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A contribution to *Cerataphis* molecular taxonomy and ecology: the Costa Rican case

Norman BRENES-CORDERO[1], Izayana SANDOVAL-CARVAJAL[1], Nicolás PÉREZ HIDALGO[2], William VILLALOBOS MULLER[1], Xavier ESPADALER[3], Mauricio MONTERO-ASTÚA[1,4]

[1]*Centro de Investigación en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica, Costa Rica*
[2]*Instituto de Biología Integrativa de Sistemas (I 2 SysBio), Universidad de Valencia-CSIC, Spain*
[3]*CREAF, Universitat Autonoma de Barcelona, Spain*
[4]*Escuela de Agronomía, Universidad de Costa Rica, Costa Rica*

Abstract

The aphid genus *Cerataphis* Lichtenstein is native to Southeast Asia although several species have been distributed to other tropical regions of the world. *Cerataphis brasiliensis* (Hempel) and *Cerataphis lataniae* (Boisduval) are of interest due to their potential as pests on palms. Those species and *Cerataphis orchidearum* (Westwood) may be morphologically confused with each other. In Costa Rica, two species have been reported, *C. brasiliensis* and *C. orchidearum*. This work aimed to contribute molecular data and ecological observations for species of *Cerataphis* present in Costa Rica. Few colonies (low frequency of occurrence) of *C. brasiliensis* and *C. orchidearum* were recorded during a survey conducted during 2014. Ten ant species were found associated to these aphid colonies. Partial sequences for the genes *COI* and *EF-1α* were obtained and compared to the few sequences available for this genus in public databases. Species identification by *COI* (barcoding) was not conclusive. Phylogenetic analyses and similarity pairwise comparisons showed a close relationship with the genus *Tuberaphis* Takahashi, including the clustering of mixed species from both genera. Yeast-like symbionts were detected by PCR in individuals of both *Cerataphis* species found in Costa Rica. Overall, the results suggest there is a need of curated sequence data representing the different *Cerataphis* species worldwide.

Key words: phylogenetics, *COI*, *EF-1α*, *Tuberaphis*, yeast-like symbiont, ant associations.

Introduction

The aphid genus *Cerataphis* Lichtenstein (Hemiptera: Aphididae, Hormaphidinae, Cerataphidini) has an Asiatic origin but is currently distributed in tropical regions worldwide (Denmark, 1965; Aoki and Kurosu, 2010). Its dissemination was possibly due to the international trade of plants (Howard *et al*., 2001). To date, nine species have been described (Remaudière and Remaudière, 1997; Qiao and Zhang, 2001). They are associated with palms, orchids, bamboos and grasses, trees from the genus *Styrax* L., and few plant species within the families Musaceae Juss., Strelitziaceae Hutch. and Zingiberaceae Martinov (Eastop, 1966; Pérez Hidalgo *et al*., 2000; Aoki and Kurosu, 2010; Villalobos Muller *et al*., 2010).

The palm aphids, *Cerataphis brasiliensis* (Hempel) and *Cerataphis lataniae* (Boisduval), are reported as two of the aphid species that colonise palms and, along with *Cerataphis orchidearum* (Westwood), are frequently confused (Denmark, 1965; Howard *et al*., 2001; Lunz *et al*., 2011). In fact, several species are considered as junior synonyms of *C. brasiliensis*, including *Cerataphis fransseni* (Hille Ris Lambers), *Cerataphis palmae* Ghesquiere, and *Cerataphis variabilis* (Hille Ris Lambers) (Russell, 1996). Moreover, *C. brasiliensis* contains two intraspecific forms, distinguished by morphological characteristics in nymphs and adults (Mews *et al*., 2008).

Two species of *Cerataphis* have been reported in Costa Rica based on morphological characters during three independent surveys conducted throughout the country: *C. brasiliensis* and *C. orchidearum* (Voegtlin *et al*., 2003; Villalobos Muller *et al*., 2010; Zamora Mejías *et al*., 2012). Samples were carefully studied by Villalobos Muller *et al*. (2010) due to the possible misidentification of *C. brasiliensis* and *C. lataniae* and they concluded that only the former species has been found in Costa Rica.

Cerataphis species are of interest for agriculture due to the possibility of becoming plant pests. *C. brasiliensis* may develop high populations in palms and cause economic damage due to reduction of plant vigour and aesthetics through yellowing of leaves around aphid feeding sites, sooty mould (*Capnodium* sp.) growth on honeydew (affecting photosynthesis potential) and stunted growth (Denmark, 1965; Reinert and Woodiel, 1974; Howard *et al*., 2001; Josephrajkumar *et al*., 2011). Coconut palms, especially of the Malayan Dwarf type, are colonized and can be heavily infested and damaged by *C. brasiliensis* (Enobakhare and Omogiate, 2000; Josephrajkumar *et al*., 2011). In Brazil, this species may cause severe injury to the Australian Royal Palm, *Archontophoenix alexandrae* (F.Mueller) (Campos-Farinha and Zorzenon, 2005); it was also reported from native Amazonian palms, *Astrocaryum vulgare* Martus and *Astrocaryum aculeatum* G. Mey (26.6% of 263 plants evaluated) and *Euterpe oleracea* Martus and *Euterpe precatoria* Martus (7.6% of 3400 plants evaluated) (Lunz *et al*., 2011). In the case of *E. oleracea*, the açaí berry palm, a current economically important fruit, *C. lataniae* was identified as a primary potential pest (Souza and Lemos, 2004).

Different species of Auchenorrhyncha and Sternorrhyncha (Hemiptera), including aphids, establish mutualistic ecological associations with ants (Hymenoptera Formicidae). This relationship is a trophobiosis, where the insect emits a sugar-rich liquid (honeydew) and the ants in return for the feeding substance provide protection from predators and parasitoids (Delabie, 2001;

Styrsky and Eubanks, 2007). A number of species of ants have been found in relationship with *Cerataphis* spp., including *Dolichoderus bispinosus* (Olivier), *Myrmicaria* sp., *Oecophylla smaragdina* (F.), *Camponotus* sp., *Anoplolepis custodiens* F.Smith, *Polyrhachis yerburyi* Forel (Stern *et al*., 1995; Ramírez *et al*., 2001; Styrsky and Eubanks, 2007). Nevertheless, reports of ant-aphid relationships in Costa Rica are limited (Espadaler *et al*., 2012).

Likewise, symbionts have an essential role in the ecology and nutrition of insects. Aphids are no exception and have primary or multi-partner relationships and secondary symbionts that historically increased the diversification of the lineage by allowing them to move into previously unexplored niches. The primary endosymbiont in most aphid species is the bacterium *Buchnera aphidicola* (Baumann *et al*., 1995; Douglas *et al*., 2006; Rothacher *et al*., 2016; Sudakaran *et al*., 2017). However, in the evolution of the tribe Cerataphidini, some species have lost the *Buchnera* endosymbiont, which was replaced by a yeast-like extracellular symbiont (YLS) found in the haemocoel and the fat body. Among the *Cerataphis* genus, several species carry the YLS, including *C. brasiliensis*, *C. lataniae*, and *C. orchidearum*. Meanwhile, two species have been found to carry *Buchnera*: *Cerataphis bambusifoliae* Takahashi and *Cerataphis vandermeermohri* (Hille Ris Lambers) (Fukatsu *et al*., 1994; Aoki and Kurosu, 2010).

The purpose of this work was to improve the information available for the genus *Cerataphis* by generating sequence data and reporting ecological observations on endosymbionts and ant associations from Costa Rican samples. The information is a contribution towards a comprehensive understanding of the molecular taxonomy and ecology of this genus.

Materials and methods

Sample collection and relative frequency estimation

Eleven aphid colonies preliminary identified as belonging to the genus *Cerataphis* were found during a general aphid survey throughout Costa Rica during 2014. *Cerataphis* individuals were obtained from different host plants in the provinces of San José, Puntarenas and Limón (table 1). Aphid samples were maintained at −35 °C in 95% ethanol and at 6 °C in 70% ethanol for molecular and morphological analyses, respectively. Morphological descriptions of the species *C. brasiliensis* and *C. orchidearum* in Costa Rica are available from Voegtlin *et al.* (2003). In the event that ants were observed within the aphid colony, ant specimens were also collected and stored at 6 °C in 70% ethanol for morphological identification.

Relative frequency (number of samples of interest / total number of samples) of the genus *Cerataphis* in relation to all the aphid samples (= colonies) was estimated for this work and two previous reports (Villalobos Muller *et al*., 2010; Zamora Mejías *et al*., 2012). Each plant host location record for a species was counted as an independent colony/sample.

DNA extraction and PCRs

DNA extraction from individual aphid specimens (n = 34) proceeded accordingly to the animal tissue protocol of NucleoSpin Tissue extraction kit (Macherey-Nagel, Germany) with a modification at the elution step: two centrifugation steps were performed, using 50 µL of elution buffer each time. We aimed to sequence three individuals per colony, however, amplification or sequencing failed on occasion and more than three individuals were processed for some colonies.

Two genes were targeted for molecular identification and phylogenetic analysis of *Cerataphis* spp. samples: i) cytochrome c oxidase subunit I (*COI*) and ii) elongation factor 1α (*EF-1α*). To amplify *COI*, primer pairs C1-J-1490 (= LepF) and C1-N-2198 (= LepR) (Hajibabaei *et al*., 2006; Miller and Halbert, 2014), or LCO1490 and HCO2198 (Folmer *et al*., 1994) were used to obtain a 658 bp fragment corresponding to region c of *COI*. Additionally, we used primers C1-J-1718 (Simon *et al*., 1994) and C1-J-2411 (Lagos *et al*., 2012), which amplified region b and partial region a of *COI* (868 bp). *EF-1α* gene was amplified using primers EF-3 and EF-6 to generate a fragment of 785 bp (Miller and Halbert, 2014) and alternatively a nested PCR protocol was used (Pérez-Hidalgo *et al*., 2012). PCR reactions were carried out using final volume of 25 µL with final concentration of 1X Dream Taq Master Mix (2X, Thermo Scientific, Lithuania), 200 nM of each primer and 5 µL of DNA. Additionally, trehalose (1% final concentration) was added to PCR reactions for *COI*. Reactions were run with the following thermocycler profiles: for primers C1-J-1490 / C1-N-2198 and LCO1490 / HCO2198: 94 °C × 1 minute; 5 × (94 °C × 40 seconds, 45 °C × 40 seconds, 72 °C × 1 minute); 35 × (94 °C × 40 seconds, 51 °C × 40 seconds, 72 °C × 1 minute); 72 °C × 5 minutes (Hajibabaei *et al*., 2005). For primers C1-J-1718 / C1-J-2411: 96 °C × 2 minutes; 40 × (95 °C × 30 seconds, 53 °C × 30 seconds, 72 °C × 2 minutes); 72 °C × 10 minutes (Lagos *et al*., 2012). For *EF-1α* gene: 94 °C × 2 minutes; 38 × (94 °C × 30 seconds, 50 °C × 1 minute, 72 °C × 30 seconds); 72 °C × 7 minutes for primers EF-3 and EF-6 (von Dohlen *et al*., 2002), and for the Nested-PCR: 94 °C × 1 minute; 40 × (94 °C × 30 seconds, 50 °C × 1 minute (52 °C for the second reaction), 68 °C × 1.5 minute); 68 °C × 7 minutes (Pérez-Hidalgo *et al*., 2012).

Sequencing and molecular identification

Amplicons of *COI* and *EF-1α* were directly sequenced by Sanger method (Macrogen, Korea) using the forward and reverse primers for each gene. The final contigs were obtained using BIOEDIT 7.0 (Hall, 1999). The resulting sequences were assigned preliminarily (July 15th, 2020) to a species by alignment using the BLAST tool of NCBI (Altschul *et al*., 1990) and the Identification Engine tool ("Search Databases" option: "Species Level Barcode Records" or "All Barcode Records on BOLD") at the Barcode Of Life Data system, BOLD (Ratnasingham and Hebert, 2007).

Pairwise similarity values (percentage) were estimated and averaged at the species and genera level for *Cerataphis* and *Tuberaphis*. An alignment including all available (to July 14th, 2020) *COI* sequences in GenBank

Table 1. Collecting data of *Cerataphis* spp. Lichtenstein 1882 colonies in Costa Rica and GenBank accession numbers of DNA sequences.

Species	Colony ID[(a)]	Locality Municipality/Province	Altitude (m a.s.l.)	Host plant	YLS[(b)] detection	GenBank accession numbers (a, …, f = aphid individuals)[(c)]	
						COI	*EF-1α*
Cerataphis brasiliensis	CR14-006[(d)]	Goicoechea, San José 9.937272°N 84.073308°W	1173	*Dypsis lutescens* (H.Wendl.) Beentje et J.Dransf.	a. + b. + c. +	a. MT266526 b. MT266527 c. MT266528	a. MT281379 b. MT281380 c. MT281381
Cerataphis brasiliensis	CR14-008	Curridabat, San José 9.912053°N 84.0215°W	1234	*Dypsis lutescens*	a. + b. + c. NT d. +	a. MT266529 b. MT266530 c. MT266531 d. MT266532	a. MT281382 b. MT281383 c. --- d. MT281384
Cerataphis brasiliensis	CR14-026	San José, San José 9.935819°N 84.073656°W	1169	*Alpinia purpurata* (Vieill.) K.Schum.	a. neg. b. + c. + d. +	a. MT266533 b. MT266534 c. MT266535 d. ---	a. MT281385 b. MT281386 c. MT281387 d. MT281388
Cerataphis brasiliensis	CR14-162	Buenos Aires, Puntarenas 9.171944°N 83.334722°W	360	*Cyrtostachys renda* Blume	a. + b. + c. +	a. MT266536 b. MT266537 c. MT266538	a. MT281389 b. MT281390 c. MT281391
Cerataphis brasiliensis	CR14-260	Pococí, Limón 10.551388°N 83.507500°W	7	*Bismarckia nobilis* Hildebr. et H.Wendl.	a. + b. + c. +	a. MT266539 b. MT266540 c. MT266541	a. MT281392 b. MT281393 c. MT281394
Cerataphis brasiliensis	CR14-305	Montes de Oca, San José 9.939722°N 84.042500°W	1229	*Dypsis lutescens*	a. NT b. NT c. NT d. + e. neg. f. +	a. MT266542 b. MT266543 c. MT266544 d. MT266545 e. MT266546 f. MT266547	a. MT281395 b. MT281396 c. MT281397 d. MT281398 e. --- f. MT281399
Cerataphis orchidearum	CR14-396	San José, San José 9.921666°N 84.078333°W	1135	*Guarianthe skinneri* (Bateman) Dressler et W.E.Higgins	a. neg. b. neg. c. neg.	a. MT266553 b. MT266554 c. MT266555	a. MT281405 b. MT281406 c. MT281407
Cerataphis orchidearum	CR14-400[(d)]	San José, San José 9.921666°N 84.078333°W	1135	*Lycaste* sp.	a. neg. b. neg. c. neg.	a. --- b. MT266556 c. MT266557	a. MT281408 b. MT281409 c. MT281410
Cerataphis brasiliensis	AE	Parrita, Puntarenas 9.529233°N 84.476502°W	2	*Cocos nucifera* L.	a. + b. neg.	a. MT266548 b. MT266549	a. MT281400 b. MT281401
Cerataphis orchidearum	ASC	San José, San José 9.921666°N 84.078333°W	1135	*Paphiopedilum* sp.	a. neg. b. neg. c. neg.	a. MT266550 b. MT266551 c. MT266552	a. MT281402 b. MT281403 c. MT281404

[(a)] An additional *C. brasiliensis* colony (CR14-261) was also collected on the palm *C. renda* at Pococí, Limón (10.551388°N 83.507500°W); but no individuals were processed for molecular analyses.
[(b)] Nested-PCR result for yeast-like symbiont detection; + = positive sample; neg. = negative sample; NT = not tested.
[(c)] --- = no amplification or sequencing failure, repeatedly.
[(d)] No ants were observed associated to these colonies during sample collection.

or BOLD for those genera was analysed in MEGA X (Kumar *et al*., 2018) to calculate genetic distance (a matrix of pairwise comparisons) using the p-distance method and 2000 repetitions. The values were transformed to percentage and subtracted from 100 to obtain an estimation of similarity.

Phylogenetic analyses

All the available sequences for *COI* (region *c* = barcoding region), *EF-1α* and a fragment expanding from 3'-end of *COI* (region *a*) through Leucine tRNA and finishing in the 5'-end of *COII* (cytochrome oxidase subunit II) (abbreviated herein *COI*-tRNA-*COII*) for *Cerataphis* and *Tuberaphis* were retrieved (July 14th, 2020) from public databases, GenBank or BOLD. Additionally, several sequences for those genomic regions were obtained for species of the genera *Astegopteryx* Karsch (Hemiptera Aphididae) and *Aleurodaphis* van der Goot (Hemiptera Aphididae), to be used as outgroup clades (supplemental material table S1). The sequences obtained herein (*COI* and *EF-1α*) and those downloaded from public databases (*COI*, *EF-1α* and *COI*-tRNA-*COII*) for each genomic region were aligned using the algorithm MUSCLE (Edgar, 2004) in MEGA X. A few sequences for each gene resulted shorter in comparison to other sequences and were eliminated. Alignments were cropped to eliminate overhangs on both ends and used for phylogenies. The final alignments consisted in, *COI*: 39 sequences and 617 nucleotide positions; *EF-1α*: 19 sequences and 1030 positions, and *COI*-tRNA-*COII*: 18 sequences and 810 positions. Appropriate model for analysis was identify with model test tool in MEGA X. Phylogenetic analyses were run using the Maximum Likelihood (ML), Neighbor Joining (NJ) and Parsimony (P) approaches in MEGA X. As a test of confidence, a bootstrap method with 2000 iterations was selected for each analysis. The Gamma parameter value used for NJ was estimated from the corresponding ML analysis. Additionally, a Bayesian analysis was also run with MrBayes v.3.2.7 (Huelsenbeck and Ronquist, 2001), using a mixed model approach and a

rate of nucleotide substitutions following a Gamma distribution with a proportion of invariable sites (GammaInv) and 10^7 generations (iterations).

Screening for the presence of symbionts

The presence of bacterial endosymbionts *Buchnera* and *Wolbachia*, and of a eukaryotic yeast-like symbiont (YLS) was determined for 30 individual aphids (table 1), except for CR14-008_c, CR14-305_a, b, and c (the corresponding DNA was consumed in previous PCR reactions). PCR reactions were carried out as described in previous section. *Buchnera* and *Wolbachia* were tested with genus-specific primers designed to amplify 16S gene fragments: Buch16S1F and Buch16S1R (Tsuchida *et al*., 2002); and W-Specf and W-Specr (Werren and Windsor, 2000), respectively. Thermocycler profile for *Buchnera* detection was 95 °C for 4 minutes; 40 × (95 °C × 30 seconds, 55 °C × 30 seconds, 72 °C × 30 seconds);72 °C × 10 minutes (Tsuchida *et al*., 2002), and for *Wolbachia* was 95 °C × 2 minutes, 2 × (95 °C × 2 minutes, 60 °C × 1 minute, 72 °C × 1 minute), 35 × (95 °C × 30 seconds, 60 °C × 1 minute, 72 °C × 45 seconds); 72 °C × 5 minutes (Werren and Windsor, 2000). Positive controls were included in all reactions, for *Buchnera* DNA samples from *Aphis sambuci* L. (AS7_B, GenBank accession number MT647615) and *Micromyzus pojanii* Cermeli et Smith (MC11_B, MT647616); and for *Wolbachia*: *A. sambuci* (6_W, MH427174), *Neophyllaphis varicolor* Miller et Halbert (NV364A_F_W, MH791167) and *Aphis citricidus* (Kirkaldy) (26_W, MH427167).

Presence of a YLS was determined using a nested-PCR with primer pairs UO1/UO3' and UOR1/UOR2 (Hongoh and Ishikawa, 2000). The consensus primers UO1 and UO3' were used for general amplification of fungal uricase genes, a 980 bp fragment. The specific amplification of uricase genes of fungal symbionts was accomplished with primer pair UOR1 and UOR2 using as template the UO1/UO3'amplification product, to obtain a 784 bp fragment (Hongoh and Ishikawa, 2000). Both rounds of amplification were run with the following thermocycler profile: 94 °C × 1 minute; 35 × (94 °C × 1 minute, 59 °C × 2 minutes, 70 °C × 2 minutes); 72 °C × 10 minutes. Most delphacids harbour yeast-like symbionts (Noda, 1977; Suh *et al*., 2001), thus DNA samples from four specimens: *Peregrinus maidis* (Ashmead), *Tagosodes* sp., and two *Sogatella* sp. were included as positive controls. Selected amplicons were sequenced as previously described for *COI* and *EF-1α*.

The sequences obtained for YLS in this work were aligned with several corresponding sequences in GenBank and a phylogenetic analysis was conducted following similar procedures and software as mentioned before, gaps and missing data deleted, ML method with Hasegawa-Kishino-Yano model and a discrete Gamma distribution - 5 categories (+*G*, parameter = 1.2538). There was a total of 499 positions in the final dataset.

Results and discussion

Two *Cerataphis* spp. are found in Costa Rica

Morphological identification confirms the occurrence of two species in Costa Rica: *C. brasiliensis* and *C. orchidearum*. *C. lataniae* has been recorded from several countries in the Americas, including the USA, Cuba, Dominican Republic, Jamaica, Colombia, Brazil, among others (CABI, 2020). However, a taxonomic study in Brazil indicated that *C. lataniae* was erroneously reported, and the correct species is *C. brasiliensis* (Lunz *et al*., 2011). Considering the ease with which the species are confused, additional studies are needed to confirm or refute the existence of *C. lataniae* from other localities in the Americas where it has been reported. Only *C. brasiliensis* and *C. orchidearum* have been reported from Honduras, Panama, Colombia, Venezuela and Guadeloupe island (Cermeli, 1989; Evans and Halbert, 2007; Quirós *et al*., 2009; Meurgey and Ramage, 2020; Simbaqueba and Cardona, 2021). To our knowledge, those three (or possibly two) species constitute the introductions of *Cerataphis* spp. to the neotropics.

Cerataphis colonies have a low frequency of occurrence in Costa Rica

The survey conducted during 2014 targeted aphid colonies in general, visiting different locations throughout Costa Rica. Only 11 colonies of 450 colonies sampled were identified as *Cerataphis* spp. (figure 1). This frequency of occurrence and data from two previous surveys in the country (table 2), allowed us to estimate a relative frequency of occurrence of *Cerataphis* colonies in relation to all aphid colonies at less than 2.5% (mean 1.8%), which suggests that this genus represents a small fraction of the aphid fauna found in Costa Rica.

Although, reports indicated *C. brasiliensis* as a potential pest in palms in Florida and Brazil (Denmark, 1965; Reinert and Woodiel, 1974; Howard *et al*., 1998; Campos-Farinha and Zorzenon, 2005; Lunz *et al*., 2010; 2011),

Table 2. Estimated relative abundance of *Cerataphis* spp. colonies in Costa Rica.

Survey	No. *Cerataphis* samples / total	Frequency	Colony resampling[(a)]	Reference
2014	11 / 450	0.0244	A	This work
2008-2009	5 / 218	0.0229	B	Zamora Mejías *et al*., 2012
2008	5 / 650	0.0077	A, B	Villalobos Muller *et al*., 2010
Total	21 / 1318	0.0159	2 colonies	

[(a)] Possible resampling of an aphid colony. Resampling inferred from a similar host-location record. Same letter indicates a possibly repeated colony between studies. A = *Alpinia purpurata*, San José, San José, and B = *Chamaedorea costaricana*, San José, San Pedro de Montes de Oca.

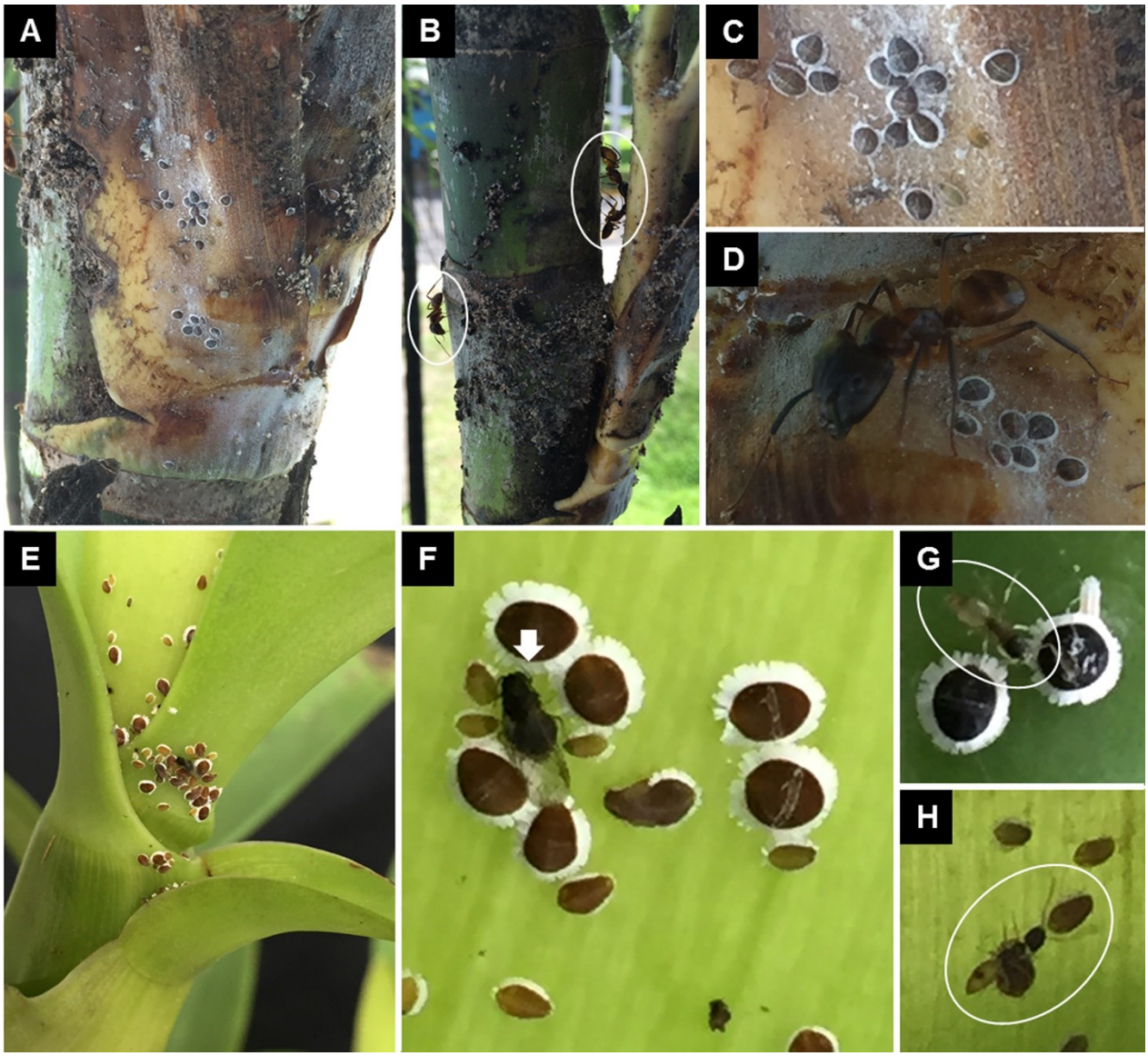

Figure 1. *C. brasiliensis* (**A-D**) colony CR14-305 on *D. lutescens* and *C. orchidearum* (**E-H**) colony CR14-396 on *G. skinneri*. Ants were observed associated to both colonies (**D** and white oval in **B**, **G** and **H**). Few winged adults were observed (white arrow in **F**).

the proportion of *Cerataphis* colonies recorded during several surveys in Costa Rica suggested a low frequency/occurrence of these aphids in the country (table 2). Moreover, we are not aware about reports from nurseries or landscaping companies indicating infestations on neither ornamental palms nor orchids.

Several ant species are associated to *Cerataphis* colonies in Costa Rica

Ants were observed walking around and within aphid colonies, and in some instances tapping with their antennae the aphids' abdomen. Therefore, the data of those observations was collected and ants identified (table 3). A total of 10 species of ants were observed associated with nine *Cerataphis* colonies (seven of *C. brasiliensis* and two of *C. orchidearum*). Two ant species, *Paratrechina longicornis* (Latreille) (Formicinae) and *Solenopsis geminata* (F.) (Myrmicinae) were found associated to three different *C. brasiliensis* colonies. Five aphid colonies were associated with a single ant species. Three *C. brasiliensis* colonies were associated with two ant species simultaneously and one colony was associated with three ant species (table 3).

Espadaler *et al.* (2012) conducted the first and only study available on aphid-ant associations for Costa Rica and by extension for the Central American region. In this study, *C. brasiliensis* and *C. orchidearum* were associated to eight and two ant species, respectively. Moreover, a literature review evidenced a tendency of *Cerataphis* to associate with diverse species of ants (supplemental material table S3). To the best of our knowledge, eight of the ten species reported herein are new ant records associated to *Cerataphis* colonies. These results and previous reports (supplemental material table S3) are in accordance with Stern *et al*. (1995) who concluded that colonies of *C. brasiliensis* (= *C. fransseni* in their paper) on secondary hosts (the samples included in this work) require to be tended by ants.

Table 3. Associations between ants and *Cerataphis* spp. observed during this work in Costa Rica.

Aphid species	Colony	Ant species[a]	*N*[b]
C. brasiliensis	CR14-008	*Camponotus striatus* (F.Smith)[NR]	1
"	"	*Pheidole radoszkowskii* Mayr[NR]	3
"	CR14-026	*Brachymyrmex heeri* Forel[NR]	1
"	"	*Wasmannia auropunctata* (Roger)	6
"	CR14-162	*Brachymyrmex obscurior* Forel[NR]	3
"	"	*Paratrechina longicornis* (Latreille)[NR]	2
"	CR14-260	*Solenopsis geminata* (F.) (black form)[NR, (c)]	14
"	CR14-261[(d)]	*Solenopsis geminata* (F.) (black form)	7
"	CR14-305	*Camponotus planatus* Roger[NR]	4
"	"	*Paratrechina longicornis* (Latreille)	1
"	"	*Solenopsis geminata* (F.) (black form)	7
"	AE	*Paratrechina longicornis* (Latreille)	1
C. orchidearum	CR14-396	*Tapinoma melanocephalum* (F.)	1
"	ASC	*Pheidole megacephala* (F.)[NR, (e)]	2

[(a)] NR = new record of an ant species with a *Cerataphis* species association.
[(b)] Number of ant individuals observed during sampling of each aphid colony.
[(c)] The genus *Solenopsis* without the identification of the species was reported associated to *C. brasiliensis* (Josephrajkumar *et al*., 2011; Lunz *et al*., 2011) (supplemental material table S3).
[(d)] Colony collected on the palm *C. renda* at Pococí, Limón (10.551388°N 83.507500°W).
[(e)] *Pheidole megacephala* has been reported previously associated with *C. brasiliensis* (Espadaler *et al*., 2012) and *C. lataniae* (Zimmerman, 1948) (supplemental material table S3).

Molecular identification with *COI* and *EF-1α* sequences was inconclusive

The genus *Cerataphis* originates from southeast Asia, as thus the few sequences available are mainly from China and India, and few from Malaysia, Taiwan or Vietnam. In this work, we obtained partial sequence data for the mitochondrial *COI* and the nuclear *EF-1α* genes (table 1) for colonies of *C. brasiliensis* and *C. orchidearum* in the neotropics. For the later species, those represent the only sequences available in public databases until the preparation of this manuscript. A total of 32 *COI* and 32 *EF-1α* sequences were obtained and were submitted to GenBank (table 1).

Using the identification tool service in BOLD with *COI C. brasiliensis* sequences from Costa Rica, the result was: "a species level match could not be made, the queried specimen is likely to be one of the following: *C. brasiliensis*, *C. lataniae*, *Tuberaphis xinglongensis*" (identification engine results with database Species Level Barcode Records). The queried sequences had matches with identities >98% with those species. Nevertheless, the highest identity hit was with *C. brasiliensis*. In the case of sequences corresponding to *C. orchidearum*, using the "Species Level Barcode Records" database, the system

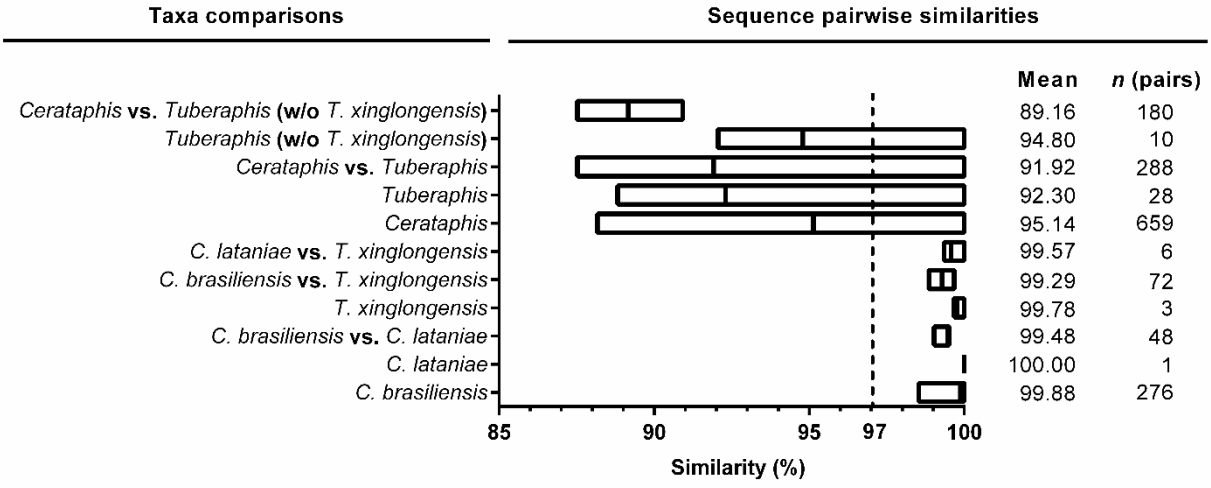

Figure 2. Average sequence similarity of pairwise comparisons for *COIc* (617 bp) for selected taxa in genera *Cerataphis* and *Tuberaphis*. Similarities calculated from a matrix of pairwise genetic distance (p-distance in MEGA X). The range (minimum to maximum value, horizontal bar), average pairwise similarity (Mean) and total number of pairwise comparisons (*n* pairs) are shown for each species or genera comparison of interest. "w/o" = without the sequences corresponding to *T. xinglongensis*.

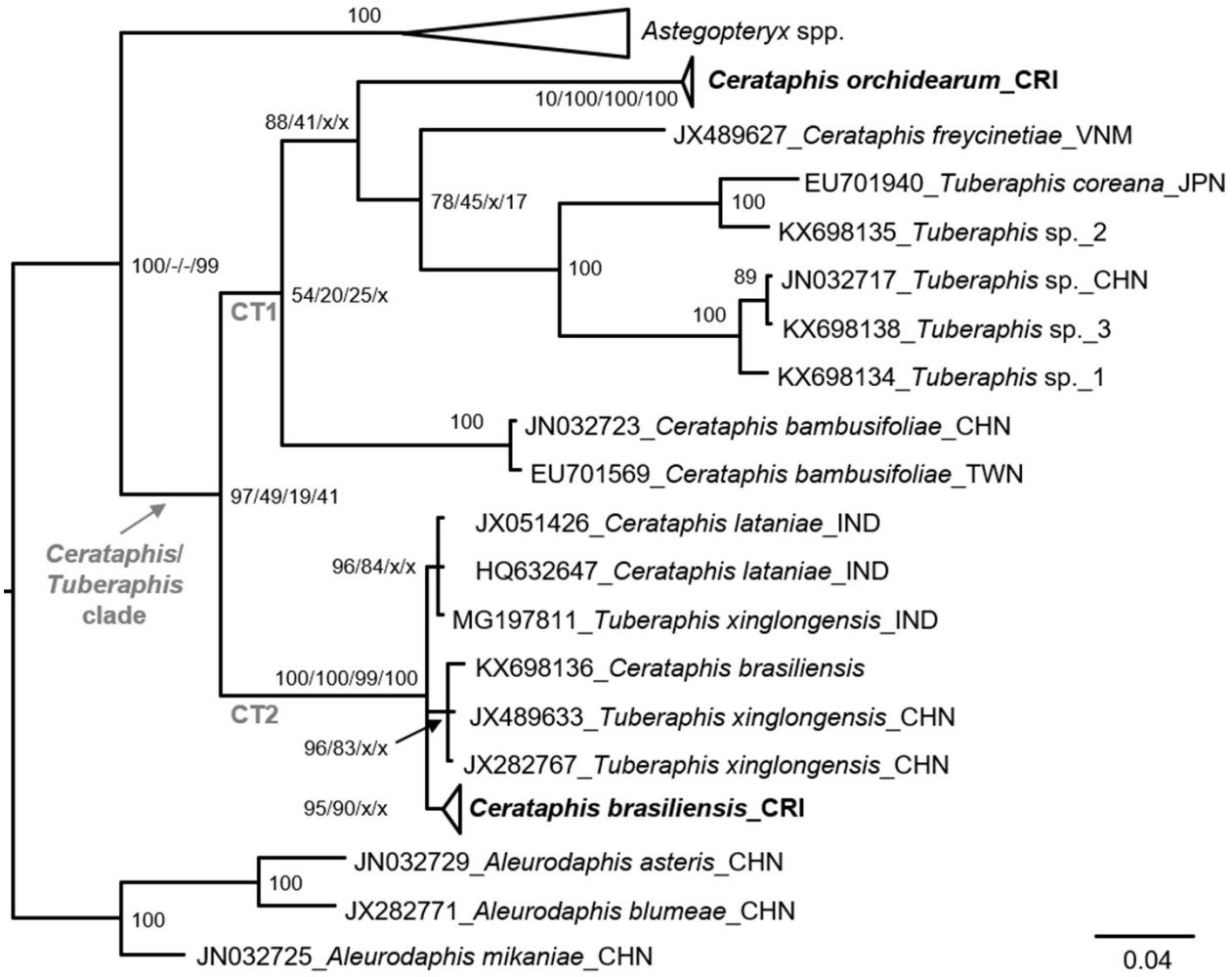

Figure 3. Phylogenetic tree for partial *COI* gene available sequences of the genera *Cerataphis* and *Tuberaphis.* Phylogeny inferred by Bayesian (B) method in MrBayes (v. 3.2.7a) with a mixed model, and a Gamma distribution with a proportion of invariable sites was used to model evolutionary rate differences among sites. The alignment was also analysed by Maximum Likelihood (ML), Neighbor Joining (NJ) and Parsimony (P) methods in MEGA X. Node confidence values (percentages): posterior probability for B and bootstrap values for ML, NJ and P are indicated for selected nodes (B / ML / NJ / P); "×" = a different node topology was obtained with the corresponding method; and hyphen ("-") = a similar node was obtained, but no bootstrap value calculated. CT1 and CT2 were used to designate two subclades containing *Cerataphis* and *Tuberaphis* sequences. Clades with Costa Rican sequences for *C. brasiliensis* (MT266526-MT266532, MT266534-MT266549) and *C. orchidearum* (MT266550-MT266555, MT266557), and a representation of the genus *Astegopteryx* (AGIRI114_17, EU701518, HQ112196, JN032708, JX489624-JX489626) were collapsed. Representative sequences of the genera *Astegopteryx* and *Aleurodaphis* were included as outgroups. Geographic location (when available) is indicated by three-letter ISO country codes: CHN, China; CRI, Costa Rica; IND, India; JPN, Japan; TWN, Taiwan; VNM, Viet Nam. Scale bar represents the number of substitutions per site.

was unable to match any records (= "no results"). Repeating the search with "All Barcode Records on Bold" database rendered a single match to *Cerataphis* ("no species, an early-release record") with 99.5% similarity, and the following hits corresponded to *Cinara* spp. with identities <92%. BLAST searches on GenBank resulted also in matches with different species or aphid genera.

Due to the conflicting results obtained in comparisons within databases, sequences available of *Cerataphis* and *Tuberaphis* were analysed by pairwise percentage of similitude; the average values within a taxon were compared between the species or genera of interest (figure 2). A 97% of sequence similarity is considered a threshold for delimitation of aphid species (Hebert *et al*., 2003; Foottit *et al*., 2008). Therefore, our starting expectation for the analysis was that pairwise comparisons and the corresponding average of several comparisons of the same species should have a value equal or greater than 97%. Conversely, pairwise comparisons between two distinct species within the same genera, should have similarity values near or below the 97% threshold. Thus, the average value of individual pairwise comparisons between two distinct genera, should be definitively below 97% similarity. Using the sequences available in databases

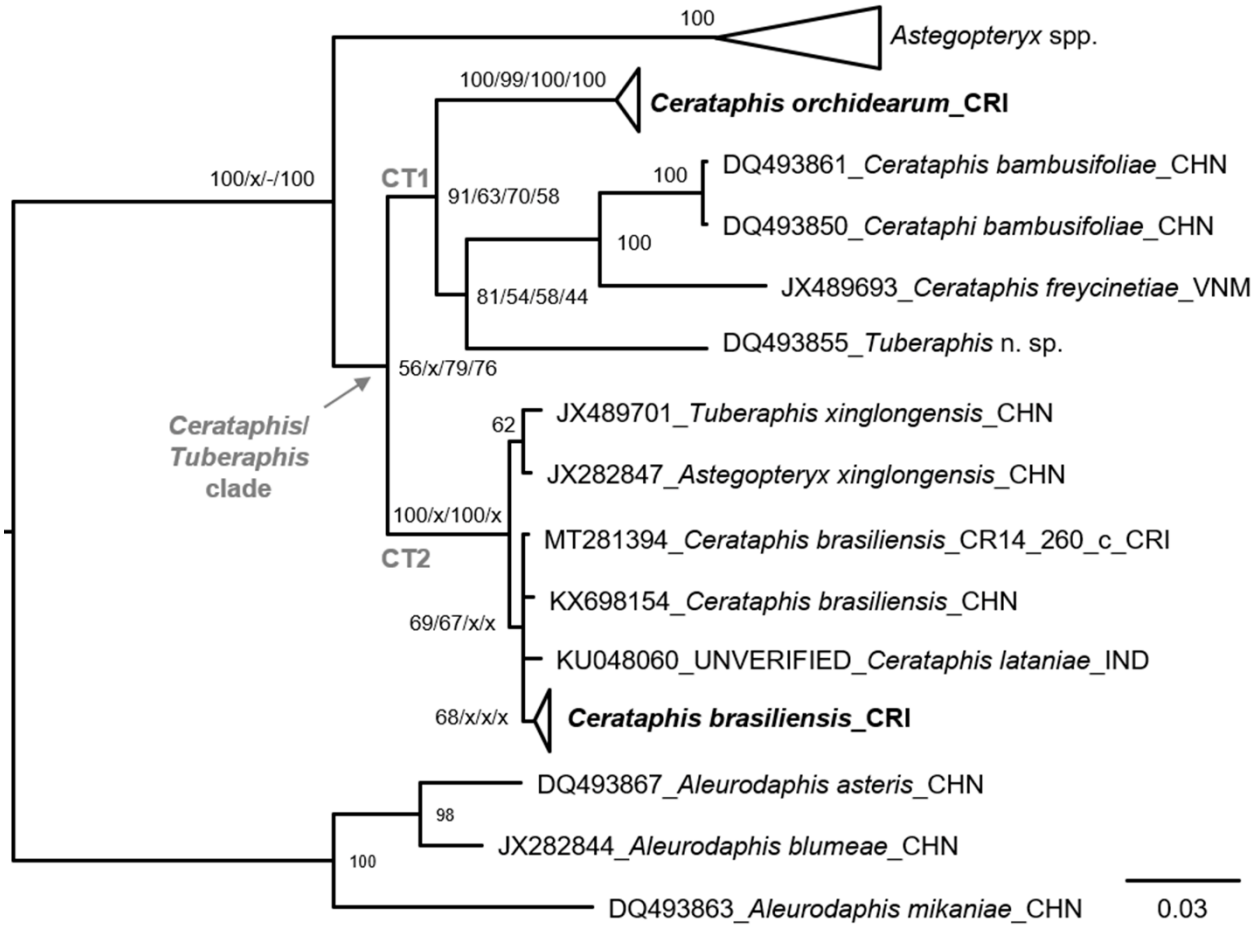

Figure 4. Phylogenetic tree for partial *EF-1α* gene available sequences of the genera *Cerataphis* and *Tuberaphis.* Phylogeny inferred by Bayesian (B) method in MrBayes (v. 3.2.7a) with a mixed model, and a Gamma distribution with a proportion of invariable sites was used to model evolutionary rate differences among sites. The alignment was also analysed by Maximum Likelihood (ML), Neighbor Joining (NJ) and Parsimony (P) methods in MEGA X. Node confidence values (percentages): posterior probability for B and bootstrap values for ML, NJ and P are indicated for selected nodes (B / ML / NJ / P); "×" = a different node topology was obtained with the corresponding method; and hyphen ("-") = a similar node was obtained, but no bootstrap value calculated. CT1 and CT2 were used to designate two subclades containing *Cerataphis* and *Tuberaphis* sequences. Clades with Costa Rican sequences for *C. brasiliensis* (MT281380, MT281381, MT281383, MT281385, MT281387, MT281389, MT281390, MT281395-MT281397, MT281399-MT281401, also includes a sequence from Spain, FM174690) and *C. orchidearum* (MT281402, MT281404-MT281408, MT281410), and a representation of the genus *Astegopteryx* (DQ493848, JX489690, MK028326, MK028330, MK028332, JX489691, JX489692) were collapsed. Representative sequences of the genera *Astegopteryx* and *Aleurodaphis* were included as outgroups. Geographic location (when available) is indicated by three-letter ISO country codes: CHN, China; CRI, Costa Rica; IND, India; VNM, Viet Nam. Scale bar represents the number of substitutions per site.

and those obtained herein, the similarity between the species *C. lataniae* (two sequences) and *C. brasiliensis* (24 sequences; 23 from this work) was higher than 99% for every pairwise comparison (figure 2). Likewise, the range of similarity values between sequences from the genus *Cerataphis* and *Tuberaphis* was from 87.52 to 100% (figure 2). Those values denoted that one or several sequences registered in the databases as a species of *Tuberaphis* spp. have similarities higher than 97% and up to 100% similarity to one or several sequences registered as a species of *Cerataphis* spp. Therefore, the results imply either i) there are *COI* sequences misidentified in the databases; ii) or, if the sequences come from correctly identified individuals, the morphological taxonomy and separation of *Tuberaphis* and *Cerataphis* is not in accordance with the molecular data, which may indicate that the *COI* region has low efficiency to resolve members of those two genera, various species are misplaced in the two genera, or the two genera should be synonymized.

Phylogenetic analyses

In most phylogenetic trees (10/12 dendrograms) generated with either *COI*, *EF-1α* or *COI*-tRNA-*COII* two sister clades are distinguished (figures 3, 4 and 5), one branch (labelled CT1) included sequences corresponding to several *Cerataphis* and *Tuberaphis* species including *C. orchidearum*. The second branch (labelled CT2) included exclusively *C. brasiliensis*, *C. lataniae* and *Tuberaphis*

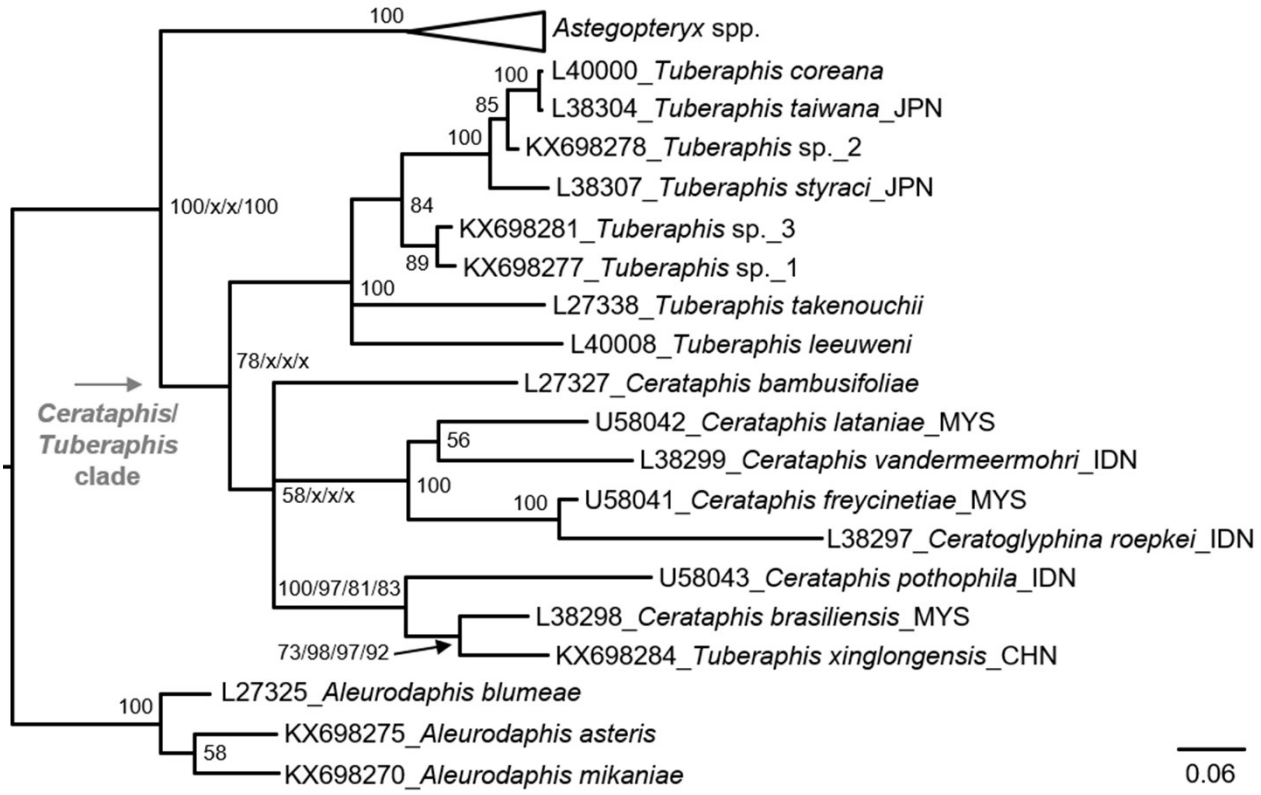

Figure 5. Phylogenetic tree for partial sequences expanding from 3'-end of *COI* (region *a*) through Leucine tRNA and finishing in the 5'-end of *COII* (*COI*-tRNA-*COII*) of the genera *Cerataphis* and *Tuberaphis.* Phylogeny inferred by Bayesian (B) method in MrBayes (v. 3.2.7a) with a mixed model, and a Gamma distribution with a proportion of invariable sites was used to model evolutionary rate differences among sites. The alignment was also analysed by Maximum Likelihood (ML), Neighbor Joining (NJ) and Parsimony (P) methods in MEGA X. Node confidence values (percentages), posterior probability for B and bootstrap value for ML, NJ and P indicated for selected nodes (B / ML / NJ / P); "×" = a different node topology was obtained with the corresponding method; and hyphen ("-") = a similar node was obtained, but no bootstrap value calculated. Representative sequences of the genera *Astegopteryx* (collapsed, includes L27324, L27326, L39992, U58038, KX698283) and *Aleurodaphis* were included as outgroups. Geographic location (when available) is indicated by three-letter ISO country codes: IND, India; JPN, Japan; MYS, Malaysia. Scale bar represents the number of substitutions per site.

xinglongensis (Zhang) sequences that were not represented in CT1. Thus, the genera *Cerataphis* and *Tuberaphis* were mixed in two branches of the dendrograms. Only in two out of 12 resulting dendrograms, the genera were found in two mutually exclusive branches, except for *T. xinglongensis* that consistently grouped (for three genetic markers evaluated), close to *C. brasiliensis* and distant to all other available *Tuberaphis* species sequences. Moreover, in three of the 12 dendrograms the *Astegopteryx* clade is not external to the *Cerataphis*-*Tuberaphis* clades (CT1 and CT2).

Unexpectedly, *C. brasiliensis* sequences (*COI* and *EF-1α*) from Costa Rica grouped together in the same main cluster with the available sequences in BOLD and/or GenBank for *C. brasiliensis*, *C. lataniae*, and *T. xinglongensis* with node support values >99% using different phylogenetic methods (figures 3 and 4). Three subgroups may be recognized in the *COI* clusters generated by Bayesian (B) and Maximum Likelihood (ML) methods (figure 3); one including the Costa Rican *C. brasiliensis* samples (node values B/ML: 95/90%), another includes *C. lataniae* (HQ632647 and JX051426) with one *T. xinglongensis* (MG197811) sequence (96/84%) and the third subgroup comprises a *C. brasiliensis* (KX698136) and two *T. xinglongensis* (JX282767 and JX489633) sequences (96/83%). Therefore, phylogenetic analyses were also inconclusive to confirm the identity of the Costa Rican *C. brasiliensis* colonies.

Additionally, the *COI* and *EF-1α* data suggested close phylogenetic relationship between the *C. lataniae* and *C. brasiliensis* species. Sequences from both species were in a separated cluster from all other sequenced *Cerataphis* and *Tuberaphis* species, but for *T. xinglongensis* (figures 3 and 4). Conversely, with the *COI*-tRNA-*COII* sequences available in GenBank, *C. lataniae* grouped more distant to *C. brasiliensis*, in a subcluster with *C. vandermeermohri*, *Cerataphis freycinetiae* van der Goot and *Ceratoglyphina roepkei* (Hille Ris Lambers) (figure 5). It is important to note that *C. lataniae* samples correspond to different individuals for each marker, and thus variation may be a consequence of intraspecific diversity; or more plausible, that in one or several cases the sequences are misidentified.

Sequences from colonies morphologically identified as

C. orchidearum from orchid hosts grouped in a separate cluster than *C. brasiliensis* and *C. lataniae* (figures 3 and 4), though macroscopically are similar to the colonies observed on palms or red ginger corresponding to *C. brasiliensis*. *C. orchidearum* sequences seem more related to *C. bambusifoliae* and *C. freycinetiae* in the phylogenetic trees.

The phylogenetic analyses highlighted several interesting points. i) While *COI* sequences from *C. lataniae* and *C. brasiliensis* showed more than 98% identity, they grouped close together but in independent subbranches in all dendrograms for *COI* or *COI*-tRNA-*COII*, suggesting that are valid different species as shown by previous morphological examinations (Russell 1996; Pérez Hidalgo *et al*., 2000). ii) In the dendrograms generated for *EF-1α*, the available sequence for *C. lataniae* in GenBank is labelled as "UNVERIFIED" (KU048060), and indeed, it consistently grouped within *C. brasiliensis* sequences, suggesting that it may be misidentified. iii) Sequences of *T. xinglongensis* did not group with other *Tuberaphis* spp. but with *C. brasiliensis* and *C. lataniae* for three different genetic markers (figures 3, 4, and 5). In the case of *COI* sequences, two *T. xinglongensis* sequences clustered with *C. brasiliensis* and a third one sequence clustered with *C. lataniae* (figure 3). Therefore, some of the *T. xinglongensis* records may be misidentified. All the data suggested that at the molecular point of view, using the three markers *COI*, *EF-1α*, and *COI*-tRNA-*COII*, this species may correspond to a member of the *Cerataphis* genus. iv) Also, our analyses confirmed previous results, *Cerataphis* and *Tuberaphis* are either sister taxa or are paraphyletic with respect to each other but represent a monophyletic clade as a whole (Stern, 1994; Huang *et al*., 2012; Chen *et al*., 2014; Xu *et al*., 2018).

C. brasiliensis and *C. orchidearum* colonies in Costa Rica have a yeast-like symbiont

None of 30 aphid individuals tested harbour neither *Buchnera* nor *Wolbachia*, according to PCR results. The expected amplicon size corresponding to the yeast-like symbiont (YLS) was observed in 18 aphid individuals. Two *C. brasiliensis* individuals (MT647617 and MT647618), one *C. orchidearum* (MW115615), and four positive controls (MT647619-MT647622) were sequenced; blast results confirmed that the amplicons match a fungal uricase gene associated to a YLS (supplemental material table S2). Therefore, our results indicated that colonies of *C. brasiliensis* and *C. orchidearum* sampled in Costa Rica harbours YLS.

The dendrogram obtained for the YLS uricase gene partial sequences (figure 6) suggested that the *C. orchidearum* symbiont is phylogenetically different from the *C. brasiliensis* one. Moreover, the latter symbiont is more related to those reported in *Tuberaphis* spp. and other hemipterans than to *C. orchidearum*. This result contributed evidence in favour of the hypothesis that multiple events of *Buchnera* replacement by a YLS have occurred, rather than a single acquisition followed by several events of recovery of the prokaryotic endosymbiont (Fukatsu and Ishikawa, 1992; 1996; Fukatsu *et al*., 1994; Xu *et al*., 2018).

Conclusions

Results herein highlight the limited number of barcoding sequences available for genera *Cerataphis* and *Tuberaphis*. There is therefore a need to sequence more individuals representing all species of *Cerataphis* and *Tuberaphis* from different geographical locations to establish robust and consistent phylogenetic relationships able to define appropriate systematics for these taxa. Also, the data suggested possible misidentified sequence records in the public databases. Therefore, the results stress the need for appropriate identification of individual samples (curation) of the few available sequences for these genera. Moreover, based on the molecular results and few sequences available in public databases, we also suggest the need for a revision of the species *T. xinglongensis*, which was recovered closely to the palm dwelling species, *C. brasiliensis* and *C. lataniae* (figures 2 to 5).

Ten ant species were found associated to *Cerataphis* spp. colonies in Costa Rica, which suggests an unspecific and common association between these aphids and ants. It was confirmed that *C. brasiliensis* and *C. orchidearum* colonies in Costa Rica carry a YLS rather than a prokaryotic endosymbiont; neither *Buchnera aphidicola* nor *Wolbachia* were detected in individual aphids from the sampled colonies.

Pairwise *COI* sequence comparisons and phylogenetic analyses herein indicated that a single barcoding gene, the mitochondrial cytochrome *c* oxidase I gene (*COIc* / *COX1*), is not enough for a robust molecular identification of *Cerataphis* and *Tuberaphis* (and perhaps by extension other taxa in the subfamily Hormaphidinae), as it has been observed in other insect cases (Foottit *et al*., 2008; Song *et al*., 2008; Lee *et al*., 2011), or that species within the two genera are misplaced or the genera should be synonymized.

There is a widespread deployment of the 5'-prime region of *COI* gene (the Folmer region = barcoding), but, in the case of aphids, it has also been used the 3'-end of *COI* together with *tRNA* and partial *COII* genes (for example: Stern, 1994; 1998; Massimino Cocuzza *et al*., 2015). Therefore, there is not a common marker o consistent use of a gene region for all samples or species of interest. Alternatively, a lack of completeness of the database may render it temporarily inaccurate (meanwhile the database is completed for a specific genus or species) (Wilson *et al*., 2011). Therefore, a set of genetic markers should be defined for aphids, *COI* and secondary markers, and consistently employed for comparable results worldwide, to accomplish a more robust and accurate molecular identification system. Work toward that direction is on progress: i) testing different loci has already been done for aphids (Lee *et al*., 2014; Lee and Akimoto, 2015), and ii) research deploying different genes, including primary endosymbiont ones, is available (Théry *et al*., 2018).

Finally, molecular data, though a powerful tool, should not be regarded as a substitute either to morphological/morphometric studies or to the trained expert; in that sense, molecular data should be one piece of an integrated approach (Song *et al*., 2008; Massimino Cocuzza *et al*., 2015).

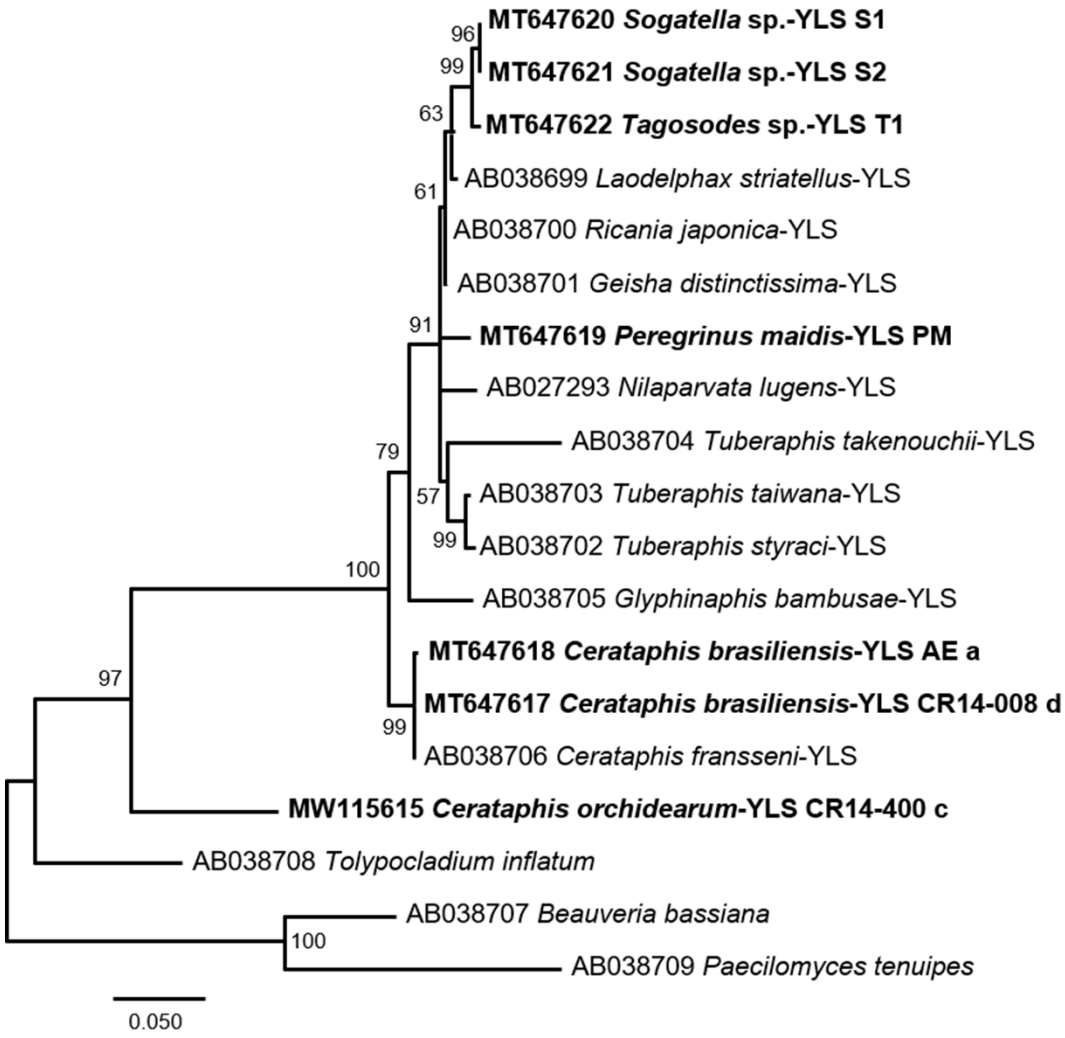

Figure 6. Phylogeny for partial region of a fungal uricase gene for YLS of Cerataphidini aphids and pathogenic related fungi. Phylogeny inferred by Maximum Likelihood method in MEGA X with a Hasegawa-Kishino-Yano model and a Gamma distribution to model evolutionary rate differences among sites. Scale bar represents the number of substitutions per site.

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Authors' addresses: Mauricio MONTERO-ASTÚA (corresponding author: mauricio.monteroastua@ucr.ac.cr), Norman BRENES-CORDERO, Izayana SANDOVAL-CARVAJAL, William VILLALOBOS MULLER, Universidad de Costa Rica, Centro de Investigación en Biología Celular y Molecular (CIBCM), San José, 11501-2060, Costa Rica; Nicolás PÉREZ HIDALGO, Instituto de Biología Integrativa de Sistemas (I 2 SysBio), Universidad de Valencia-CSIC, Spain; Xavier ESPADALER, CREAF, Universitat Autonoma de Barcelona, Spain.

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