipidae). *In* The biology of gall-inducing arthropods. United States Forest Service General Technical Report NC-199. *Edited by* G. Csóka, W.J. Mattson, G.N. Stone, and P.W. Price. United States Department of Agriculture, Washington, DC, United States of America. Pp. 247–260.

Porco, D., Rougerie, R., Deharveng, L., and Hebert, P. 2010. Coupling non-destructive DNA extraction and voucher retrieval for small soft-bodied Arthropods in a high-throughput context: the example of Collembola. Molecular Ecology Resources, **10**: 942–945.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution, **25**: 1253–1256.

Price, P.W., Fernandes, G.W., and Waring, G.L. 1987. Adaptive nature of insect galls. Environmental Entomology, **16**: 15–24.

Ratnasingham, S. and Hebert, P.D.N. 2007. BOLD: the barcode of life data system (http://www.barcodinglife.org). Molecular Ecology Notes, **7**: 183–189.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., *et al*. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology, **61**: 539–542.

Santos, A.M.C., Besnard, G., and Quicke, D.J. 2011. Applying DNA barcoding for the study of geographical variation in host-parasitoid interactions. Molecular Ecology Resources, **11**: 46–59.

Schönrogge, K., Stone, G.N., and Crawley, M.J. 1995. Spatial and temporal variation in guild structure: parasitoids and inquilines of *Andricus quercuscalicis* (Hymenoptera: Cynipidae) in its native and alien ranges. Oikos, **72**: 51–60.

Schönrogge, K., Stone, G.N., and Crawley, M.J. 1996. Abundance patterns and species richness of the parasitoids and inquilines of the alien gall former *Andricus quercuscalicis* Burgsdorf (Hymenoptera: Cynipidae). Oikos, **77**: 507–518.

Sheffield, C.S., Hebert, P.D.N., Kevan, P.G., and Packer, L. 2009. DNA barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies. Molecular Ecology Resources, **9**: 196–207.

Shorthouse, D.P. 2012. SimpleMappr, an online tool to produce publication-quality point maps [online]. Available from www.simplemappr.net [accessed 3 April 2012].

Shorthouse, J.D. 1973. The insect community associated with rose galls of *Diplolepis polita* (Cynipidae, Hymenoptera). *Quaestiones Entomologicae*, **9**: 55–98.

Shorthouse, J.D. 1988. Occurrence of two gall wasps of the genus *Diplolepis* (Hymenoptera: Cynipidae) on the domestic shrub rose, *Rosa rugosa* Thunb. (Rosaceae). The Canadian Entomologist, **120**: 727–737.

Shorthouse, J.D. 2010. Galls induced by cynipid wasps of the genus *Diplolepis* (Hymenoptera: Cynipidae) on the roses of Canada's grasslands. *In* Arthropods of Canadian grasslands (volume 1): ecology and interactions in grassland habitats. *Edited by* J.D. Shorthouse and K.D. Floate. Biological Survey of Canada, Ottawa, Ontario, Canada. Pp. 251–279.

Shorthouse, J.D., Leggo, J.J., Sliva, M.D., and Lalonde, R.G. 2005. Has egg location influenced the radiation of *Diplolepis* (Hymenoptera: Cynipidae) gall wasps on wild roses? Basic and Applied Ecology, **6**: 423–434.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., and Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America, **87**: 651–701.

Smith, M.A., Bertrand, C., Crosby, K., Eveleigh, E.S., Fernandez-Triana, J., Fisher, B.L., *et al*. 2012. Wolbachia and DNA barcoding insects: patterns, potential, and problems. Public Library of Science One, **7**: e36514.

Smith, M.A., Fisher, B.L., and Hebert, P.D.N. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. Philosophical Transactions of the Royal Society of London B: Biological Sciences, **360**: 1825–1834.

Smith, M.A., Rodriguez, J.J., Whitfield, J.B., Deans, A.R., Janzen, D.H., Hallwachs, W., *et al*. 2008. Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. Proceedings of the National Academy of Sciences of the United States of America, **105**: 12359–12364.

Smith, M.A., Wood, D.M., Janzen, D.H., Hallwachs, W., and Hebert, P.D.N. 2007. DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. Proceedings of the National Academy of Sciences of the United States of America, **104**: 4967–4972.

Smith, M.A., Woodley, N.E., Janzen, D.H., Hallwachs, W., and Hebert, P.D.N. 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proceedings of the National Academy of Sciences of the United States of America, **103**: 3657–3662.

Stille, B. 1984. The effect of host plant and parasitoids on the reproductive success of the parthenogenetic gallwasp *Diplolepis rosae* (Hymenoptera; Cynipidae). Oecologia, **63**: 364–369.

Stone, G.N., Schönrogge, K., Atkinson, R.J., Bellido, D., and Pujade-Villar, J. 2002. The population biology of oak gall wasps (Hymenoptera: Cynipidae). Annual Review of Entomology, **47**: 633–668.

Sun, X.-J., Xiao, J.-H., Cook, J.M., Feng, G., and Huang, D.-W. 2011. Comparison of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fig wasp species and the effects of *Wolbachia* on host mtDNA evolution and diversity. BMC Evolutionary Biology, **11**: 86.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, **28**: 2731–2739.

Werren, J.H., Baldo, L., and Clark, M.E. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nature Reviews Microbiology, **6**: 741–751.

Will, K.G., Mishler, B.D., and Wheeler, Q.D. 2005. The perils of DNA barcoding and the need for integrative taxonomy. Systematic Biology, **54**: 844–851.

Xiao, J.H., Jia, J.G., Murphy, R.W., and Huang, D.W. 2011. Rapid evolution of the mitochondrial genome in chalcidoid wasps (Hymenoptera: Chalcidoidea) driven by parasitic lifestyles. Public Library of Science One, **6**: e26645.