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Phylogenomic insights and geographic distribution of the New World genus *Amphibolips* Reinhard (Hymenoptera: Cynipidae, Cynipini) using ultraconserved elements

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Abstract

In recent years, new wasp species and genera of Cynipidae have been described, and their species delimitation and evolutionary relationships have been supported using molecular markers. However, few studies have included comprehensive and extensive sampling of specimens across the complete distribution of a single genus. In this study, we analysed the phylogenetic relationships of the genus *Amphibolips* throughout its range using ultraconserved elements (UCEs). We collected 520 adult wasps from 401 sites in various geographic locations, predominantly in Mexico, and used 78 wasps for UCE analysis. Our results demonstrate that *Amphibolips* forms a monophyletic clade. *Amphibolips* can be further subdivided into four genetically well-differentiated clades. Each clade had morphological traits that could be collectively characterised. Species delimitation using molecular sequencing highlights the taxonomic complexity involved in separating and assigning species using exclusively morphological criteria. This approach offers an opportunity to refine current morphological taxonomic criteria or propose new criteria to establish more accurate classifications. The rich diversity of host species and geographic regions in Mexico represents a biodiversity hotspot for *Amphibolips*. The number of undescribed species remains high. This study facilitates the determination of appropriate boundaries for putative new *Amphibolips* species.

KEYWORDS
Amphibolips, Cynipidae, New World, phylogenomics, ultraconserved elements

INTRODUCTION

Taxonomic identification based on the morphological characteristics of species in highly diverse groups of organisms does not allow the recognition of complex species differentiation processes, such as in the case of cryptic species (Fišer et al., 2018; Padial et al., 2010). Speciation studies have therefore greatly benefited from next-generation sequencing (NGS) techniques (Coates et al., 2018). In recent years, DNA enrichment of ultraconserved elements (UCEs) has been extensively used for discovering evolutionary or population-level processes due to the inclusion of highly conserved regions (95% to 100%) of genomes shared among taxa (Bejerano et al., 2004; Faircloth et al., 2012; Stephen et al., 2008; Woolfe et al., 2005) and the feasibility of obtaining DNA fragments from fresh and degraded material, facilitating the inclusion of taxa with many loci (Faircloth et al., 2012). UCEs also offer the possibility of recovering sequences of loci used in studies prior to the use of NGS techniques (usually Sanger), allowing the inclusion of species available in public genetic libraries, such as GenBank (Branstetter & Longino, 2019). Recent developments in Cynipoidea phylogeny, particularly within Cynipidae, have tested the utility of the UCE markers to improve our understanding of the natural classification of complex groups of species (Blaimer et al., 2020; Hearn et al., 2024; Ward et al., 2022; Zhang et al., 2020). These advances have improved the understanding of the processes associated with speciation events, particularly in the relationship between host species within each clade.

The family Cynipidae includes more than 1000 species that have had a high diversification rate in close association with their host plants (Buffington et al., 2020; Liljeblad et al., 2008; Melika & Abrahamson, 2002; Melika, Nicholls, et al., 2021; Melika, Pujade-Villar, et al., 2021; Nieves-Aldrey, 2001; Stone et al., 2002; Ward et al., 2022). Most species of the family Cynipidae, particularly Cynipini, induce galls with a great complexity (Hernández-Soto et al., 2015; Kariñho-Betancourt et al., 2019, 2020). Studies on particular genera within Cynipini across their entire distribution are scarce, which limits our understanding of the mechanisms driving species diversification (Cook et al., 2002; Cooke, 2018; Driscoe et al., 2019; Nicholls et al., 2017; Ward et al., 2022; Zhang et al., 2021).

Taxonomic studies have advanced the classification within the tribe Cynipini, including descriptions of new genera and species and the identification of synonymous species based on morphological traits (Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2020; Melika, Nicholls, et al., 2021; Melika, Pujade-Villar, et al., 2021; Nicholls et al., 2018; Nieves-Aldrey et al., 2012; Nieves-Aldrey et al., 2021; Zhang et al., 2021). These efforts require the use of modern tools to clarify some of the remaining taxonomic controversies among closely related groups of wasps. Molecular data allow us to recognise greater cryptic species diversity, particularly within morphologically complex genera that have been considered paraphyletic or polyphyletic (Cooke, 2018; Nicholls et al., 2017). To explore these species complexes, we studied the genus *Amphibolips* Reinhard (Cynipini), which is restricted to the American continent and is associated with a specific section of oak species (Fagaceae: *Quercus* L., Section *Lobatae* Loudon). Mexico represents one of the main centres of diversification of oaks in the world, with more than 160 oak species, including 109 endemics (Valencia-A, 2004). Most oak species in the *Lobatae* section have diversified in Mexico, with 76 species of which 61 are endemic (Valencia-A, 2004). Cynipid gall wasps showed a great diversity associated to Mexican oaks (Pascual-Alvarado et al., 2017). In particular, *Amphibolips* induces some of the most charismatic and conspicuous galls, ranging from hollow galls with a central larval chamber suspended by radiating fibres, to juicy and extremely sour gall tissues that are thought to deter potential predators (Guiguet et al., 2023). Significant progress has been made in the description and species delimitation of the species within this genus over the past 15 years (Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2020, 2021, 2023; Medianero & Nieves-Aldrey, 2010; Melika et al., 2011; Nicholls et al., 2022; Nieves-Aldrey et al., 2012; Pujade-Villar et al., 2018). However, discrepancies have arisen among authors regarding species boundaries, with differing emphases on which morphological characters should (or should not) be used to delineate species. This has led to the proposal of synonyms or new species that may be controversial due to the different criteria on morphological traits used by taxonomic authorities or the limited number of individuals examined.

We excluded species from the *'niger'* complex, as their relationship with other *Amphibolips* species remains uncertain. While Cuesta-Porta et al. (2023) placed a species from each complex in a basal clade, this result should be interpreted with caution due to the limited taxon sampling. Some morphological characteristics of the galls and adults in the *'niger'* complex have led to the possibility that they may belong to a different genus (Cuesta-Porta et al., 2020, 2023; Nieves-Aldrey et al., 2012). We consider that it is necessary to include a larger number of species from the *'niger'* complex to determine whether they belong to the genus *Amphibolips* or represent a sister genus.

Fifty-nine species of *Amphibolips* have been described in North and Central America, ranging from Canada to Panama. Most of these species were found in the Nearctic region, but recent descriptions of new species showed that the greatest diversity occurs in Mexico, hosting both Nearctic and Neotropical species, with many species in the transition zones between these two biogeographic regions (Martínez-Romero et al., 2022). Among the Mexican species of *Amphibolips*, 27 are endemic to Mexico, one is shared with Panama and two are shared with the United States (Burks, 1979; Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2020, 2021, 2023; Medianero & Nieves-Aldrey, 2010; Melika & Abrahamson, 2002; Melika et al., 2011; Nieves-Aldrey et al., 2012; Pujade-Villar et al., 2018).

Before 1937, only two species of *Amphibolips* were described in Mexico (*A. palmeri* Bassett and *A. niger* Beutenmüller) (Bassett, 1890; Beutenmüller, 1911, 1917). In 1937, Kinsey described nine species, six of which were grouped in the *'niger'* complex; the remaining three (*A. dampfi* Kinsey, *A. nassa* Kinsey and *A. fusus* Kinsey) were not grouped in any complex. Melika et al. (2011) described two new species, *A. zacatecaensis* Melika and Pujade-Villar and *A. hidalgoensis* Pujade-Villar and Melika. Parallel to the *'niger'* complex proposed by Kinsey (1937), a second group named the *'nassa'* complex was proposed that included six species, *A. palmeri*, *A. dampfi*, *A. nassa*,

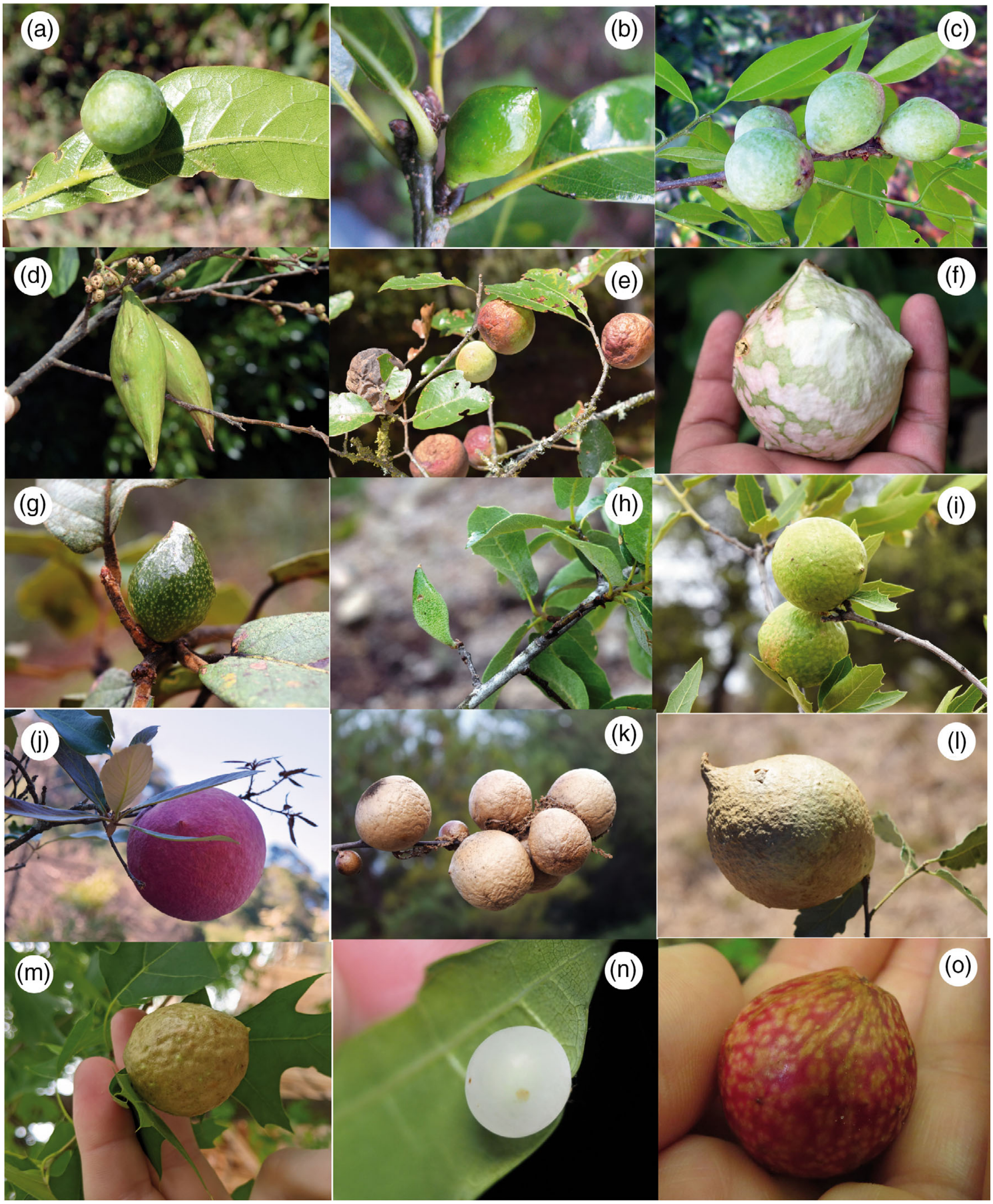

FIGURE 1 Galls of *Amphibolips* species. (a) *A. salicifoliae* Medianero and Nieves-Aldrey. (b) *A. aliciae* Medianero and Nieves-Aldrey. (c) *A. castroviejoi* Medianero and Nieves-Aldrey. (d) *A. magnigalla* Nieves-Aldrey and Castillejos-Lemus. (e) *A. kinseyi* Nieves-Aldrey and Castillejos-Lemus. (f) *A.* sp. nov. Oaxaca. (g) *A. durangensis* Nieves-Aldrey and Maldonado. (h) *A. fusus* Kinsey. (i) *A.* nr *trizonata* Ashmead. (j) *A.* sp. nov. Jalisco. (k) *A. hidalgoensis* Pujade-Villar and Melika. (l) *A. zacatecaensis* Melika and Pujade-Villar. (m) *A. confluenta* Harris (courtesy of Adam Kranz). (n) *A. nubilipennis* Harris (courtesy of Adam Kranz). (o) *A. quercusjuglans* Osten Sacken (courtesy of Adam Kranz).

A. hidalgoensis, *A. zacatecaensis* and *A. fusus*. Nieves-Aldrey et al. (2012) described seven new species outside of those in the '*niger*' group, raising the number of known species to 13. However, it is noteworthy that the '*nassa*' complex, as described by Nieves-Aldrey et al. (2012), did not adequately include the extant species diversity outside of the '*niger*' group, as the description omitted the anterior wing coloration pattern, which was important for some of the species described by Kinsey, such as *A. dampfi*, *A. fusus* and *A. nassa* (Nieves-Aldrey et al., 2012). Pujade-Villar et al. (2018) described *A. cibriani* Pujade-Villar as a new species within the '*nassa*' group. In 2020, two papers described eight new species of wasps of the '*nassa*' complex (Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2020, 2021):

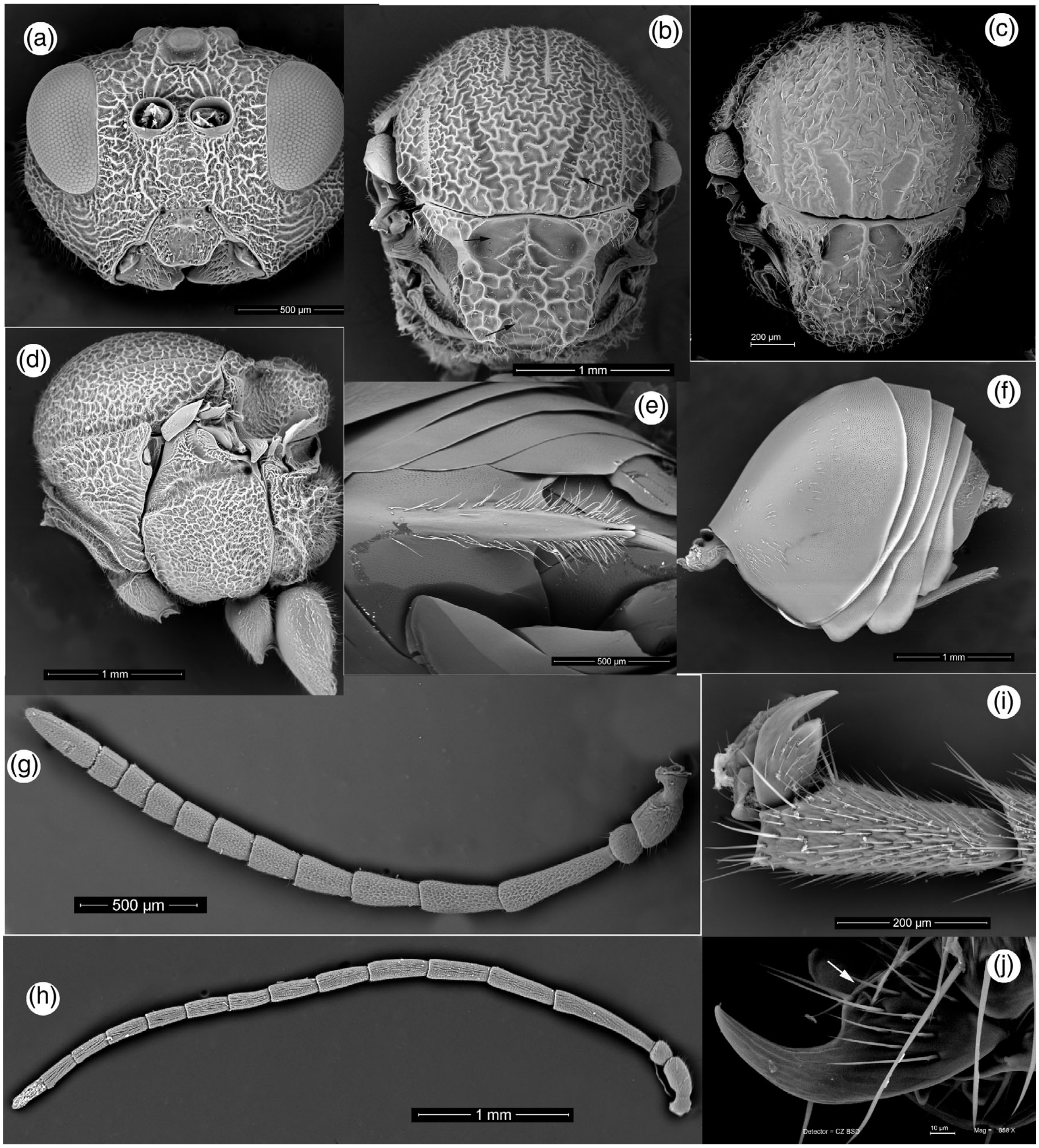

FIGURE 2 Diagnostic morphological characters of *Amphibolips* species; SEM photographs. (a) Head anterior view of *Amphibolips* nr *trizonata* Ashmead. (b) Mesosoma dorsal view of *A. castroviejoi* Medianero and Nieves-Aldrey. (c) Mesosoma dorsal view of *A. salicifoliae* Medianero and Nieves-Aldrey. (d) Mesosoma lateral view of *A.* nr *trizonata*. (e) Hypopygium ventral view of *A. castroviejoi*. (f) Metasoma lateral view of *A.* nr *trizonata*. (g) Female antenna and (h) male antenna of *A.* nr *trizonata*. (i) Metatarsal claw of *A.* nr *trizonata* and (j) of *A. salicifoliae*.

A. magnigalla Nieves-Aldrey and Castillejos-Lemus, *A. nigrialatus* Nieves-Aldrey and Castillejos-Lemus, *A. kinseyi* Nieves-Aldrey and Castillejos-Lemus, *A. bassae* Cuesta-Porta and Pujade-Villar, *A. bromus* Pujade-Villar and Cuesta-Porta, *A. rulli* Pujade-Villar and Cuesta-Porta, *A. turulli* Pujade-Villar and Cuesta-Porta and *A. comini* Cuesta-Porta and Pujade-Villar. In 2023, the most recent species, *A. ufo* Cuesta-Porta and Pujade-Villar, which belongs to the *'niger'* complex was described (Cuesta-Porta et al., 2023). Cuesta-Porta et al. (2020) delimited the species of the *'nassa'* complex more adequately with new characters but these were still restricted to species from Mexico and Panama. The study also synonymised *A. malinche* Nieves-Aldrey and Pascual with *A. hidalgoensis* due to their similarity (Cuesta-Porta et al., 2020).

Here, we analysed *Amphibolips* wasps outside the *'niger'* complex (sensu Kinsey, 1937). These wasps induce galls in buds, stems or leaves and rarely in acorns. They are usually globose or spindle-shaped and detachable (Figure 1), with a spongy parenchyma surrounding a central larval cell, occasionally supported by radiating filaments (Beutenmüller, 1909; Kinsey, 1937; Melika & Abrahamson, 2002). The species outside of the *'niger'* complex can be

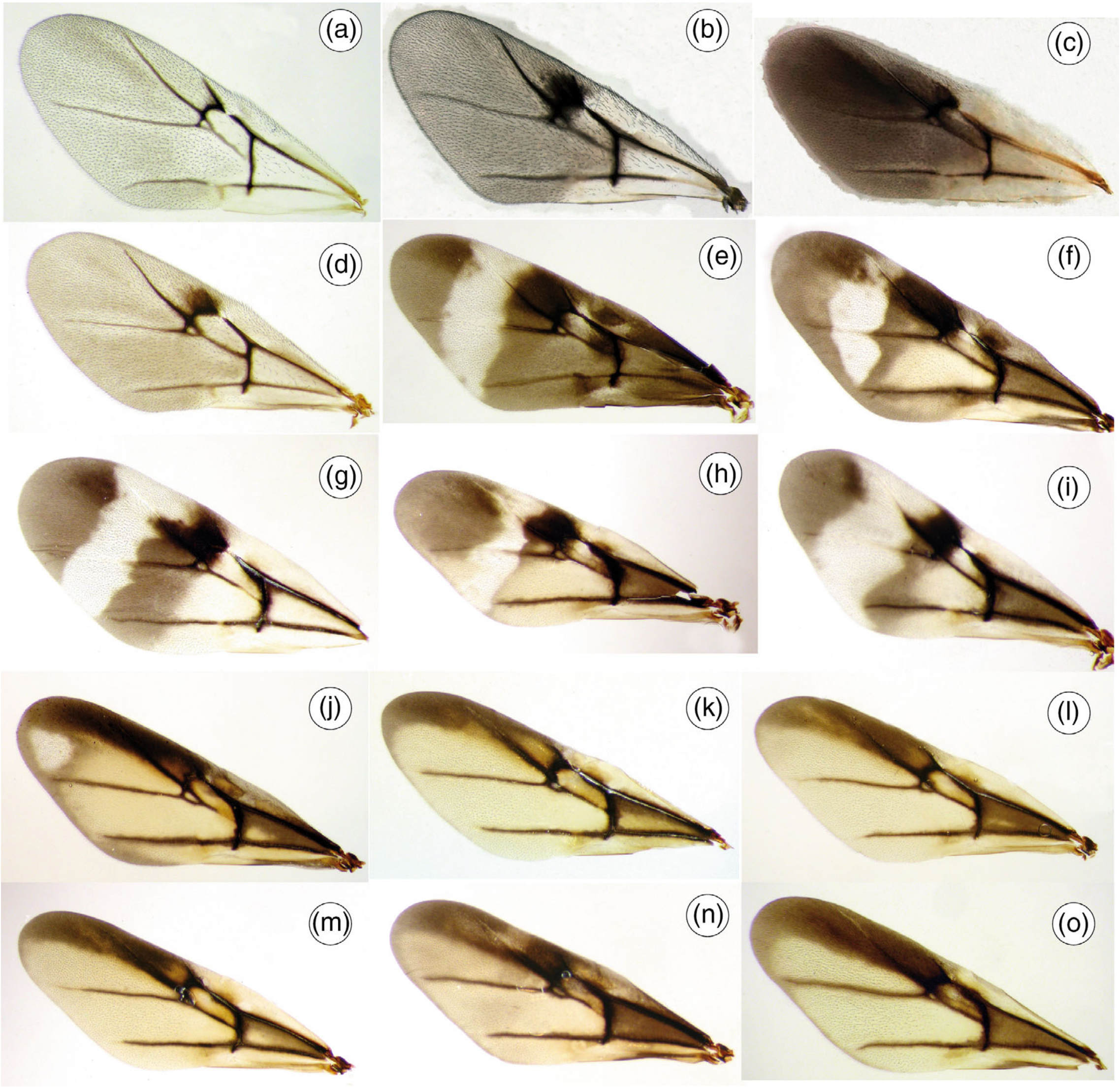

FIGURE 3 Forewings of female *Amphibolips* species. (a) *A. salicifoliae* Medianero and Nieves-Aldrey. (b) *A. confluenta* Harris (modified from Ward et al., 2022). (c) *A. nubilipennis* Harris (modified from Ward et al., 2022). (d) *A. aliciae* Medianero and Nieves-Aldrey. (e) *A. castroviejoi* Medianero and Nieves-Aldrey. (f) *A. kinseyi* Nieves-Aldrey and Castillejos-Lemus. (g) *A. fusus* Kinsey. (h) *A. durangensis* Nieves-Aldrey and Maldonado. (i) *A.* nr *trizonata* Ashmead. (j) *A.* sp. nov. Oaxaca. (k) *A. jaliscensis* Nieves-Aldrey and Pascual. (l) *A. hidalgoensis* Pujade-Villar and Melika. (m) *A. michoacaensis* Nieves-Aldrey and Maldonado. (n) *A.* sp. nov. Jalisco. (o) *A. zacatecaensis* Melika and Pujade-Villar.

easily recognised by the following diagnostic characters (Figure 2): antenna with 12 to 14 segments in females and 15 to 16 segments in males; robust body with a strong coarse reticulate sculpture, notauli not well marked, mesoscutellum often emarginate posteriorly; metasomal tergites punctate posteriorly; metatarsal claws with a large secondary basal tooth; forewings usually more or less smoked with spots, bands or completely obscured (Figure 3); radial cell open; ventral spine of the hypopygium usually long and pointed apically, and without setae forming an apical tuft (Medianero & Nieves-Aldrey, 2010; Melika & Abrahamson, 2002; Melika et al., 2011). In addition to these common characters between species outside the '*niger*' complex, specific characters can differentiate the individual species. For example, one species is multilocular (*A. quercuspomiformis* (Bassett)), and some species lack basal lobes on the metatarsal claws (*A. quercuspomiformis* and *A. salicifoliae* Medianero and Nieves-Aldrey; Figure 2j). Another interesting aspect of the '*nassa*' complex (sensu Melika et al., 2011) is that some species have widely separated geographic distributions (Cuesta-Porta et al., 2020). For example, *A. aliciae* Medianero and Nieves-Aldrey is distributed in Panama and Puebla (Mexico), and *A. quercuspomiformis* occurs in California (USA) and Guanajuato (Mexico) in different host species and climates.

Cynipid taxonomy and systematics are mainly based on morphology (Liljeblad et al., 2008; Lobato-Vila & Pujade-Villar, 2021; Pujade-Villar et al., 2013; Ronquist, 1999), but some recent studies have used molecular data to support taxon delimitation (Fang et al., 2020; Medianero et al., 2021; Nieves-Aldrey et al., 2021; Ward et al., 2020, 2024; Zhang et al., 2021; Zhang, László, et al., 2019). In particular, within *Amphibolips*, evidence of morphological variability has been reported for some species

(Cuesta-Porta et al., 2020), but many species remain described with a single wasp and without genetic evidence (Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2020). The remarkable diversity of oak species of the *Lobatae* section in Mexico, along with their propensity to hybridise between sympatrically distributed species (González-Rodríguez et al., 2004; Hipp et al., 2020; McCauley et al., 2019; Ramos-Ortiz et al., 2016; Tovar-Sánchez et al., 2008; Tovar-Sánchez & Oyama, 2004, 2006; Valencia-A, 2004), provides an opportunity to explore the speciation within cynipids. Thus, the objective of this study was to analyse the evolutionary relationships between species outside the '*niger*' complex of *Amphibolips*. Here, we present the first phylogenomic study of the Cynipini genus *Amphibolips* across most of its geographic range.

METHODS

Taxon sampling

Galls were collected in Mexico between 2017 and 2019, in Panama between 2008 and 2009 and in the United States in 2020. In situations where the same type of galls appeared on different oak species in the same locality, we collected both galls to further compare the wasps involved. We obtained 520 adult wasps from 401 sites, predominantly in Mexico, across the Trans Mexican Volcanic Belt, the Sierra Madre Oriental and the Sierra Madre Occidental (Figure 4). As outgroups, we included *Erythres* Kinsey sp. and *Zopheroteras* Ashmead sp. associated with the *Lobatae* section and *Odontocynips hansoni* Pujade-Villar associated with the *Quercus* section. The galls were collected directly from the host trees and transferred to the facilities of the Universidad Nacional Autónoma de México (UNAM). The galls were placed in plastic containers with ventilation until wasps emerged. Once the wasps emerged from their galls, they were placed in 99% ethanol in refrigeration-resistant plastic containers. These tubes were kept refrigerated until DNA extraction. Species identification was based on the *Amphibolips* keys available for Mexico (Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2021; Melika et al., 2011; Nieves-Aldrey et al., 2012). When the morphology of the wasps and their galls was clear, the species name was retained. In cases where the specific assignment of the wasps was not clear, different assignments were used, such as indeterminate, new or related to similar species.

DNA extraction and sequencing of ultraconserved elements

Cynipid sequencing encompassed 81 species, including 78 *Amphibolips* species and three outgroups (Table S1). For *Amphibolips*, each of the

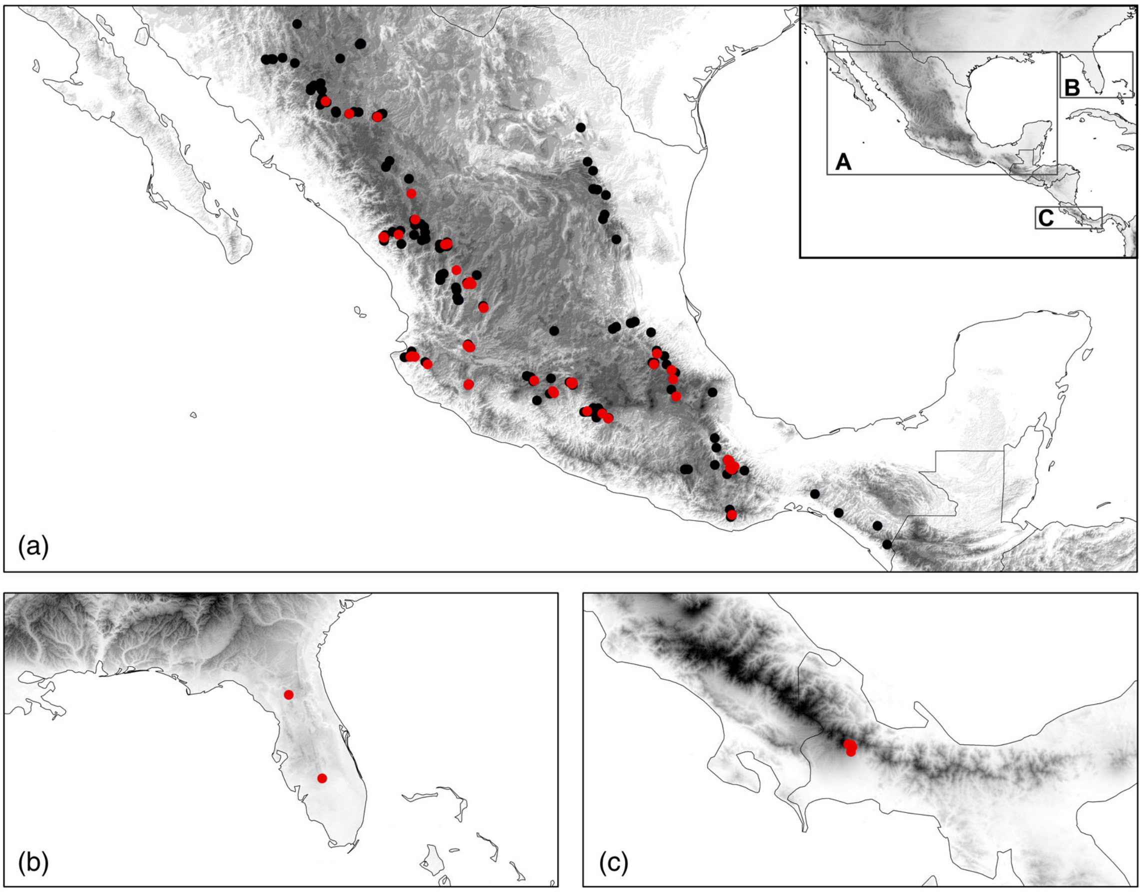

FIGURE 4 Map of localities sampled to carry out this study (all circles). The red circles represent the localities that were used for the phylogenomic analysis with UCEs. (a) Locations in Mexico; (b) locations in the USA; (c) locations in Panama.

species or morphospecies that was collected in different host plants were included. The entire wasp body was used for DNA extraction when there were many individuals of the same identified species or morphospecies. When dealing with unique specimens, only two legs on the right side were used to maintain the morphological integrity of the wasp. In some cases, the remaining material from scanning electron microscopy dissections was used for amplification. The voucher specimens were deposited in the facilities of the Museo Nacional de Ciencias Naturales in Madrid, Spain; the Laboratorio de Ecología Genética y Molecular of ENES-Morelia, at UNAM, Mexico; and the National Museum of Natural History, USA. DNA extraction was performed with the help of the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's specifications. The quality of the extractions was checked through 1% agarose gel electrophoresis. Concentration and purity tests were performed with a Qubit 3.0 (Life Technologies) and a NanoDrop 2000 (Thermo Scientific). UCE enrichment and sequencing on a NovaSeq 6000 instrument (Illumina) were performed by Daicel Arbor Biosciences (Ann Arbor, USA) with the Hymenoptera 2.5Kv2P probe set (Branstetter et al., 2017) following the standard pipeline (see Zhang, Williams, & Lucky, 2019).

The Phyluce pipeline was used to process the UCE loci (version 1.7.1, Faircloth, 2016), except where indicated. The raw reads, adaptors, primers and low-quality bases were trimmed with Illumiprocessor (Faircloth, 2013), a tool that uses Trimmomatic (Bolger et al., 2014). We decided to perform the de novo assembly directly with SPAdes (Bankevich et al., 2012; Prjibelski et al., 2020) instead of using the Phyluce pipeline due to the inconsistencies in the sum of the viable sequences to be paired plus the sequences in singletons (most samples had more than three million reads). We modified the search for UCE loci by changing the minimum percent identity to 82% instead of the normal value (80%) to avoid contaminants, as recommended by Bossert and Danforth (2018). The UCE loci were aligned with MAFFT v7 (Katoh & Standley, 2013) and trimmed following the steps of the Phyluce pipeline, including the use of Gblocks (Castresana, 2000) using the following settings: -b1 0.5, -b2 0.5, -b3 12 and -b4 7. We used Spruceup v.2022.2.4 (Borowiec, 2019) to remove outlier sequences when concatenated arrays of UCE loci were already available.

Our analyses included eight species of *Amphibolips* and nine outgroup species previously studied with UCEs (Blaimer et al., 2020; Ward et al., 2022). The eight species of *Amphibolips* were included to examine relationships including species from northern Mexico. Outgroup species from the *Lobatae*-associated clade, as identified by Ward et al. (2022), were included to provide phylogenetic context, and *Dryocosmus kuriphilus* Yasumatsu was used to root the tree (Blaimer et al., 2020). Details on the samples obtained from other studies are summarised in Table S2.

Phylogenomic analyses

We performed analyses on three distinct datasets of UCE loci located in at least 50%, 80% and 85% of the individuals to assess the consistency across varying levels of genomic representation. Phylogenetic trees were constructed from the entire dataset (98 individuals including 86 specimens of *Amphibolips* and 12 outgroup specimens) using concatenated matrices, applying different analytical methods for each dataset. The resulting data matrices contained 1671, 1118 and 789 loci, respectively. The total lengths of the alignments for these datasets were 1,418,957, 1,033,879 and 770,814 bp, respectively.

To assess the impact of different data partitioning strategies on phylogenetic inference, we generated maximum likelihood (ML) trees with IQ-TREE v 2.2.2.6 (Minh et al., 2020) using two partitioning schemes for each data matrix. This approach allowed us to compare the congruence between the resulting trees and evaluate the robustness of our phylogenetic conclusions. First, sequence evolution models were calculated for each UCE locus using ModelFinder (Kalyaanamoorthy et al., 2017) in IQ-TREE. Following Naser-Khdour et al. (2019), we generated phylogenetic trees removing the partitions that violated the assumptions of the sequence substitution models proposed in IQ-TREE (with the –symtest-remove-bad command). Second, we partitioned the UCE loci with the Sliding-Window Site Characteristics using Entropies method (SWSC-EN, Tagliacollo & Lanfear, 2018) to calculate the best partitioning scheme with PartitionFinder2 (Lanfear et al., 2017) with the help of the 'rclusterf' algorithm. When the best sequence substitution model was available, IQ-TREE analyses were performed with 1000 ultrafast bootstraps (UFBoot; Hoang et al., 2018) and 1000 SH-likelihood ratio tests (SH-aLRT; Guindon et al., 2010). Nodes with support values of ultrafast bootstraps ≥95 and SH-likelihood ratio tests ≥80 were considered robust. To reduce false-positives (branches wrongly receiving 100% support), we used the GENESITE option for resampling partitions and then sites within resampled partitions (Gadagkar et al., 2005; Seo et al., 2005). To reduce the risk of overestimating support values with ultrafast bootstraps due to possible model violations (Minh et al., 2013), we added a step in IQ-TREE using a hill-climbing nearest neighbour interchange (Hoang et al., 2018). Individual trees were generated for each UCE locus using multispecies coalescence analysis on IQ-TREE trees analysed with ModelFinder. We used ASTRAL-III (Zhang et al., 2017) to infer the coalescence phylogeny of species from the gene trees. Branch support was assessed using posterior local probability (Zhang et al., 2017). Branches with ≤10% ultrafast bootstrap support in each UCE locus tree were collapsed using Newick utilities (Junier & Zdobnov, 2010), resulting in polytomies to replace weakly supported branches, a method proven to enhance phylogenetic accuracy (Zhang et al., 2017). To streamline our ASTRAL analyses, we focused on comparing the topologies derived from the 80% and 50% matrices, given the strong congruence among the underlying IQ-TREE maximum likelihood trees.

To evaluate the consistency across results, we generated species trees using IQ-TREE for concatenated matrices. In each analysis, we removed partitions that failed to meet specific model assumptions, as was done in the prior analysis. The partition models and sequence evolution for each matrix were determined with ModelFinder. Branch support was assessed with 1000 replicates of ultrafast bootstrap and SH-likelihood ratio tests in IQ-TREE.

Phylogenetic trees were visualised in FigTree (Rambaut, 2012), and subsequent edits were made with Adobe Photoshop for image adjustments and Illustrator for vector graphics enhancements.

Concordance analyses

Incomplete lineage sorting frequently complicates phylogenetic inference, especially in recently divergent species or populations with substantial differences from those with deep phylogenetic divergence (Maddison & Knowles, 2006). To assess the impact of incomplete lineage sorting in our study, we employed the gene concordance factor (gCF) and the site concordance factor (sCF) in IQ-TREE tool (Minh et al., 2020; Mo et al., 2023). These metrics complement traditional branch support measures, such as bootstrapping, by providing a comprehensive depiction of the associations among loci and sites (Minh et al., 2020). We analysed the concordance factors derived from UCE loci trees constructed with IQ-TREE and subsequently used them in the ASTRAL analyses.

Molecular species delimitation

To accommodate our extensive dataset and computational limitations, we chose two molecular species delimitation methods that are computationally efficient, requiring minimal user input. This approach allowed us to conduct a comprehensive analysis of species boundaries without sacrificing the accuracy of our results. The resulting phylogenies were compared with data on wasp morphology, host species and geographic distribution. Given the consistent results obtained across various phylogenetic analyses, we opted for the 80% completeness matrix. This matrix offers a robust dataset that includes a substantial proportion of the available loci, while mitigating the potential biases introduced by excessive missing data.

For the Bayesian Poisson Tree Processes (bPTP, Zhang et al., 2013), we performed an analysis with 10,000,000 Markov chain Monte Carlo (MCMC) generations, a thinning interval of 100, a sample size of 10,000 and a burn-in of 10% (0.1). The consensus tree for this analysis was obtained from IQ-TREE using the SWSC-EN partition method.

Species bOundry Delimitation using Astral (SODA, Rabiee & Mirarab, 2020) is a method that employs a multispecies coalescent model (MSC) and incorporates polytomy tests implemented in ASTRAL (Sayyari & Mirarab, 2018). For this analysis, we used previously generated gene trees in which the best models for UCE loci were selected using ModelFinder. Branches with ultrafast bootstrap support ≤10% were collapsed using the Newick utilities package. These gene trees correspond to those passing the symmetry test, which examines partitions violating certain assumptions about sequence evolution models. A guide tree (Figure S8) was used in the analysis, considering the results of the concordance analysis. We also assessed the potential influence of missing species alignments on molecular species delimitation, thus alternatively using a 98% completeness matrix (44 loci) to evaluate how this alters the outcome (Lähteenaro et al., 2024). For this analysis, we employed the same commands as in the ML analyses, treating each UCE as a partition, collapsing nodes with ≤10% ultrafast bootstrap support and enabling the symmetry test. The guide tree remained unchanged for this analysis.

RESULTS

Sequence processing

We obtained 669,988–10,478,654 raw sequence reads per taxon (median: 3,932,053), including the sequences obtained from published studies. We assembled these raw sequence reads with SPAdes into 10,202-504,293 contigs per taxon (median: 133,999). After we captured the 2590 target UCE loci, we obtained 263 to 1628 loci, with a median count of 1528 loci and a median length of 958 bp (see Table S3 for detailed capture and assembly information).

Following alignment and trimming, we analysed three datasets with 50%, 80% and 85% completeness. Overall, the phylogenetic trees resulting from IQ-TREE were well supported (Figures 5 and S1–S9). In general, most of the nodes demonstrated high statistical support for species relationships. However, there was variation in tree topologies between different datasets and analytical approaches (ML and coalescence of species), particularly at nodes with lower support, as measured by SH-aLRT and UFBoot.

We selected the 80% matrix for our analysis because it provided a proportionate representation of the loci across the studied species. The SWSC-EN partitioning scheme used to depict the tree with IQ-TREE has been recommended as the most suitable for this type of marker (Tagliacollo & Lanfear, 2018). The ML trees inferred with IQ-TREE resembled those from the MSC analysis performed with ASTRAL, albeit with some differences, as detailed in Figures S10 and S11.

The position of genera related to *Amphibolips*

In all cases, we used *Dryocosmus kuriphilus* to root the trees based on recent phylogenetic studies (Blaimer et al., 2020; Ward et al., 2022). Considering that most of the included wasp species belong to a clade exclusively associated with the *Lobatae* section of *Quercus*, *Odontocynips hansoni* (associated with the *Quercus* section) was also included. In ASTRAL, in both cases, two monophyletic clades consistently emerged: one comprising all outgroup wasps and the other comprising the *Amphibolips* species (Figures S10 and S11). Trees with *O. hansoni* as the sister taxon to other wasps generally showed higher support levels that did those suggesting a clade comprising *Callirhytis quercusoperator* (Osten Sacken) + *Erythres* sp., except for the tree derived from the 85% matrix without partitions (Figure S3). IQ-TREE analyses resulted in the formation of two large clades: one consisting of outgroup

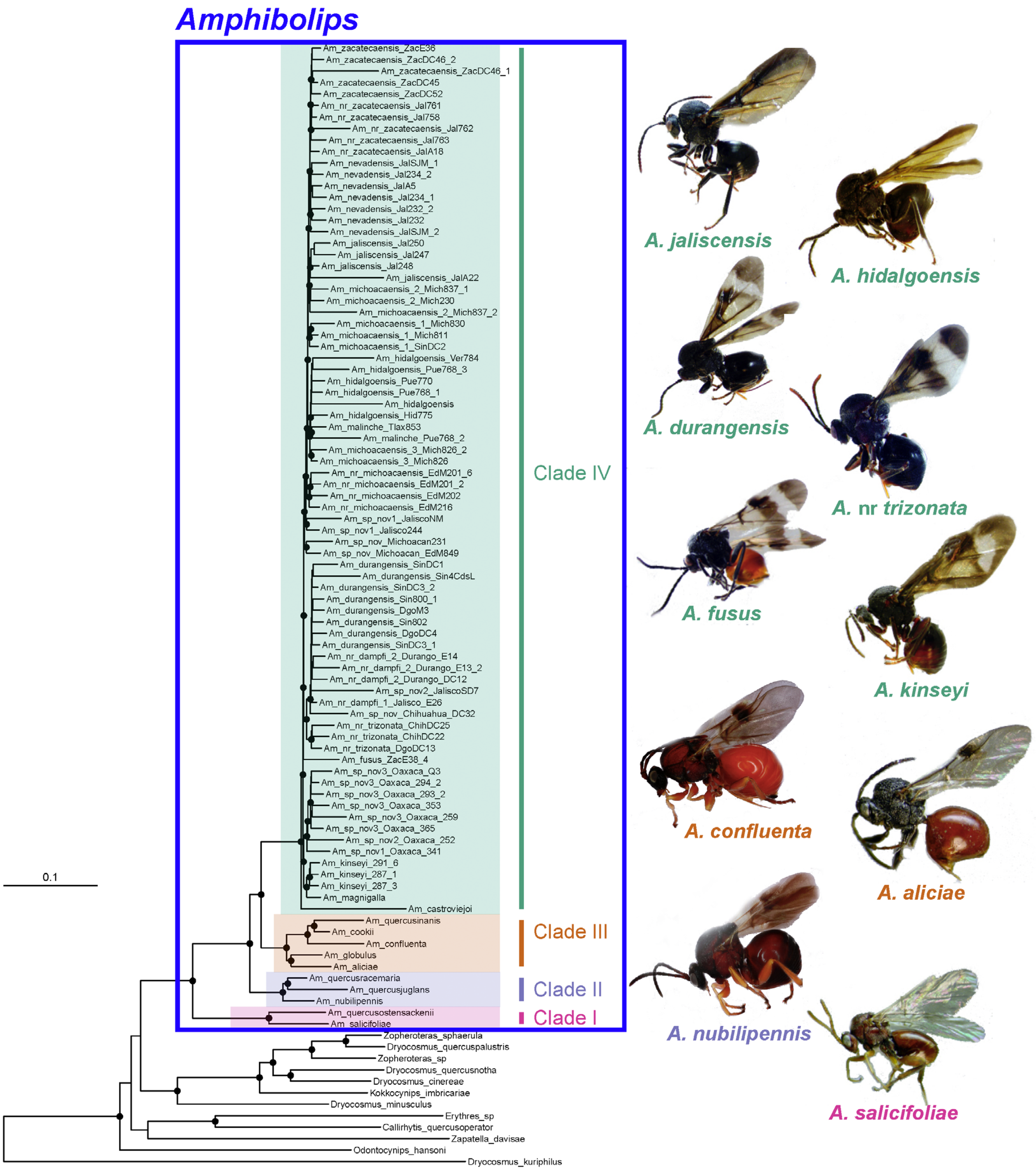

FIGURE 5 Phylogeny of *Amphibolips*. IQ-TREE tree result of Maximum Likelihood analysis using the SWSC-EN partitioning scheme with the 80% matrix (details in the Methods section). The clade corresponding to the genus *Amphibolips* is inside the blue box. Within the genus *Amphibolips*, the four clades proposed here are highlighted in different colours and numbered according to the text. Nodes with black dots indicate support values of UFBoot >95 and SH-aLRT >80. The habitus images of *Amphibolips* species are named according to the colour of the clade to which they correspond. Photos of *A. confluenta* and *A. nubilipennis* courtesy of Andrew Forbes lab.

species and the other encompassing the entire genus *Amphibolips* (Figure 5). The topology of the outgroup species of the first clade was maintained across all analyses, although the support values varied (Figures S1–S11). Within this clade, species from the genera *Dryocosmus* Giraud, *Kokkocynips* Pujade-Villar and Melika and *Zopheroteras* exhibited varying compositions.

Phylogenetic relationships of *Amphibolips* using maximum likelihood (ML)

The species currently classified under *Amphibolips* formed a monophyletic group, displaying a consistent general arrangement across various phylogenetic trees (Figures S1–S11). Species from the United States and Panama maintained their relationships and retained their positions as sister species to the Mexican species group in all analyses. Variations among trees primarily concerned the most closely related wasps within the Mexican clade, which exhibited less morphological and/or genetic differentiation. Specifically, within the Mexican species, those from Oaxaca formed their own monophyletic group, termed the Oaxacan clade (Figure 6), which is sister to the other Mexican wasps. In the Oaxacan clade, two distinct subclades consistently emerged, irrespective of the analysis method. One of these subclades comprises the recently described species *A. magnigalla* and *A. kinseyi* (Castillejos-Lemus et al., 2020) and represents the sister group to the other subclade. The latter contains species that have been recently discovered and are pending taxonomic classification. There were slight variations in some of the most closely related species of this Oaxacan clade among individuals of the same species. The phylogenetic position of *A. fusus* was consistently and unambiguously resolved as sister species to the remaining *Amphibolips* species, with the exception of those constituting the Oaxacan clade (Figure 6). To our knowledge, our study represents the rediscovery of this species from a locality proximate to its type locality and original host plant in which it was initially described (Kinsey, 1937). Two major wasp clades emerged: one confined to the Sierra Madre Occidental and another spanning the Trans-Mexican Volcanic Belt (TMVB), including southern portions of the SMOc (Figure 6). The phylogenetic relationships of closely related wasps within the SMOc, TMVB and Oaxacan clades demonstrated some degree of incongruence across different analyses. Notably, within the latter clade, individuals morphologically identified as *Amphibolips michoacaensis* Nieves-Aldrey and Maldonado were distributed across different branches, demonstrating that morphological similarity does not necessarily indicate close genetic kinship between wasps.

Phylogenetic relationships of *Amphibolips* using multispecies coalescent model (MSC)

The MSC trees generated by ASTRAL showed consistency across the 50% and 80% of the data matrices (Figures S10 and S11). LocalPP branch support was generally high (≥0.7) for the deepest nodes and tended to be lower (<0.7) for many terminal nodes. The phylogenetic arrangement of the terminal branches for the species from the United States and Panama was consistent in both analyses, corroborating the IQ-TREE results. The separation of the Mexican *Amphibolips* species into two clades, one being the Oaxacan clade and the other encompassing all remaining species, was maintained. The relationships of some of the wasps changed on terminal branches in the Oaxacan clade; however, all individuals involved were from the same species. These changes were similar to those obtained for the different trees with IQ-TREE. The position of *A. fusus* was the same in all cases, although with less support in the ASTRAL results (Figures S10 and S11). The sister taxon of *A. fusus* was divided into two clades in both cases, as in the case of the IQ-TREE results. The most important change within the clade was in the *Amphibolips* wasp from Jalisco (Jal247). This species is the sister taxon of a large group of wasps almost exclusive to the TMVB in the 50% matrix. However, in the 80% matrix, it shifted to a smaller group from Jalisco and Michoacán (Figures S10 and S11). Other changes between the trees for this clade were very small. Relationships within the exclusive SMOc clade also showed minor changes between analyses.

Differences between ML and MSC analyses

Although the overall tree topologies from the ML (IQ-TREE) and MSC (ASTRAL) analyses generally aligned, notable discrepancies emerged. Among the outgroup species, the main difference was the inclusion of *O. hansoni* as part of a clade together with *C. quercusoperator* + *Erythres* sp., which subsequently formed the same clade together with *Zapatella davisae* Buffington and Melika in the ASTRAL-III tree (Figure S11). The remainder of the wasps from other genera (*Dryocosmus* (except *D. kuriphilus*), *Zopheroteras* and *Kokkocynips*) formed a clade equal to that obtained in all the IQ-TREE analyses. For the clade distributed in the TMVB, the most important difference occurred with *A. hidalgoensis*, which shifted from a close association with Hidalgo and Puebla wasps from the IQ-TREE results to being a sister taxon of a broader group spanning SMOc and TMVB. Despite these changes, the foundational relationships within the clades, often involving conspecific individuals, remained without major changes. In the SMOc-exclusive clade, a major change was observed for *A.* aff. *dampfi* from Jalisco (E26). IQ-TREE placed it as a sister taxon to wasps from Jalisco, Durango and Sinaloa, while ASTRAL-III estimates it as sister taxon to a single wasp from Durango. Terminal branch positioning varied to some degree between the two analytical strategies.

Gene and site concordance

In general, despite having high support values (SH-aLRT and UFBoot), gene and site concordance (gCF, percentage of loci supporting a node and sCF, percentage of informative sites supporting a node) factors had moderate to low values (<0.7) (Figures S12 and S13). The gCF values, mainly on the shallow nodes, were usually lower than the sCF values, and the sCF values provided sufficient support for the nodes in the tree. Nodes that had both low gCF and sCF values matched the lowest support values obtained for the trees with SH-aLRT and UFBoot. The values may indicate that there are regions within UCEs that have some type of conflicting signal (Minh et al., 2020), changing some of the relationships between the different analyses. This aspect is more evident considering the differences in the results from the

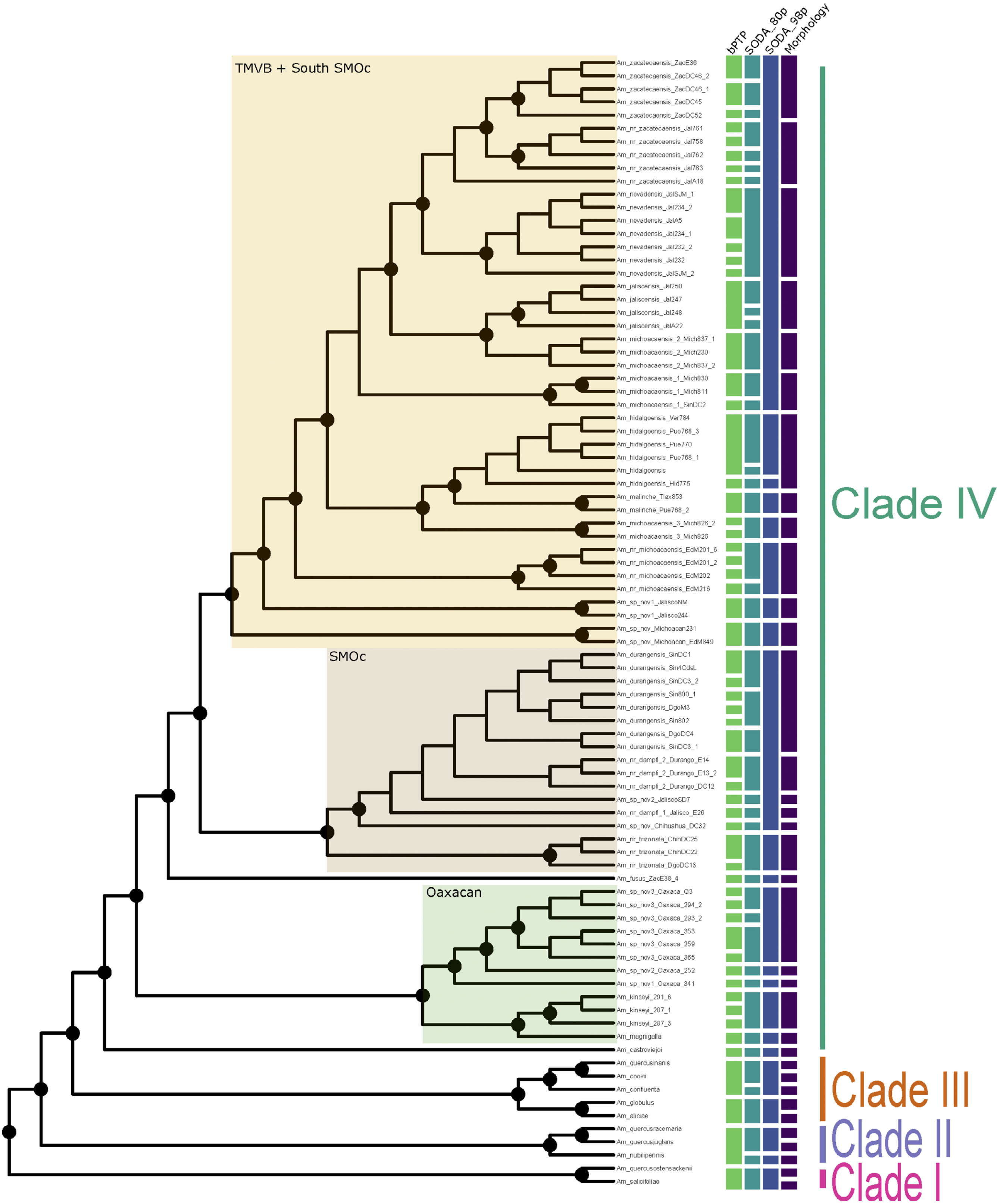

FIGURE 6 Cladogram of the genus *Amphibolips*. Cladogram extracted from the tree in Figure 5, excluding outgroups for clarity. The bars to the far right indicate the clades mentioned in the text. The results of molecular species delimitation and morphological analysis are specified to the right of the cladogram. Three geographic groups of species are highlighted according to how they are mentioned in the text: Oaxacan, SMOc and TMVB + South SMOc. Nodes with black dots indicate support values of UFBoot >95 and SH-aLRT >80.

MSC analysis (Figures S10 and S11) with respect to those from the IQ-TREE tree (Figure 5).

Identifying novel clades within the *Amphibolips* genus

Within *Amphibolips*, four groups of species can be observed. Clade I (Figure 5), the most basal node within *Amphibolips*, consists of two species, one from the United States (*A. quercusostensackenii* (Bassett)) and one from Panama (*A. salicifoliae*). Clade II comprises three species of wasps from the United States and Canada (*A. nubilipennis* (Harris), *A. quercusjuglans* (Osten Sacken) and *A. quercusracemaria* (Ashmead)). Clade III is composed of five species, one from Panama (*A. aliciae*) and four from the United States (*A. globulus* Beutenmüller, *A. confluenta* (Harris), *A. cookii* Gillette, and *A. quercusinanis* (Osten Sacken)). Clade IV encompasses the remainder of the Mexican species and one species from Panama (*A. castroviejoi* Medianero and Nieves-Aldrey), representing the clade with the greatest number of species.

Molecular species delimitation

Morphologically, some wasps were difficult to assign to a known species or exhibited intermediate traits between those of the two most similar described species. These morphological differences can occur between wasps from the same collection site or even from the same host tree (Table S1). Other wasps were assigned to known species with wide distributions, such as *A. michoacaensis*. A small number of wasps displayed morphological characteristics that did not align with any previously documented species, thus suggesting that they could be classified as undescribed species. We attempted to delimit the *Amphibolips* wasps using molecular data, and the results are summarised in Figure 6.

Despite using different strategies to test species delimitation hypotheses, there were some similarities in the results obtained using bPTP and SODA. The bPTP programme was designed to align with the phylogenetic species concept, accounting for a larger number of substitutions. In contrast, SODA utilises ASTRAL gene trees to propose species delimitation, which is influenced by gCF (an indicator that some nodes are supported by a low number of loci). To mitigate this influence, we employed the IQ-TREE guide tree (Figure 5), considering that relationships were better supported by standard bootstrap values. In both analyses, the wasps identified as *A. michoacaensis* and *A. zacatecaensis* were not the same species and were split into multiple distinct species. There was a notable disparity in the number of identified *Amphibolips* species between the two methods and among different SODA replicates, with bPTP recognising 58 putative species and SODA recognising 46 and 22 putative species, separately. In the case of bPTP, only 20 putative species had more than one wasp. Among the noteworthy results consistently observed in both analyses, *A. salicifoliae*, originally described from Panama, clustered within the same clade as *A. quercusostensackenii*, a Nearctic species distributed throughout Eastern North America. Similarly, *A. aliciae*, another Panamanian species, exhibited close affinity with *A. globulus*, which is also a Nearctic species. Conversely, recently described species, such as *A. castroviejoi* from Panama and *A. magnigalla* from Oaxaca, Mexico, were confirmed as distinct species. Additionally, *A. fusus* emerged as a clearly delineated species, separate from the others. Wasps allied to *A. hidalgoensis* present an atypical case: bPTP suggested two species, whereas SODA suggested three species with the 80% matrix, and two species (identical to bPTP) with the 98% matrix.

DISCUSSION

Congruence between phylogenetic methods

Bootstrap values, while informative about sampling variance of a branch, do not provide insights into the number of loci or sites supporting a particular clade. Concordance factors (gCF and sCF) complement bootstrap values by indicating the proportion of loci or sites supporting a given node. Overall, observed patterns across the phylogenetic trees were consistent. Considering the shifts in relationships between the IQ-TREE (Figure 5) and ASTRAL (Figures S10 and S11) results, the gene and site concordance factors suggest that the maximum likelihood analysis offers greater consistency. We assume this is based on the premise that low gCF values imply a scarcity of loci supporting some nodes, while low sCF values suggest that some nodes are primarily supported by informative sites. The clearest example is the Jalisco wasp (Jal247), which occupies contrasting positions in both ASTRAL results. The results of the concordance factor test (Figures S12 and S13) corroborate that the loci supporting the relationship of this species are few, with most of the evidence provided by informative sites. According to the localPP value proposed by Sayyari and Mirarab (2016), values below 33% may be due to chance, suggesting that the reduction in loci could be related to the decrease in localPP in the test with the 80% matrix, possibly due to the loss of informative sites. In our case, the phylogenetic tree derived from the IQ-TREE maximum likelihood analysis therefore provides the best approximation for the evolutionary relationships of *Amphibolips* species. Bootstrap values in our maximum likelihood tree (Figure 5) generally showed standard thresholds suggesting a robust overall topology, although concordance factors (gCF and sCF) (Figures S12 and S13) provide limited support for certain relationships.

Concordance between molecular species delimitation and morphological taxonomy

Molecular species delimitation methods can produce variable results (Prebus, 2021), and our analyses with bPTP and SODA exemplify this. While bPTP consistently identified a greater number of putative species, SODA, particularly with the 98% matrix, tended to underestimate species diversity. Given the contrasting results, we adopted a conservative approach to species delimitation, as represented by the 35 morphological species in Figure 6. By integrating morphological data, host associations, emergence timing and geographic distribution, we aimed to provide a more comprehensive assessment of species boundaries. The oversplitting observed in bPTP may be influenced by factors such as gene flow (Luo et al., 2018), while the undersplitting in SODA could be attributed to the reduced number of loci. These results underscore the need for caution when interpreting the outputs of any single delimitation method. Although we did not propose taxonomic changes in this study, our results suggest the importance of considering multiple lines of evidence for understanding species diversity in *Amphibolips* and Cynipidae.

Monophyly of *Amphibolips*

Kinsey (1920) proposed the relationships of the major groups of Cynipidae and included four species of *Amphibolips* to assess their morphological differentiation. He recognised the congruence in their morphology and maintained them within the same genus. Although Kinsey was already acquainted with *A. quercuspomiformis*, he regarded this species as part of *Andricus* Hartig but recognised its shared morphological traits with *Amphibolips* species. Kinsey and Ayres (1922) included *Callirhytis* Foerster under *Andricus*. Subsequent studies incorporating DNA samples from cynipid wasps, including samples of *Amphibolips*, provided indications of its possible monophyly (Andersen et al., 2021; Cooke, 2018; Cuesta-Porta et al., 2023; Drown & Brown, 1998; Nicholls et al., 2017; Nieves-Aldrey et al., 2021; Ward et al., 2022). *Amphibolips* has a unique and exclusive association with oaks of the *Lobatae* section, which are restricted to the American continent. Regardless of the data or methods of analysis, all our results consistently support the monophyly of the genus *Amphibolips*.

Considering the results of this study, the '*nassa*' complex (Cuesta-Porta et al., 2020; Melika et al., 2011) needs to be reduced or adjusted to a more precisely distributed clade of species so that a grouping can be proposed that better corresponds to the natural classification of clades. Revisiting Nieves-Aldrey et al.'s (2012) proposition regarding the morphological similarities among Mexican species and Cuesta-Porta et al.'s (2020) attempt to categorise the '*nassa*' complex, particularly focusing on Mexican and Panamanian species through morphological characters, we suggest aligning the '*nassa*' complex with clade IV. It is essential, however, to distinguish between naming a complex based on taxonomic criteria (Claridge, 2017; Reid, 1970) and naming a clade that may encompass various complexes, as exemplified by *Amphibolips zacatecaensis* in clade IV.

Amphibolips clade I

In the case of *Amphibolips* clade I, represented by *A. quercusostensackenii* and *A. salicifoliae*, a prominent shared characteristic is the similarity of their galls (Figure 1a). Both species develop galls on the adaxial leaf surface of their host species. These galls are fragile, and the larval chamber is supported by radiating filaments. Another shared trait between these species is their hyaline forewings, with the absence of dark spots, bands or infumate areas (Figure 3a). *Amphibolips quercusostensackenii* (Bassett, 1863) inhabits *Quercus rubra* L., *Q. ilicifolia* Wangen. and *Q. coccinea* Muenchh., while *A. salicifoliae* is associated with *Q. salicifolia* Née. These two wasp species have dissimilar host species situated within different subsections (Manos & Hipp, 2021). In the *Amphibolips* '*niger*' complex review (Cuesta-Porta et al., 2023), *A. quercusostensackenii* is identified as a sister species of *A. ufo*. In turn, they are sister species of *A. quercuspomiformis*, the sole species with multilocular galls and lacking basal lobe on the tarsal claws. This trait is unique within the genus, shared with *A. salicifoliae* (Figure 2j) but not with *A. quercusostensackenii* (Cuesta-Porta et al., 2023). Cuesta-Porta et al. (2023) suggested the potential separation of genera within this clade is plausible. The '*niger*' complex has been viewed as an anomaly within the *Amphibolips* genus (Kinsey, 1937) or as potential alternate sexual generations (Nieves-Aldrey et al., 2012). Broader species and individual inclusion may aid in clarifying their systematic placement and potential separation from the genus *Amphibolips*.

Amphibolips clade II

Clade II is composed exclusively of *Amphibolips* species from north of Mexico (*A. nubilipennis*, *A. quercusjuglans* and *A. quercusracemaria*). These Nearctic species share some similarities. For instance, their galls closely resemble those of clade I, as noted by Mayr in 1881. However, these galls fall into two categories—those formed on leaf blades (Figure 1n) and those that develop on acorns (a feature not very common in *Amphibolips*, but seen in *A. quercusjuglans*; Figure 1o). The galls of these three species contain fleshy parenchyma, giving them a slightly firmer texture when touched and less fragile texture when dried.

Cook (1909) suggested that the structure of *A. quercusjuglans* is akin to that of species such as *A. gainesi* Bassett or *A. trizonata* Ashmead (Figure 1i). However, none of these species mentioned by Cook (1909), except *A. quercusjuglans*, have undergone molecular studies. Beutenmüller's (1909) annotation is intriguing, as it highlights that the galls of *A. quercusjuglans* wilt when they dry, unlike those of *A. gainesi*, even though they share many common features, including their formation from *Quercus* acorns. The *Amphibolips* clade II species belong to group C of galls in Cook's proposal (1909), specifically subdivisions B and C. Cook's (1909) observation of *A. quercusjuglans* and *A. quercusracemaria* seems accurate, with Beutenmüller's (1909) differentiation highlighting the similarity among the three species in the clade with succulent galls and few radiating filaments, leading to wilting when dried. It is important to consider the possibility of these galls corresponding to alternate generations (Ward et al., 2022) or differences of gall formation in different plant organs. The wings may be slightly darkened, leaving a small uncoloured section (*A. quercusracemaria*, Ashmead, 1881) or having a darker spot at the distal section of the wing (*A. nubilipennis*, Harris, 1841; Figure 3c). The wing spot pattern of *A. quercusjuglans* is relatively similar to that of *A. nubilipennis* (Walsh & Riley, 1869). The host plants are more closely related to each other, falling within the three subsections of *Lobatae* proposed by Manos and Hipp (2021) for the eastern United States. *A. quercusjuglans* has been observed on oak species from all three subsections, *A. nubilipennis* on two subsections (except *Palustres* (Trelease)), and *A. quercusracemaria* on one subsection (*Phellos* (G. Don)).

Amphibolips clade III

In the case of clade III of *Amphibolips*, the similarities are less evident than in the previous clades. For example, the galls of the US species are semispherical (except for *A. globulus*, which is somewhat spindle-shaped), while those of the Panamanian species are spindle-shaped (Figure 1b). The most extreme case is represented by *A. confluenta* (Figure 1m), which has a long history of synonyms and proposals regarding its alleged alternate generation (for a general overview, see Beutenmüller, 1909). The morphology of adults, external characteristics of galls and phenology of this species change among localities, dates and host species (a summary of this history is well explained on the Gallformers website, https://gallformers.org/gall/580). In general, the galls in this group have tiny protuberances; however, occasionally, they can have a smooth external texture. These protuberances are usually of a slightly different colour than the gall itself (galls are usually light green; the Panamanian species is darker), that is, a lighter, darker or distinct shade depending on the species. When smooth, the scattered points can still be seen, similar to the protuberances but appearing as spots. The only species that sometimes does not follow this pattern is *A. confluenta* (Figure 1m). Generally, they are slightly firmer to the touch compared to the galls in clade I and feel less succulent than those in clade II, although there are differences within this clade as well. Importantly, within clade III, there are two divisions—one grouping *A. aliciae* and *A. globulus*, and the other comprising the three remaining US species. The galls of *A. aliciae* and *A. globulus* are the only spindle-shaped galls (Figure 1b), while the others are spherical (Figure 1M). Spindle-shaped galls tend to be stiffer, although this varies depending on the developmental stage. Except for *A. confluenta* galls, galls tend to have thicker epidermises, larger intercellular spaces within the parenchyma, and thick radiating filaments towards the larval chamber. In *A. confluenta*, these filaments are often thinner, the epidermis is thinner and the parenchyma tends to consist of spongy tissue, which makes it difficult to observe the filaments (sometimes until the spongy tissue degrades). Among some shared characteristics within these species, all species have a red metasoma (with *A. globulus* being the darkest), display the same spot pattern at the base of the radial cell (Figures 3b,d), and have a rounded posterior mesoscutellum according to their original descriptions. However, in at least two species (*A. globulus* and *A. aliciae*), a slight posterior median impression that interrupts the rounded margin can be observed.

Host plants are somewhat different among the species in clade III. The most geographically distinct host species is *A. aliciae*, so it has no close relationship to the other clade III species. Among the US species, various *Quercus* species have been found, but they are mainly limited to two subsections, *Phellos* and *Coccineae* (Trelease). The only exception to this pattern is *A. confluenta*, which has been proposed for *Q. palustris* Muenchh., highlighting the importance of including different populations of this species (in addition to the form *A. quercusspongifica* (Osten Sacken)) to evaluate the relationships of this species with its close relatives.

Amphibolips clade IV

Amphibolips clade IV includes Mexican species, except for *A. castroviejoi* from Panama. The extensive sampling effort allowed us to recover a significantly diverse collection of galls and wasps from different localities not previously reported (Figure 4). Some of these species resemble the described species, although they present slight variations that complicate the certainty of their species classification. Other wasps seem to belong to novel species that bear no resemblance to any known entities (Table S1). Here, we report *A. fusus* once again, after previously having reports only of its galls (Figure 1h). The positioning of this species in the phylogeny underscores the importance of adequate sampling to represent the phylogenetic diversity of the genus, allowing for a better understanding of species relationships and their potential associations with their natural history.

Clade IV is restricted to the region from Mexico to Panama and is closely associated with oak species identified as *Erythromexicana* by Hipp et al. (2020). Recent species descriptions within the genus *Amphibolips* imply that species diversity in Mexico may exceed the reported diversity in the United States and Canada (Castillejos-Lemus et al., 2020; Cuesta-Porta et al., 2020, 2021, 2023; Melika et al., 2011; Nieves-Aldrey et al., 2012; Pujade-Villar et al., 2018), showing similarities with diversification events observed in their host plants (Hipp et al., 2020).

Wing coloration patterns in clade IV

All species in clade IV exhibited a wing pattern with males occasionally having an entirely smoky wing devoid of distinct light bands (Figure 3). This pattern is distinct from those of clades I and III, but could be seen as a modification of the pattern observed in clade II (Figure 3c).

Some species are characterised by a dark band running along the anterior margin of the hind wing, extending from the base to the apex and reaching the wing margin at the level of the radial cell and up to the wing tip (Figure 3k–o). Another group of species displays a generalised dark coloration over much of the wing, with patterns marked by hyaline bands within the dark areas of the wing, which can either be bands spanning the wing from both margins or restricted to specific areas depending on the species (Figure 3e–g). There are species with wing spot patterns that may vary between these two coloration patterns, at times resembling a blend of both (Figure 3h,i). Wasps from the Oaxacan group bear a striking resemblance to the wing coloration pattern present in *A. castroviejoi* (Figure 3j). The wings of the SMOc-exclusive group exhibited a spot pattern similar to that of *A. durangensis* Nieves-Aldrey and Maldonado (Figure 3h,i). The clade predominantly found in the TMVB exhibits a hyaline posterior wing and retains coloration only in the anterior wing band (Figure 3k–o).

Mesoscutellum shape and middle mesoscutellar incision in clade IV

In a new species from Durango and Chihuahua (related to *A. trizonata*), the posterior edge of the mesoscutellum is nearly rounded with a slight impression in the centre (Figure 2b). In contrast, in the recently described species *A. magnigalla*, *A. kinseyi* and *A. nigrialatus*, the posterior edge of the mesoscutellum is deeply incised, and the deep incision almost reaches the scutellar foveae. Castillejos-Lemus et al. (2020) mentioned a pronounced middle posterior depression of the mesoscutellum for wasps that they had the opportunity to observe. The appearance of the middle posterior depression of the mesoscutellum should be further investigated in a taxonomic review of clade IV of *Amphibolips*.

Gall patterns in clade IV

The galls of clade IV were not catalogued by Cook (1909), but he included a Mexican species (*A. palmeri*) in his group C, subdivision c (group C combines gall characteristics of prominent fibrous filaments and large intercellular spaces near the larval chamber with varying degrees of succulence in subdivisions, showing a gradient of succulence). We have observed numerous galls in Mexico, and those of subdivision c are uncommon in Mexico (Castillejos-Lemus pers. obs.). Judging by the distribution of the wasp and the gall cited for *A. palmeri* and the similarity highlighted by Beutenmüller between this and *A. trizonata*, we believe that this assignment by Cook is erroneous. We present evidence supporting the similarity of *A. trizonata* gall to that of Mexican species (Figure 1i). Consequently, we assert that within our study, galls in clade IV exhibit a tendency toward two distinct types: semispherical galls with spongy parenchyma (Figure 1e,f,i–l) or spindle-shaped galls with a firm-textured parenchyma (similar to the thick epidermis of spindle-shaped galls in clade III; Figure 1g,h). A potential intermediate form may be represented by galls with a combination of spongy parenchymal tissue and a thicker epidermis (Figure 1c). Some of these galls, especially spherical galls, often have thin radiating filaments that are challenging to observe due to the presence of spongy tissue (similar to *A. magnigalla*; Figure 1d). Although galls from other clades are present south of the United States, our observations indicate their significantly lower abundance in this region. The external appearance and structure of most galls in Mexico closely resemble those of the spongy galls of *A. confluenta* (Figure 1m).

Species complexes in the genus *Amphibolips*

An intriguing aspect of our results, including molecular species delimitation, is the positioning of various species groups within what is considered the '*nassa*' complex, such as *A. zacatecaensis*, *A. hidalgoensis*, *A. durangensis* and *A. michoacaensis*. These species are part of a group that shares many common morphological characteristics. Species differentiation has been challenging due to the significant similarity among species. For example, we included a species from the region where *A. malinche* was originally described that displays features similar to those of *A. michoacaensis*, leading us to retain this species. According to a criterion for minimal species estimation in *Amphibolips* (to avoid excessive species splitting, Christenhusz, 2020), based on our analyses this wasp (Tlax853) is conspecific with a wasp exhibiting features of *A. hidalgoensis* (Pue768_2) but also showing intermediate traits towards *A. malinche*. There are four individuals in this clade of wasps related to *A. hidalgoensis*, including the wasp from which the species was originally described (Melika et al., 2011; Ward et al., 2022). These four putative species exhibit intermediate traits of those of *A. hidalgoensis*, *A. michoacaensis* and *A. malinche* or a combination thereof. It seems that *A. malinche* does belong to another species that partly shares its distribution with *A. hidalgoensis*. We propose that *A. malinche* should still be considered a valid species based on our analyses.

The putative species obtained from the SODA analysis based on the 98% completeness matrix deserve mention as well. The most extreme cases pertain to *A. durangensis* and *A. zacatecaensis*. It is highly probable that some wasps correspond to the same species (*A. durangensis*) but are from alternate generations; thus, careful evaluation is needed to determine whether wasps belong to different generations or to an affiliated species. In the case of *A. zacatecaensis*, there is a clustering of many wasps, suggesting that this species may be synonymous with other wasps described from sites near the original species. If this is indeed the case, it is highly likely that *A. nassa* should be the valid name, and several species of *Amphibolips* should be synonyms of this. In this regard, perhaps this should be an option for what should be termed the '*nassa*' species complex, avoiding the inclusion of all other species that are clearly morphologically and genetically distinct.

CONCLUSION

In this study, we tested a comprehensive phylogenetic hypothesis covering a diverse array of species within the genus *Amphibolips* across its expansive range in North America and Central America. This in-depth exploration not only enhances our understanding of the evolutionary dynamics within the genus but also sets the stage for future investigations into the ecological and biological implications of these phylogenetic relationships. By providing a robust foundation for further studies, our findings contribute significantly to the broader context of cynipid wasp research and advance our understanding of the intricate biodiversity patterns in specific regions.

The controversies around the '*niger*' and '*nassa*' species complexes of *Amphibolips* can be resolved by following the criteria proposed by Nieves-Aldrey et al. (2012), which specifically pertain to Mexican species of *Amphibolips* and their differentiation from those of north of Mexico. A more natural approach might be to suggest that the '*nassa*' complex of species corresponds to the species in clade IV of our phylogeny or, more specifically, to the group of species clustered in one of our results around *A. zacatecaensis*.

A wide range of programmes and models have been proposed for molecular species delimitation. This study employs this approach to explore potential species boundaries within *Amphibolips*, recognising the ongoing debate about optimal methodologies. Rather than providing a definitive solution, we aim to offer a framework for integrating molecular data with other evidence sources when considering taxonomic revisions. Expanding our taxon sampling would facilitate more comprehensive phylogenetic analyses, enabling tests of hypotheses about gall development (Cook, 1909), the diversification patterns potentially linked to host plant associations (Hipp et al., 2020) and the impact of forest fragmentation on gall wasp communities (Maldonado-López et al., 2015, 2016). By understanding regional species diversification, we can identify potential centres of speciation in Mexico (Pascual-Alvarado et al., 2017).

AUTHOR CONTRIBUTIONS

Dohuglas Eliseo Castillejos-Lemus: fieldwork (main); molecular methods and phylogenetic analysis; writing (lead). **Jose-Luis Nieves-Aldrey:** fieldwork; identification of samples and taxonomy; writing (revision); **Yuanmeng Miles Zhang:** phylogenetic analysis (supporting); writing (revision); **James A. Nicholls:** phylogenetic analysis (supporting); writing (revision); **Enrique Medianero**: fieldwork, contributed samples; **Alejandra Rougon-Cardoso**: phylogenetic analysis (supporting), writing (revision); **Graham N. Stone:** writing (revision); **Ken Oyama**: funding acquisition, conceptualisation (lead), writing (revision).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The raw sequencing data generated in this study are made publicly accessible through the NCBI Short Read Archive under BioProject accession number PRJNA1149323. Data matrices and phylogenetic trees used or generated in the analyses are available on Dryad at https://doi.org/10.5061/dryad.3ffbg79tk.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Oak gall wasps (Hymenoptera: Cynipidae: Cynipini) used in this study.

Table S2. Gall wasps UCE data from previous studies that were used in this study.

Table S3. Assembly statistics for UCE contigs.

Figure S1. Maximum Likelihood tree obtained with IQ-TREE using the 50% completeness matrix without partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S2. Maximum Likelihood tree obtained with IQ-TREE using the 80% completeness matrix without partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S3. Maximum Likelihood tree obtained with IQ-TREE using the 85% completeness matrix without partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S4. Maximum Likelihood tree obtained with IQ-TREE using the 50% completeness matrix considering each UCE as partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S5. Maximum Likelihood tree obtained with IQ-TREE using the 80% completeness matrix considering each UCE as partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S6. Maximum Likelihood tree obtained with IQ-TREE using the 85% completeness matrix considering each UCE as partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S7. Maximum Likelihood tree obtained with IQ-TREE using the 50% completeness matrix using the SWSC-EN method to obtain the partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S8. Maximum Likelihood tree obtained with IQ-TREE using the 80% completeness matrix using the SWSC-EN method to obtain the partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S9. Maximum Likelihood tree obtained with IQ-TREE using the 85% completeness matrix using the SWSC-EN method to obtain the partitions. The values on the nodes correspond to SH-aLRT and UFBoot.

Figure S10. Coalescence phylogenetic tree obtained with ASTRAL-III using the 50% completeness matrix. The values on the nodes correspond to posterior local probability.

Figure S11. Coalescence phylogenetic tree obtained with ASTRAL-III using the 80% completeness matrix. The values on the nodes correspond to posterior local probability.

Figure S12. Gene and site concordance (gCF and sCF) factors test obtained with IQ-TREE using the 50% completeness matrix. The values on the nodes correspond to SH-aLRT, UFBoot, gCF, and sCF, respectively.

Figure S13. Gene and site concordance (gCF and sCF) factors test obtained with IQ-TREE using the 80% completeness matrix. The values on the nodes correspond to SH-aLRT, UFBoot, gCF, and sCF, respectively.

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