New Species Diversity Revealed from Molecular and Morphological Characterization of Gall-Inducing Calophya spp. (Hemiptera: Calophyidae) from Brazilian Peppertree

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New species diversity revealed from molecular and morphological characterization of gall-inducing *Calophya* spp. (Hemiptera: Calophyidae) from Brazilian peppertree

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Brazilian peppertree (*Schinus terebinthifolia* Raddi; Sapindales: Anacardiaceae), native to South America, is considered one of the worst upland invasive species in Florida (Schmitz et al. 1997). Rodgers et al. (2012) estimated that approx. 283,000 ha in Florida have been invaded by this weed. Its aggressive growth, in addition to allelopathic properties, results in displacement of native vegetation and changes in plant community structure (Morton 1978; Morgan & Overholt 2005). Two genetic lineages of Brazilian peppertree invaded Florida (haplotypes A and B) and extensively hybridized since their arrival (Williams et al. 2005, 2007). In the USA, Brazilian peppertree also is present in Texas, Alabama, California, Georgia, and Hawaii (EDDMapS 2014). Mechanical methods and herbicide applications are used for controlling Brazilian peppertree (Gioeli & Langeland 1997; Langeland 2001). However, these methods are unsuitable for some natural areas due to the potential for collateral damage to native species (Doren & Jones 1997), and costly maintenance programs are required to prevent regrowth (Koepp 1978). There is general agreement among public and private land managers that an ecologically-based IPM plan is needed to provide an environmentally sustainable, cost-effective, and permanent solution to Florida's Brazilian peppertree problem (Cuda et al. 2006).

Gall-inducing psyllids in the genus *Calophya* (Hemiptera: Calophyidae) are specialist herbivores, several of which are associated with trees in the genus *Schinus* in South America (Burckhardt & Basset 2000). In Brazil, *Calophya terebinthifolii* Burckhardt & Basset and *C. latiforceps* Burckhardt induce open-pit galls on the leaves of Brazilian peppertree in the states of Santa Catarina and Bahia (Burckhardt & Basset 2000; Burckhardt et al. 2011), respectively. Adults lay eggs on margins and veins of new leaves, and after 7 to 9 d, crawlers hatch and initiate gall induction on the adaxial side of the leaves. Developmental time of *C. terebinthifolii* and *C. latiforceps* from egg to adult emergence is approximately 40 d (Christ et al. 2013; Diaz et al. 2014). Damage by *C. latiforceps* nymphs is characterized by yellowing, deformation, and abscission of leaves (Diaz et al. 2014). The psyllid damages plants by reducing leaf performance and thereby affecting plant growth. In laboratory trials, psyllid attack decreased photosynthesis by 59%, chlorophyll content by 10%, leaf area by 30%, and leaf biomass by 13% compared with uninfested trees (R. Diaz, unpublished data). Host specificity tests revealed that nymphs can develop only on Brazilian peppertree, and therefore *Calophya* spp. are considered promising potential biological control agents of Brazilian peppertree in Florida.

The large latitudinal range of the *Calophya*–Brazilian peppertree association in Brazil (> 2000 km) stimulated questions about local adaptation to specific Brazilian peppertree genotypes (Cuda et al. 2012) and variation in cold tolerance. Between 2012 and 2014, we collected 50–100 individuals of *Calophya* spp. from trees in the states of Bahia, Espírito Santo, and Santa Catarina. The insects in Bahia were collected from haplotype B and K plants (Diaz et al. 2014). The trees from which *Calophya* spp. were collected in Espírito Santo and Santa Catarina were not genetically characterized but, based on the distributional information in Mukherjee et al. (2012), were likely haplotype A plants. The United States Department of Agriculture requires description of candidate weed biological control agents, which can include both morphological and molecular methods (USDA/APHIS/PPQ 2003). Therefore, the goal of this study was to characterize the *Calophya* populations maintained in the quarantine facility in Florida by using morphological and genetic methods. The *Calophya* populations examined originated from the following locations: a) Salvador: 12.908°S, 38.336°W, city of Salvador, Bahia State (Florida State Collection of Arthropods [FSCA] voucher E2013-3192-1); b) Camboriú: 26.921°S, 48.640°W city of Balneario Camboriú, Santa Catarina State (FSCA voucher E2014-5749-1); c) Carapina: 20.213°S, 40.229°W, city of Carapina, Espírito Santo State (FSCA voucher E2013-6744-1); and d) Ubu: 20.786°S, 40.579°W, city of Ubu, Espírito Santo State (FSCA voucher E2013-6743-1). Leaves containing 5th instars were placed in sturdy Ziploc® bags containing moist paper towels. Upon arrival at the quarantine facility, newly emerged adults were placed on Brazilian peppertree saplings and the 4 colonies were

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maintained separately. Insect collections and their export from Brazil were conducted under permit 12BR008156/DF from the Ministry of Environmental Resources of Brazil (Ministerio do Meio Ambiente). Importation into the United States was under USDA/APHIS/PPQ permit P526P-12-00304.

Based on morphology, adults from populations Ubu, Carapina, and Salvador were identified as *C. latiforceps* and adults from population Camboriú as *C. terebinthifolii* by Susan Halbert (Ubu, Carapina, and Salvador) and Daniel Burckhardt (Salvador). Adults were identified using descriptions from Burckhardt and Basset (2000) and Burckhardt et al. (2011), and reference vouchers from the Florida State Collection of Arthropods. Adults from populations Ubu, Carapina, and Salvador are yellow (Fig. 1A) and from Camboriú are yellow (abdomen) and black (head and thorax) (Fig. 1B). Examination of 5th instar nymphs revealed further differences. Individuals from the Ubu population had a continuous black band along the dorsal side of the head, thorax, and abdomen (Fig. 1C), whereas 5th instars of psyllid populations from Carapina and Salvador lacked dark markings (Fig. 1D). Fifth instars of the Camboriú population had 2 black bands on the dorsal side (Fig. 1E).

Deoxyribonucleic acid (DNA) was extracted from 10 individuals pooled from each population; *C. terebinthifolii*, *C. latiforceps* Salvador, Carapina, and Ubu. Degenerate cytochrome oxidase I (COI) gene primers were designed from an alignment of psyllid mitochondrial sequence submissions downloaded from GenBank that corresponded to *Pachypsylla venusta* (Osten-Sacken), *Diaphorina citri* Kuwayama, *Cacopsylla pyri* L. and *Bactericera cockerelli* (Sulc). The *P. venusta* complete mitochondrial genome (GenBank Accession: AY278317.1) was used as a reference for this alignment. The alignment was done using Sequencher 5.1 software (Gene Codes Corp., Ann Arbor, Michigan, USA). Regions with low complexity were chosen to design degenerate PCR primers for the amplification of a phylogenetically useful fragment of the COI gene (one that matches sequences from many psyllid COI genes already in GenBank). The primer sequences and the base positions relative to the *P. venusta* whole mitochondrial genome accession are as follows: Psyllid-COI-F1, 5'-GCA/CGGAGGA/TGGAGACCA/TAT-3', located at base positions 658–667; and Psyllid-CO1-R1, 5'-AGGAAA/TTTCAGAA/GTAT/ACTATG-3', located as base positions 1495–1515.

The designed primers were used in 20 µL PCR reactions conducted using 50 ng of total psyllid genomic DNA isolated from a batch of 10 psyllids of each collection using the Promega Wizard® SV Genomic DNA purification system (Promega, Madison, Wisconsin, USA). The reaction was performed in a 20 µL final volume using 18 µL of Platinum PCR supermix (Life Technologies, Grand Island, New York, USA) with 1 µL of DNA template (50 ng total template DNA) and primer concentrations of 250 nM each. Gradient 3-step PCR was performed with annealing temperatures ranging from 40 to 60 °C using the following thermal cycling conditions: 94 °C 2 min.; (94 °C 30 sec; 40–60 °C 30 sec) × 35 cycles; 72 °C 10 min. Products were analyzed by agarose gel electrophoresis, and a band corresponding to the size of the expected product (~858 bp) was observed at annealing temperatures of 44 °C for the

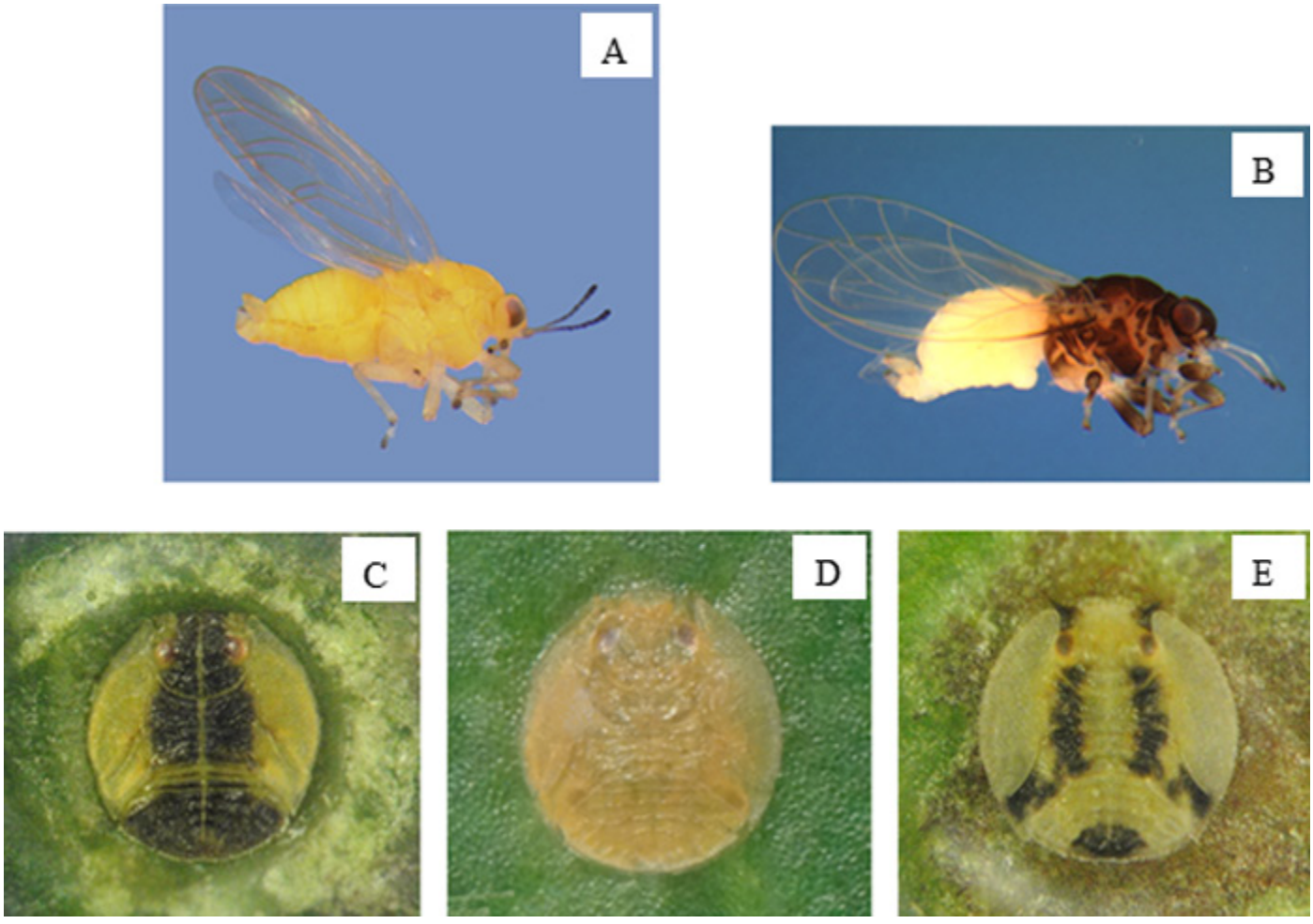

Fig. 1. Adult male *Calophya* from Ubu, Carapina, and Salvador populations (A), adult male *Calophya terebinthifolii* (B), 5th instar from Ubu (C), *Calophya latiforceps* from Salvador and Carapina (D) and *C. terebinthifolii* (E).

Table 1. Raw (below the diagonal) and model corrected (above the diagonal) pair-wise percent sequence divergences between *Calophya* populations.

	Population			
Population	Carapina	Salvador	Ubu	Terebinthifolii
Carapina		1.4-2.0	8.2-9.3	28.5-33.3
Salvador	1.3-1.8		8.2-9.7	29.7-38.7
Ubu	6.4-7.0	6.4-7.2		28.4-40.0
Terebinthifolii	13.1-13.5	13.2-14.0	13.0-14.0	

Salvador, Camboriú, and Carapina populations and 40 °C for the Ubu population. The excised fragment was purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel, Bethlehem, Pennsylvania, USA). The purified fragment was cloned into pCR®2.1-TOPO using the TOPO®TA Cloning Kit (Life Technologies, Grand Island, New York, USA) and transformed into One Shot® DH5α TOP10 chemically competent cells (Life Technologies, Gand Island, New York, USA), and the cells were plated onto Luria–Bertani (LB) medium containing kanamycin (50 µg/mL) and X-gal (40 µL per plate of a 40 mg/mL stock) as describe in the manufacturer's protocol. Twenty-four white colonies from each of the 4 populations (96 colonies total) containing inserts were transferred to 1 mL of LB liquid medium (containing 50 µg/mL kanamycin) each in a well of a 96-deep-well plate, grown overnight with shaking at 37 °C, and the bacteria were pelleted by centrifugation at 5,300 × *g* for 5 min. DNA was isolated from the pellet using a QIAprep 96 Turbo Miniprep Kit (Qiagen, Valencia, California, USA) and provided for bidirectional Sanger sequencing to an in-house sequencing core facility. Sequences were analyzed for quality, vector and primer sequence trimming, and for alignment using Sequencher 5.1 software (Gene Codes Corp., Ann Arbor, Michigan, USA). Bidirectional sequence data was obtained from 93 of the 96 colonies with the 3 failures due to either lack of colony growth in the 96-well plate or failed sequence reaction.

Sequences were contiged, traces inspected, and base calls edited using Sequencher v. 4.8 software. From the 93 sequences analyzed, 33 unique 817 bp sequences were observed, 11 each from Ubu and Salvador, 6 from Carapina, and 5 from *C. terebinthifolii*. One sample from Carapina had a frame-shift causing single nucleotide deletion (816 bp, excluded from analyses and submitted as a pseudogene to GenBank). The remaining sequences encoded the expected COI protein sequence. The consensus sequence from each population plus the most divergent Ubu clone was used for phylogenetic inference. The best-fit model of sequence evolution was determined using the Bayesian Information Criterion in jModelTest (Posada et al. 2008), and phylogenetic analysis was conducted using MRBAYES 3.2 (Ronquist et al. 2012) using 2 independent runs of 4 chains. Convergence of the posterior was assessed using the average standard deviation of split frequencies between runs (< 0.01), and samples prior to convergence were discarded as burn-in.

Monophyly of Ubu was well supported (100% of trees) and Ubu was > 6% divergent from Carapina + Salvador (Table 1). Mound et al.

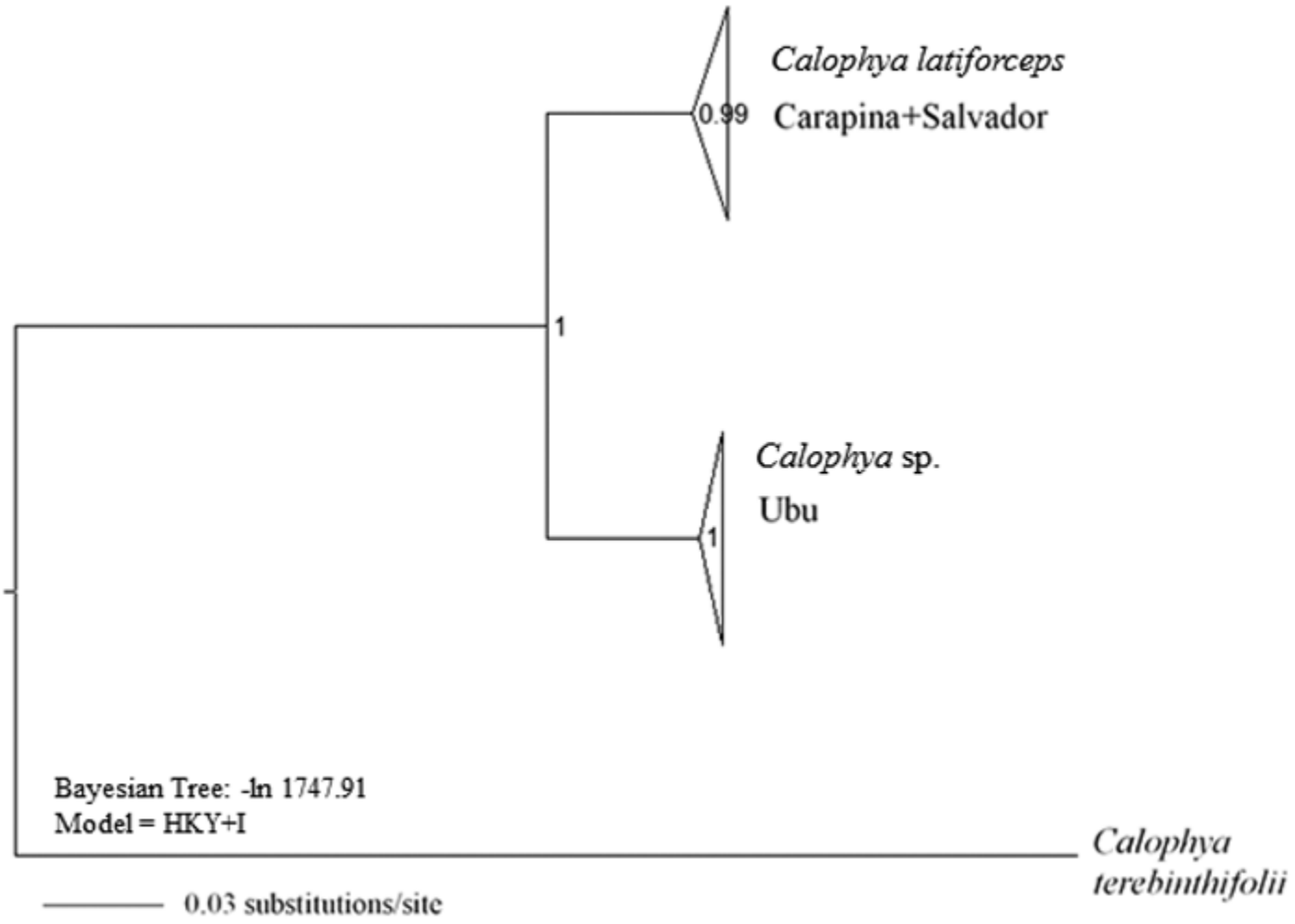

Fig. 2. Phylogenetic tree of *Calophya* spp. collected in Brazil.

(2010) described a new species of *Pseudophilothrips* based on slight morphological differences and a 4.4% molecular divergence from a previously described species. Ubu has a strikingly different nymphal coloration although there were no distinguishing features in adults. The combination of divergent color pattern in nymphs and moderate molecular divergence at the COI gene is consistent with the conclusion that Ubu represents a new species, closely related to but distinct from *C. latiforceps* (Fig. 2). To confirm Ubu's specific status, future studies will evaluate whether crossings with *C. latiforceps* produce viable offspring. Finally, *C. terebinthifolii* was 13–14% divergent from the other 3 populations, which is consistent with the 13.2–13.5% divergence reported by Burckhardt et al. (2011) between *C. latiforceps* and *C. terebinthifolii*. DNA sequences have been deposited in GenBank (Accessions KM234280–KM234312).

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Summary

Four populations of *Calophya* spp. (Hemiptera: Calophyidae) collected in Brazil were characterized using molecular and morphological methods. Examination of adults revealed the presence of 2 morphotypes, which were identified as *C. latiforceps* and *C. terebinthifolii*. However, morphological examination of 5th instar nymphs detected differences within *C. latiforceps*, with a population from Ubu, Espírito Santo, being distinct from the other 2 populations. Molecular characterization of the mitochondrial CO1 gene supported the presence of a new species of *Calophya*.

Key Words: natural enemy; Brazilian peppertree; leaflet gall inducer; phylogenetic analysis; weed biological control

Sumario

Se caracterizaron cuatro poblaciones de *Calophya* spp. (Hemiptera: Calophyidae) recolectadas en Brasil usando métodos moleculares y morfológicos. El examen de los adultos reveló la presencia de 2 morfotipos que se identificaron como *C. latiforceps* y *C. terebinthifolii*. Sin embargo, el examen morfológico de las ninfas del quinto estadio detectó diferencias dentro de *C. latiforceps*, con una población de Ubu, Espirito Santos de ser distinta de las otras dos poblaciones. La caracterización molecular del gen mitocondrial CO1 apoyó la presencia de una nueva especie de *Calophya*.

Palabras Clave: enemigos naturales, pimentero brasileño, criador de agallas en las hojas, análisis filogenética, control biológico de malezas

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