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UCE data reveal multiple origins of rose gallers in North America: Global phylogeny of *Diplolepis* Geoffroy (Hymenoptera: Cynipidae)

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ABSTRACT

Gall wasps in the genus *Diplolepis* Geoffroy are specialized herbivores that induce galls exclusively on roses. Despite their wide distribution across the Holarctic, little is known about their evolutionary history. Here we present the first phylogenomic tree of global *Diplolepis* reconstructed using Ultraconserved Elements (UCEs), resulting in a robust phylogeny based on 757 genes. Results support the existence of two principal clades: a Nearctic stem-galler clade, and a Holarctic leaf-galler clade that further splits into two Palearctic groups and one Nearctic group. This topology is congruent with a previous study based on the mitochondrial gene *COI*, an unexpected result given the common occurrence of mitonuclear discordance in closely related oak gall wasp lineages. Most *Diplolepis* species were recovered as reciprocally monophyletic, with some notable exceptions such as the *D. polita* and the *D. ignota* complex, for which species boundaries remain unresolved. Historical biogeographic reconstruction was unable to pinpoint the origin of *Diplolepis*, but confirms two independent incursions into the Nearctic. Ancestral state reconstruction analysis highlights the conservatism of gall location on the host plants, as shifts to different host organs are relatively rare. We suggest that *Diplolepis* were originally leaf gallers, with a Nearctic stem-galler clade undergoing a major plant organ switch onto rose stems. Host organ switch or reversal is uncommon, which suggests a level of conservatism. Our study showcases the resolving power of UCEs at the species level while also suggesting improvements to advance future Cynipoidea phylogenomics. Our results also highlight the additional sampling needed to clarify taxonomic relationships in the Nearctic and eastern Palearctic regions.

1. Introduction

Gall wasps in the family Cynipidae are specialized herbivores that induce galls – plant structures that provide high-quality nutrition and protection from fluctuations in microclimatic changes and natural enemies (Shorthouse and Rohfritsch, 1992; Stone and Schönrogge, 2003). Many of the ~ 1500 species of gall wasps are capable of manipulating plant tissues into producing species-specific galls, which can be regarded as extended phenotypes of the wasps (Stone and Cook, 1998). Members of tribes Cynipini and Diplolepidini are also capable of producing galls with modifications found nowhere else on the plant, such as internal air spaces, multiple larval chambers, nectar, resin, dummy chambers, and spines (Bailey et al., 2009; Nicholls et al., 2017; Ronquist and Liljeblad, 2001; Stone and Cook, 1998). These traits are thought to be highly adaptive, as they can reduce attacks by natural enemies throughout gall development (Bailey et al., 2009; László and Tóthmérész, 2013; Stone and Cook, 1998; Stone and Schönrogge, 2003),

Most of the diversity in gall wasps (~1000 species) occurs within Cynipini, with around 50 genera associated with Fagaceae (Ronquist et al., 2015). By comparison, the relatively small tribe Diplolepidini has only two genera: approximately 50 species are found in *Diplolepis* Geoffroy and 10 species in *Liebelia* Kieffer, all of which induce galls on roses (*Rosa* L. spp., Rosaceae) (Abe et al., 2007; Beutenmüller, 1907; Güçlü et al., 2008; Melika, 2006; Pujade-Villar et al., 2020; Shorthouse, 2010; Wang et al., 2013; Zhang et al., 2019a). Galls of these species

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occur on the leaves, stems, fruits (hips), or adventitious shoots of roses and can range from smooth to spiny, integral to detachable, and single-chambered or multi-chambered (Beutenmüller, 1907; Ronquist and Liljeblad, 2001; Shorthouse, 2010).

Diplolepis is Holarctic in distribution, and the six western Palearctic *Diplolepis* species are relatively well studied and easily identified (Güçlü et al., 2008; Lotfalizadeh et al., 2006; Melika, 2006). A total of nine eastern Palearctic species are recorded but the actual number is likely much higher given the high diversity of rose species (Abe et al., 2007; Pujade-Villar et al., 2020; Vyrzhikovskaja, 1963; Wang et al., 2013). No current Nearctic identification key exists for the 31 species of *Diplolepis*, despite having the highest number of species and being well collected (Burks, 1979; Shorthouse, 2010). Beutenmüller (1907) separated the Nearctic species based on gall morphology; however, the galls of some species can be structurally modified by the actions of *Periclistus* Förster (Cynipidae: Diastrophini) inquilines (Brooks and Shorthouse, 1998; Shorthouse, 1980, 1998). Previous attempts to explain the phylogeny of *Diplolepis* using single-gene data largely divided the genus into a clade of Nearctic stem gallers and a clade of Holarctic leaf gallers (Plantard et al., 1998b; Zhang et al., 2019a). However, interpreting phylogeny based on a limited number of genes can be misleading, especially when mitonuclear discordance as a result of heteroplasmy, numts (nuclear pseudogene copies of mitochondrial loci), and incomplete lineage sorting can be a common source of taxonomic problems, as has occurred in oak gall wasps and other arthropods (Magnacca and Brown, 2010; Nicholls et al., 2012; Rokas et al., 2003; Song et al., 2008).

The field of arthropod systematics has been revolutionized by the development and increased availability of tools designed to capture genomic DNA through targeted enrichment of ultraconserved elements (UCEs, Faircloth et al., 2015), and the most recent Hymenoptera probe sets can capture up to as many as 2590 UCE loci from each specimen (Branstetter et al., 2017). Additionally, studies have shown this method can be used on museum specimens upwards of 100 years old (Blaimer et al., 2016; Derkarabetian et al., 2019), and can capture DNA from microhymenoptera using non-destructive extraction protocols (Cruaud et al., 2019). The increased accessibility and versatility of UCEs has resulted in an explosion of studies using this method for resolving deep level relationships and shallow level species delimitations (reviewed in Zhang et al., 2019b). The goal of this study is to leverage the phylogenomic signals generated using UCE data to reconstruct a robust global *Diplolepis* phylogeny, and to test evolutionary hypotheses in a phylogenetic context. In particular, we ask (1) Are the sites of gall initiation by *Diplolepis* constrained by their phylogenetic relationships? and (2) What is the biogeographic origin of Diplolepidini and, does it coincide with host plant origin? As *Rosa* spp. are thought to have originated from the Asia or North America (Chen et al., 2020; Fougere-Danezan et al., 2015), we hypothesize the rose gallers originate from one of these two regions and have subsequently radiated into the Holarctic, resulting in the distribution that we see today.

2. Materials & methods

2.1. Taxon sampling

Diplolepis specimens were selected to represent the greatest possible degree of genetic variation for each species based on host, geographic distance, and morphological variation, mirroring the approach of the Zhang et al. (2019a) study. In the cases of *D. rosae* (L.) and *D. nervosa* (Curtis) which have been introduced to North America from Europe along with their hosts, we included samples from both the native and introduced range (Zhang et al., 2019a). Freshly collected specimens (by CL, ZL, and TI) in conjunction with museum samples deposited at the Smithsonian National Museum of Natural History (NMNH, by JDS, YMZ, and others) were identified to species whenever possible using gall morphology and, in cases where confident identification could not be made, morphospecies were assigned. Our data include 48 sequenced UCE libraries from specimens with an emphasis on the Nearctic and Western Palearctic *Diplolepis* species. A single specimen of *Liebelia fukudae* (Shinji) was also included as the outgroup for *Diplolepis*.

2.2. UCE sequence data collection

DNA extraction and library preparation were conducted in the Laboratories of Analytical Biology (LAB) at NMNH in Washington D.C., USA. DNA was extracted from vouchers destructively using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA), with sample collection dates ranging from 1982 to 2019. The protocol from Branstetter et al. (2017) was followed for capturing and enriching UCE loci. We fragmented the DNA to an average fragment distribution of 400–600 bp using a Qsonica Q800R sonicator (Qsonica LLC, Newton, CT, USA). Libraries were constructed from the sheared genomic DNA using Kapa Hyper Prep library preparation kits (Kapa Biosystems Inc., Wilmington, MA, USA), and custom, 8 bp dual-indexing TruSeq adapter barcodes (Glenn et al., 2019) to either end of each fragment, and then amplified the barcoded libraries using PCR. Following PCR amplification, DNA concentration of genomic libraries was measured on a Qubit 2.0 fluorometer and visualized via gel electrophoresis to assess quality.

The post-PCR libraries were purified in a “speedbead” clean-up step using a generic SPRI substitute (Rohland and Reich, 2012), and then combined together in pools of equimolar concentrations, with final concentrations of 127–170 ng/μl. The MYcroarray MYbaits protocol (Blumenstiel et al., 2010) was followed for in-solution target enrichment of the pooled DNA libraries but instead used a 1:4 (baits : water) dilution of the custom Hymenoptera 2.5Kv2P probes (ArborBiosciences, Ann Arbor, MI, USA) developed by Branstetter et al. (2017). The hybridization reaction of the RNA probes to the sequencing libraries was performed at 65 °C for 24 h. All enriched library pools were bound to streptavidin beads (Dynabeads MyOne Streptavidin T1, Life Technologies, Inc., Carlsbad, CA, USA) and washed.

Each pool was quantified and verified to ensure enrichment success using qPCR (CFX96 Touch, Bio-Rad Laboratories, Hercules, CA, USA), and then combined into a single pool-of-pools, which was size-selected to 300–500 bp using a BluePippin (SageScience, Beverly, MA, USA). Sample quality was checked using high sensitivity D1000 tape on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). The final pool-of-pools was sent to Novogene Corporation Inc. (Sacramento, CA) and sequenced on an Illumina NovaSeq 6000 (150-bp paired-end, Illumina Inc., San Diego, CA, USA).

2.3. UCE data processing

PHYLUCE v1.6.8 Tutorial I pipeline (Faircloth, 2015) was used for quality control, assembly, and UCE processing with default settings except for the following: the cleaned reads were assembled using SPAdes v3.14.0 (Bankevich et al., 2012). Alignments were trimmed using a wrapper script of Gblocks (Castresana, 2000) using the following settings: b1 = 0.5, b2 = 0.5, b3 = 12, b4 = 7. Matrices at 50% (1247 loci), 75% (757 loci), and 90% (158 loci) completeness were tested individually, before selecting the 75% complete matrix as our final dataset which balances the number of genes and topological accuracy. A total of 196,520 sites were removed from the data matrix due to potential alignment errors using the Python script Spruceup with default settings and 95% lognormal distribution (Borowiec, 2019). The summary statistics of the matrix were calculated using AMAS (Borowiec, 2016). Additionally, fragments of mitochondrial DNA *COI* were extracted from the UCE contigs using the PHYLUCE script *phyluce_assembly_match_contigs_to_barcodes* as validation of species identities from Zhang et al. (2019a). Trimmed reads for all generated sequences in this study are available from the National Center for Biotechnology Sequence Read Archive (SRA; BioProject ID PRJNA632631), and DNA barcodes are available on GenBank (MT858716–MT858743).

2.4. Phylogenomic analysis

Phylogenomic analysis was conducted under the maximum likelihood (ML) criterion with IQ-TREE v2.03 (Minh et al., 2020a), with two partitioning schemes. The first is partitoning based on loci (n = 757), and the second by Sliding-Window Site Characteristics (SWSC) of site entropy (Tagliacollo and Lanfear, 2018) which reduced the data into 356 partitions using PartitionFinder2 (Lanfear et al., 2016). To assess nodal support, we performed a Shimodaira-Hasegawa approximate likelihood-rate test (SH-aLRT, Guindon et al., 2010) with 1000 replicates using the "-alrt" flag, and 1000 ultrafast bootstrap replicates (UFBoot2, Hoang et al., 2017) using "-bb", along with "-bnni" flags to reduce risk of overestimating branch supports. Only nodes with support values of SH-aLRT ≥ 80 and UFBoot2 ≥ 95 were considered robust. The species tree was inferred under the multi-species coalescent model (MSC) in ASTRAL-III v5.7.3 (Zhang et al., 2018), using local posterior probabilities (LPP) to assess nodal support (Sayyari and Mirarab, 2016), with ≥ 0.95 considered as strong support. Unpartitioned, unrooted gene trees of each locus were estimated in IQ-TREE2 using "-S" on the charset file generated by PHYLUCE along with the data matrix, with the best models of nucleotide substitution selected in ModelFinder with "-m MFP" (Kalyaanamoorthy et al., 2017). Additionally, the gene and site concordance factors (gCF/sCF) of the MSC tree were tested to quantify genealogical concordance among our 698 genes using the species tree and gene trees generated earlier following Minh et al. (2020b). The output trees were visualized in R v4.0 (R Core Team, 2020) using the packages ggtree v2.2.0 and treeio v1.12.0 (Wang et al., 2020; Yu et al., 2017).

2.5. Ancestral state reconstruction

Ancestral state reconstruction (ASR) of the *Diplolepis* host organ shift was coded as three discrete characters based on the position of mature galls on the host plants (leaf/petiole/bud, stem, and hip). The leaf/petiole/bud galls were assigned as one state, as these galls are initiated as a result of eggs deposited on the surface of leaflets in unopened leaf buds (Shorthouse et al., 2005). Stem galls are initiated from eggs deposited along the sides of apical meristems (Shorthouse et al., 2005). Hip galls are initiated within the developing fruit (hips) (Pujade-Villar and Plantard, 2002). A combination of multiple states was used for the galls found on multiple organs (e.g. *D. rosae* coded as A + B, as the galls are found on both leaves and stems). Joint stochastic character mapping (Huelsenbeck et al., 2003) was performed using the R package phytools v.0.7.2 function "make.simmap" (Revell, 2012). An ordered transient model was determined as the best fit model using "fitPolyMk" function and the Akaike weights (ER, SYM, and ARD models were also tested), and implemented with 1000 stochastic character maps that were summarized to produce posterior probabilities for each node.

The ancestral range was reconstructed based on current geographic regions (Palearctic and Nearctic). The DEC model (Ree and Sanmartín, 2018) was considered the best model using Akaike weights through BioGeoBEARS v1.1.1 (Matzke, 2013) and visualized in RASP v4.0 (Yu et al., 2020). Additional models tested included DIVALIKE and BAYAREALIKE, but given the controversy regarding the "+J" jump dispersal model (Matzke, 2014; Ree and Sanmartín, 2018), we did not implement this parameter in our search.

3. Results

3.1. Phylogenomic analysis

After filtering out samples with low numbers of contigs captured (< 200), the number of samples used in the downstream analyses was reduced to 36 specimens representing 21 species of *Diplolepis*. The matrix consisted of 316334 bp, with 29.4% missing data, 85,737 variable sites (27.1%) and 32,814 parsimony informative sites (10.4%). All species were confirmed to be correctly identified using the *COI* barcode slices in comparison with full *COI* sequences generated by Zhang et al. (2019a).

Both ML (Figs. 1 and S1) analyses and MSC (Fig. 2) resulted in nearly identical topology, the only difference being *D. nebulosa* (YMZ074) is recovered as sister to *D. variabilis* (YMZ089 and YMZ129) in ML analyses, while in under the MSC framework YMZ089 is recovered as the sister to the other two. In all analyses, *Diplolepis* was recovered as a monophyletic group, and the genus is further separated into the Nearctic stem-galler clade [*D. californica* (Beutenmüller), *D. nodulosa* (Beutenmüller), *D. oregonensis* (Beutenmüller), *D. spinosa* (Ashmead), and *D. triforma* Shorthouse & Ritchie] and the Holarctic clade that consists of both leaf and hip gallers. This Holarctic clade is further divided into 3 subclades, comprising the Palearctic leaf 1 [*D. fructuum* (Rübsaamen), *D. mayri* (Schlechtendal), *D. rosae*, and *D. spinosissimae* (Giraud)]; Palearctic leaf 2 clades [*D. eglanteriae* (Hartig), *D. japonica* (Walker), and *D. nervosa*]; and the Nearctic leaf gallers. The Nearctic leaf gallers are further divided into the *gracilis* complex (*D. gracilis* (Ashmead), *D. ignota* (Osten Sacken), *D. nebulosa* (Bassett), and *D. variabilis* (Bassett)]; the *fusiformans* complex [*D. fusiformans* (Ashmead) and *D. rosaefolii* (Cockerell)]; and the *bassetti* complex [*D. bassetti* (Beutenmüller), *D. bicolor* (Harris), and *D. polita* (Ashmead)]. All clades were strongly supported by UFBoot2, SH-aLRT, and LPP, albeit some gene and site discordance is observed (Fig. 2). Most of the *Diplolepis* species with more than one sample were recovered as monophyletic, except for *D. polita* in both analyses (Fig. 1) and *D. variabilis* in the MSC tree (Fig. 2).

3.2. Ancestral state reconstruction

Based on the ordered transient model, the ancestral host organ used by *Diplolepis* was likely leaves (Figs. 3 and S2). The split between stem and leaf gallers largely follows the division between Nearctic stem gallers and the Holarctic leaf gallers. Both clades are conservative in shifting to other organs, although the Holarctic leaf-galler clade showed more transitions, comprising shifts to rose hips (*D. oregonensis*, *D. fructuum*), and reversal from leaf to stem galling in *D. fusiformans*. The ancestral range, based on the DEC model analysis, was inconclusive (shown as Holarctic) for *Diplolepis* (node 42) and Diplolepidini (node 43) (Fig. 4). The Nearctic species had two independent origins, one from the Nearctic stem-galler clade (node 41), and a secondary shift from the Holarctic leaf gallers to the Nearctic leaf gallers (node 30).

4. Discussion

4.1. Biogeography and host organ shift

Based on our data, the biogeographic history of *Diplolepis* is more nuanced than previously thought, which is not surprising given the complicated history behind the distribution of their host plants. We suspect that characteristics of wild roses, such as the fact that they are early successional species with adaptations for dispersal, occurrence of multiple growing points in the form of new meristems and leaf buds, long-lived, high clonability, and the abundance of immature leaves throughout the growing season, provide ample opportunities for *Diplolepis* to oviposit. Recent studies suggest that *Rosa* diverged from its sister group Potentilleae around 50–60 million years ago (Chen et al., 2020; Xiang et al., 2017), with the genus being 10–30 million years old (Chen et al., 2020; Fougere-Danezan et al., 2015). The genus *Rosa* likely originated in Asia or North America, with most extant American species being the result of re-colonization from Asia through the Bering Land Bridge (Chen et al., 2020; Fougere-Danezan et al., 2015). All known Nearctic *Diplolepis* species are found on species of *Rosa* in the Section *Cinnamomeae,* which arrived in North America around 16 million years ago (Fougere-Danezan et al., 2015). The only published Diplolepidini fossil, *Diplolepis vetus* (Cockerell), is a compression fossil of an isolated

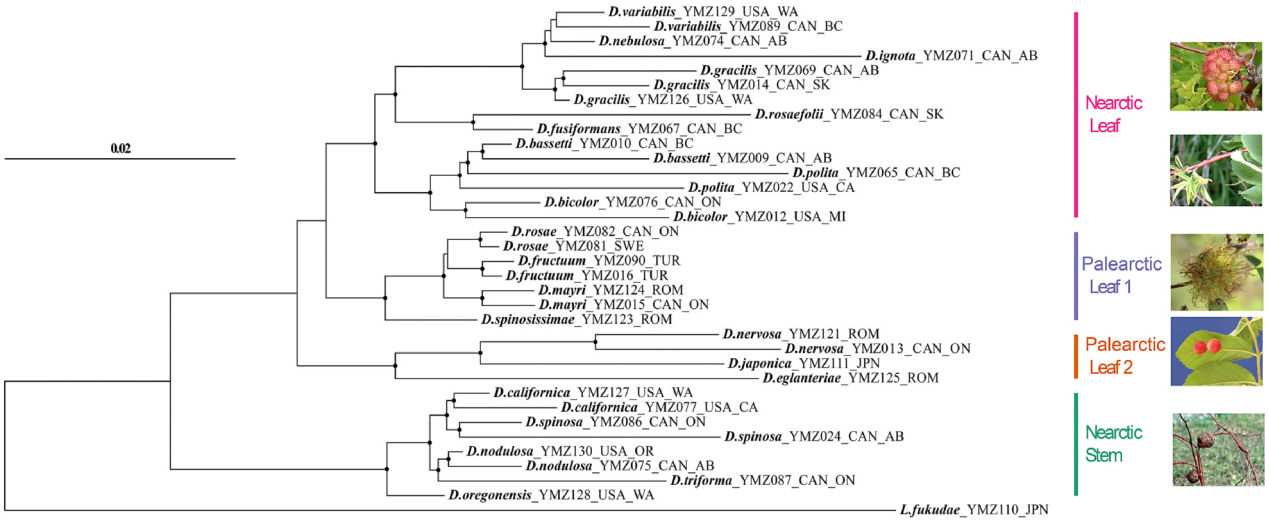

Fig. 1. Maximum-likelihood phylogeny of *Diplolepis*, based on the 75% complete SWSC partitioned UCE matrix. Node support is provided in ≥ 80% SH-aLRT and ≥ 95% UFBoot values. Photos of representative galls from top to bottom: *D. polita* (JDS), *D. fusiformans* (CL), *D. rosae* (LZ), *D. japonica* (TI), and *D. spinosa/D. triforma* (JDS).

forewing from the UK dating to approximately 37.2–33.9 million years ago, which cannot be confidently placed within the genus (Antropov et al., 2014; Pujade-Villar and Peñalver, 2019). In this study we were unable to perform divergence dating and historical biogeographic analyses for *Diplolepis* because of the absence of fossil records and the poor resolution of Cynipidae phylogenetic relationships (Liljeblad and Ronquist, 1998; Ronquist et al., 2015).

Ancestral state reconstruction revealed that *Diplolepis* is relatively conservative when it comes to shifts in host organs (Fig. 4), in contrast to the evolution of oak gallers in which shifts to different organs on the same species of host plant are common. This was unexpected since the galls of cynipids on both roses and oaks house similar natural enemies, which are thought to be the primary driver for gall structures and anatomy, as well as host shifts in gall wasps (Bailey et al., 2009; Cook et al., 2002; Stone and Cook, 1998). Both the rose and oak gallers have similar hymenopteran parasitoid communities mostly consisting of seven chalcidoid families: Eulophidae, Eupelmidae, Eurytomidae, Ormyridae, Megastigmidae, Torymidae, and Pteromalidae (Askew et al., 2006; Bailey et al., 2009; Csóka et al., 2005; László and Prázsmári, 2019; Mete and Lotfalizadeh, 2019; Shorthouse, 2010; Stone et al., 2002; Zhang et al., 2014, 2017). The host plant shift, or host plant organ shift is thought to be an evolutionary strategy to escape from these natural enemies, which in turn has led to the speciation and diversification of the gall wasps and their natural enemies (Bailey et al., 2009; Csóka et al., 2005; Hayward and Stone, 2005). Future comparative studies between the rose gall natural enemies with that of their oak gall wasp counterparts could also help in resolving this disparity between rates of gall location shifts in the two tribes.

The diversity of *Diplolepis* has been impacted by the variety of sites in which galls can be initiated and the larvae can gain control of organ morphogenesis, and is evolutionarily constrained by gall induction location. All members of the Nearctic stem-galler clade initiate integral (non-detachable) or detachable galls on the stems or adventitious shoots of their host plant by oviposition into the leaf primordia of the apical meristem (Shorthouse et al., 2005). By contrast, the Holarctic leaf-galler clade includes members that mostly induce galls on the rose leaves by ovipositing onto leaflets folded within leaf buds (Shorthouse et al., 2005). A notable exception is *D. fusiformans*, which differs from other rose stem gallers by inducing small, integral galls on current year stems (Shorthouse et al., 2005). The sister taxon of *D. fusiformans* is *D. rosaefolii*, which along with *D. lens* Weld, are the smallest *Diplolepis*. However, given the morphological similarities in the adults (YMZ/CL/JDS, pers. obsv.), it is possible that all three species are either conspecifics and are capable of inducing galls on multiple tissues, or they are recently diverged lineages along the species continuum (Zhang et al., 2019a). The Holarctic leaf-galler clade also includes *D. oregonensis* and *D. fructuum*, two species that induce galls within rose hips. The process of *D. oregonensis* gall induction is unknown; however, as this species can also induce galls on leaf buds, the process is likely different from that by which *D. fructuum* induces galls from several types of tissues within flowers and hips (walls of hips and ovaries) inside the fruit (Güçlü et al., 2008; Pujade-Villar and Plantard, 2002).

The diversity and taxonomy of *Diplolepis* galls is woefully undersampled and understudied in the eastern Palearctic region, and these omissions could potentially bias the results of our ancestral state reconstruction. Many of the recently described Chinese *Diplolepis* species, except *D. abei* (Pujade-Villar et al., 2020), were collected from Malaise traps and their galls remain unknown (Wang et al., 2013). Some *Diplolepis* species described from central Asia have not been studied since their original publication (Vyrzhikovskaja, 1963). The eastern Palearctic is also where the majority of *Liebelia*, the sister taxa of *Diplolepis* are found (Belizin, 1957; Vyrzhikovskaja, 1963). Sampling of both genera in this region is critical to understanding the diversity of Diplolepidini.

Due to the lack of eastern Palearctic species, we were not able to conclusively determine the biogeographic origin of *Diplolepis*. Nevertheless, we were able to identify at least two independent dispersal events for the Nearctic leaf and stem clades. We suggest that *Diplolepis* had a recursive history, in which North American lineages dispersed back to Asia. Other authors have suggested that the Diplolepidini are an early offshoot within the evolution of Cynipidae (Ronquist et al., 2015), and perhaps, even within Cynipoidea (Blaimer *et al.*, in prep., MLB, pers. obsv.). This placement would suggest the *Rosa* connection of Diplolepidini represents a period of early evolutionary experimentation in choice of host plants (which also includes Fagaceae and Asteraceae in other cynipid lineages). We suggest Diplolepidini originated in the eastern Palearctic, as the *Liebelia* sister taxon of *Diplolepis* is almost exclusively found here (one western Palearctic species, *Liebelia cavarae* Kieffer, is known from Sardinia, Italy) (Abe et al., 2007). Other studies have also hypothesized this region to be the origin of Cynipini (Abe et al., 2007), and Cynipidae as a whole (Ronquist and Liljeblad, 2001; Ronquist et al., 2015). We suspect that more gall sampling in this region will likely reveal many new species that will be valuable in testing our hypothesis.

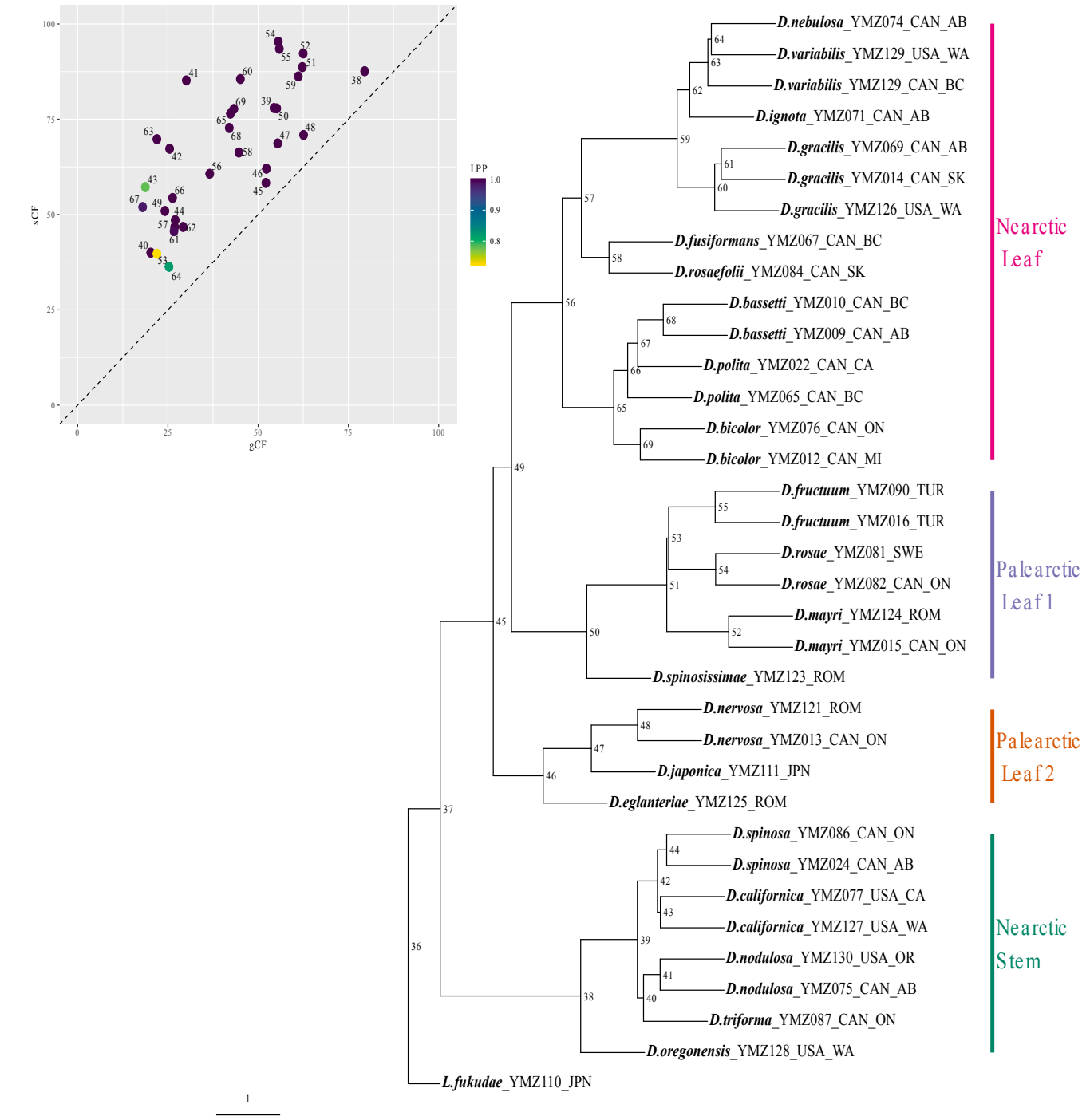

Fig. 2. Multispecies coalescence phylogeny of *Diplolepis*, based on the 75% complete UCE matrix. Node numbers are shown in phylogeny. The inset figure shows the gene and site concordance factors in relation to local posterior probabilities at each node.

4.2. Phylogeny and taxonomy of Diplolepis

Our UCE phylogenomic tree generated from 757 genes in this study is nearly identical to the topology of previous work using only *COI* (Zhang et al., 2019a). This is surprising, given the high levels of mitonuclear discordance observed in the Cynipini oak gallers (Nicholls et al., 2012; Rokas et al., 2003). A major difference between Cynipini and Diplolepidini is that the former exhibits alternation between a morphologically distinct (in adult and gall morphology) spring sexual and a fall asexual generation (Hearn et al., 2019). The alternation of generations within Cynipini could help retain ancestral polymorphism due to the larger population sizes. However, it could also be argued with two generations each year, all else being equal, Cynipini should sort ancestral polymorphism at twice the rate of univoltine lineages. By contrast, *Diplolepis* are strictly univoltine, and while many *Diplolepis* species have few to no males as result of *Wolbachia* infection and reproduce via parthenogenesis (Field et al., 1999; Plantard et al., 1998a; Schilthuizen and Stouthamer, 1998), no alternations between sexual and asexual reproduction occurs within this group. The cause of mitonuclear difference within Cynipini is likely the result of hybridization followed by preferential backcrossing to one of the parents. This is suggested by the fact that co-distributed Cynipini share mitochondrial *COI* barcodes that are diagnostic of refugia, rather than of species (Nicholls et al., 2012), but comparative molecular studies on other

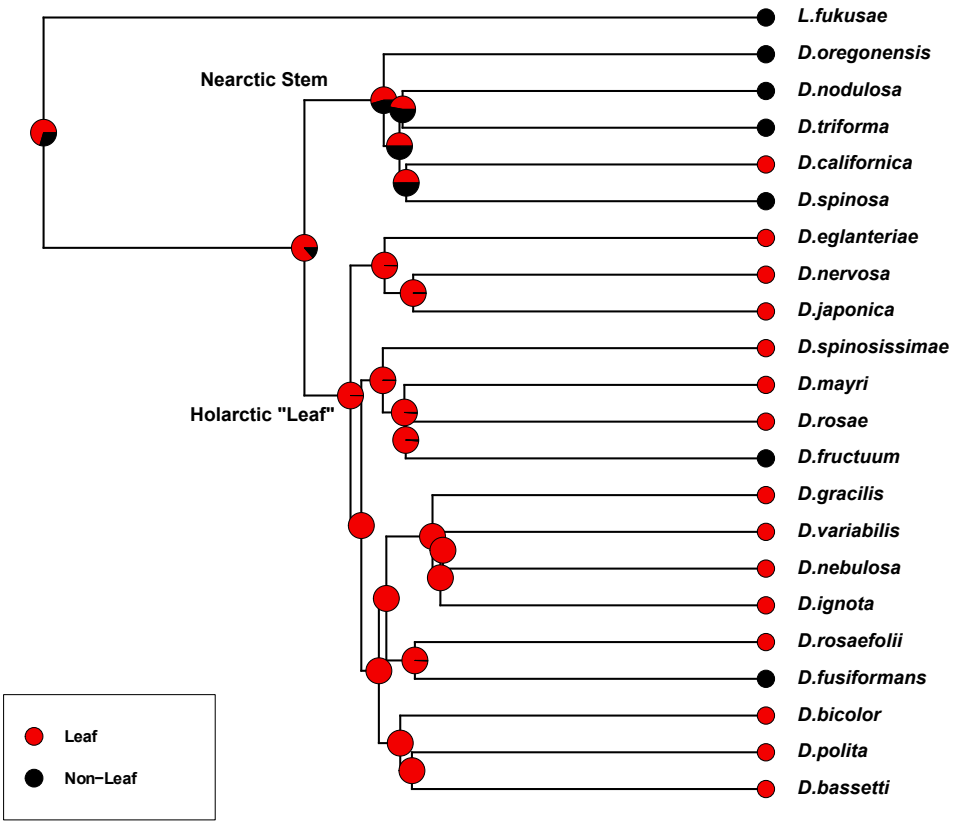

Fig. 3. Ancestral state reconstruction of the plant organs attacked by *Diplolepis*, using an unordered transient model. Leaf includes petiole and bud gall, while non-leaf includes stem and hip galls.

larger cynipid tribes without alternation of generations such as Diastrophini are needed to determine whether or not the differences in life history strategy is the cause of the discordance. Another potential group of interest is Synergini, where at least one European species, *Synergus umbraculus* (Olivier), does not show significant mitonuclear discordance (Stone et al., 2017). However, studies of additional co-distributed species would be needed to see if Synergini show the same issues as European Cynipini. Congruence between UCE and *COI* data within *Diplolepis* is encouraging as there are no signs of mitonuclear discordance that would confound the resulting phylogeny. A taxonomic revision of this group can likely generate large scale *COI* or nuclear data at lower cost for accurate species delimitation if UCE data cannot be obtained easily.

Previous studies of *Diplolepis* have grouped the Nearctic stem gallers together based on a distinctive morphological character: the synapomorphy of a flange on the hind femora (Plantard et al., 1998b; Zhang et al., 2019a). However, careful examination of additional specimens suggests that this character might be restricted to certain members of the clade (Zhang, unpublished data). Additionally, the UCE data confirmed the taxonomic uncertainties in a few species. In the Holarctic leaf-gallers clade, *D. polita*, for example, was not recovered as reciprocally monophyletic with respect to *D. bassetti*. *Diplolepis polita* is a widespread species occurring across Canada and the USA, while *D. bassetti* is restricted to western North America (Shorthouse, 2010). Both species produce spherical single-chambered galls (often densely clustered), but those of *D. polita* are covered with weak spines while those of *D. bassetti* are covered with long mossy tendrils. Given that the specimens of *D. polita* collected across its known range exhibit subtle morphological differences (for example in coloration, JDS, pers. obsv.), the presence of cryptic species is possible and warrants further investigation. Additionally, *Rosa acicularis* Lindley is a Holarctic species that ranges across Canada, Northern USA, Japan, Russia, Mongolia, and Sweden, so it is possible that Nearctic species like *D. polita* can also occur in these relatively under-sampled Palearctic regions such as far eastern Russia and Mongolia. In addition, *D. ignota*, *D. nebulosa*, and *D. variabilis* are suspected to be conspecifics based on small morphological (Beutenmüller, 1907) and molecular differences (Zhang et al., 2019a). The galls of all three species are spherical, and are found on the underside of leaves (Shorthouse, 2010), and the purported subtle differences in gall morphology could be the result of phenotypic plasticity.

4.3. Optimize UCE capture success in Cynipidae

Previous studies have demonstrated that UCE targeted enrichment protocols can be used on older pinned and ethanol preserved museum specimens (Blaimer et al., 2016; Derkarabetian et al., 2019). The result was somewhat variable within our samples despite using a destructive DNA extraction protocol to maximize DNA yield. Many older samples of *Diplolepis* used in this study were collected by JDS who preserved them in 70% ethanol at room temperature and often with many individuals per vial. Although the common means of preservation at the time, the low ethanol concentration did not preserve genomic DNA. Including data for samples with high number of missing loci can result in long branch lengths (YMZ065, YMZ071, YMZ084), and while this does not seem to affect taxonomic relationships, these long branches can confound downstream analyses such as divergence timing, historical biogeography, ancestral state reconstruction, and diversification rates. We advise caution in future studies of gall wasps and other smaller organisms, and researchers should prioritize newer specimens to ensure high loci capture success. Additionally, the highest number of loci captured was 1752 out of the 2590 possible loci from the Hym-v2 probe sets, a mere 68% success rate despite high DNA input quality. This was not surprising given that the probe set was originally designed without an exemplar from Cynipoidea (Branstetter et al., 2017), and has been shown to have lower capture success for Parasitica (Bossert and Danforth, 2018). Given the tremendous potential of UCE

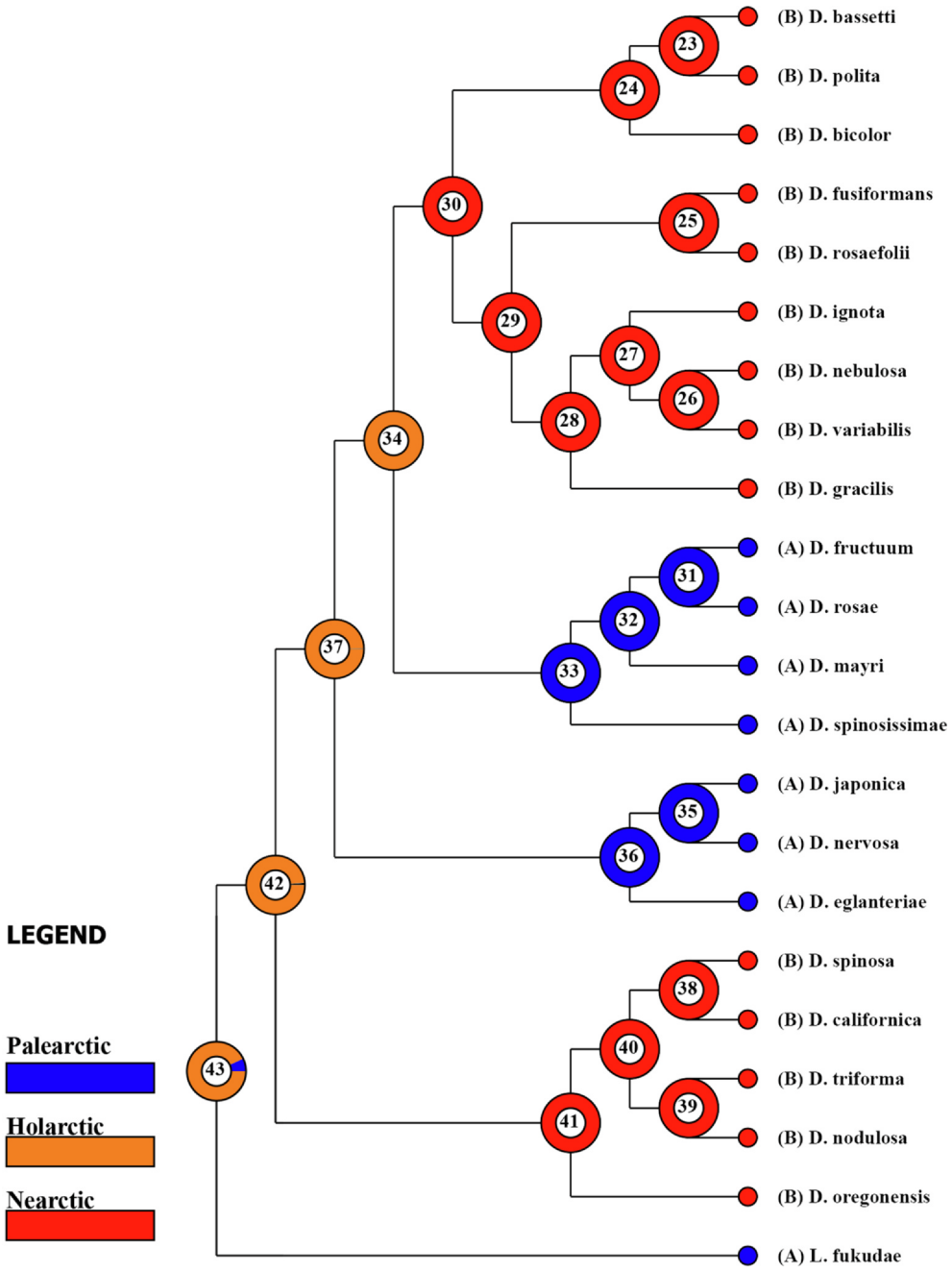

Fig. 4. Historical biogeographic reconstruction of *Diplolepis* distribution pattern, using the DEC model. Number inside circle indicates node number, and letter in brackets at the tip label represents current distribution (A = Palearctic, B = Nearctic).

phylogenomics across this hyper-diverse and understudied group of insects, there is a vital need for a modified probe set designed using additional genomes from Parasitica (many superfamilies within Proctotrupomorpha currently do not have published genomes) to increase capture success.

4.4. Conclusions

This phylogenomic study of the genus *Diplolepis* suggests the ancestral host organ of rose gall wasps was leaves, but many issues remain unresolved, such as ancestral biogeography, co-evolutionary relationship with roses, and the impacts of natural enemies on selection for gall structures that protect inducer larvae. Our study provides a robust phylogenetic framework to test such hypotheses in the future, and highlights the need for a thorough taxonomic revision of the genus *Diplolepis*, especially those species found in the eastern Palearctic region. Ideally, an integrative approach that combines historical sampling locations, molecular data, and morphological study of extant and fossil specimens from museum collections to adequately address the tritrophic evolutionary complexity of roses, *Diplolepis* species, and their natural enemies.

CRediT authorship contribution statement

Y. Miles Zhang: Conceptualization, Data Curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft. **Matthew L. Buffington:** Data curation, Investigation, Resources, Supervision, Validation, Writing - review & editing. **Chris Looney:** Conceptualization, Investigation, Methodology, Resources, Validation,

Data curation, Writing - review & editing. **Zoltán László:** Conceptualization, Investigation, Methodology, Resources, Validation, Data curation, Writing - review & editing. **Joseph D. Shorthouse:** Investigation, Resources, Validation, Data curation, Writing - review & editing. **Tatsuya Ide:** Data curation, Resources, Investigation, Validation, Writing - review & editing. **Andrea Lucky:** Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106949.

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