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Adult identity crisis in *Leucothrips* (Thysanoptera: Thripidae) associated with the tropical ornamental plant *Codiaeum variegatum* (Euphorbiaceae)

Thomas L. Skarlinsky^{1,*}, Paul Rugman-Jones², Joe Funderburk³, Richard Stouthamer², and Carlos Sanabria Ujueta⁴

Abstract

Frequent US port of entry quarantine interceptions of unidentifiable larval *Leucothrips* (Thysanoptera: Thripidae) species in association with *Codiaeum variegatum* (L.) Rumph. ex A. Juss. (Euphorbiaceae) ornamental plants from Costa Rica, initiated research to determine if these thrips were an invasive threat to US agriculture. Larval and adult *Leucothrips* were collected from the new growth leaves of *C. variegatum* and other plants in Florida and Costa Rica. There were no morphological differences among adult specimens from different hosts in Florida and Costa Rica; all identified as *Leucothrips furcatus* Hood by comparison with type specimens. However, morphological differences in larvae associated with these adult specimens were evident. DNA sequences of the mitochondrial cytochrome oxidase c subunit 1 gene (*COI*), and 2 regions of the nuclear ribosomal cistron (rRNA; the complete internal transcribed spacer 2 [*ITS2*], and a section of the 28S large subunit rRNA) were used to verify that larvae and adults collected at the same site were the same species. Molecular data revealed species level divergence congruent with larval morphological differences.

Key Words: thrips; immature; molecular; morphology

Resumen

Intercepciones frecuentes de cuarentena en los puertos de entrada de larvas de especies de *Leucothrips* (Thysanoptera: Thripidae) no identificables en asociación con plantas ornamentales de *Codiaeum variegatum* (L.) Rumph. ex A. Juss. (Euphorbiaceae) importadas de Costa Rica, inició una investigación para determinar si estos trips son una amenaza invasiva a la agricultura estadounidense. Larvas y adultos de *Leucothrips* fueron recolectados de las nuevas hojas de crecimiento de *C. variegatum* y otras plantas en la Florida y Costa Rica. No hubo diferencias morfológicas entre especímenes adultos de diferentes hospederos en la Florida y Costa Rica; Todos fueron identificados como *Leucothrips furcatus* Hood en comparación con los especímenes tipo. Sin embargo, hubo diferencias morfológicas evidentes en larvas asociadas con estos especímenes adultos. Se utilizaron secuencias de ADN de la subunidad 1 de la citocromo oxidasa mitocondrial del gen (*COI*) y 2 regiones del cistron ribosómico nuclear (ARNr, el espaciador transcrito interno completo 2 [*ITS2*] y una sección del ARNr de la subunidad grande 28S) para verificar que las larvas y los adultos recolectados en el mismo sitio eran de la misma especie. Los datos moleculares revelaron divergencia de nivel de especie congruente con las diferencias morfológicas larvales.

Palabras Clave: thrips; inmaduro; molecular; morfología

Leucothrips (Thysanoptera: Thripidae: Dendrothripinae) represents an ill-defined genus of minute white thrips, most similar to the neotropical genus *Halmathrips* Hood and to the more cosmopolitan *Pseudodendrothrips* Schmutz (Mound 1999). Currently, *Leucothrips* consists of 5 species (Mound & Tree 2016). The females of *Leucothrips pictus* Hood, *Leucothrips piercei* Morgan and *Leucothrips nigripennis* Reuter can be morphologically distinguished by 3 unique character states: *L. nigripennis* has uniformly shaded brown forewings, *L. pictus* has 8 antennal segments, and *L. piercei* possesses simple sensory cones on antennal segments III and IV (Mound 1999). However, females of the remaining 2 species, *Leucothrips furcatus* Hood and *Leucothrips theobromae* Priesner are much more difficult to distinguish, both having 7 antennal segments, unshaded forewings, and forked sensory cones on

antennal segments III and IV (Mound 1999). Indeed, morphologically they differ only by the absence or presence, respectively, of a crimson spot between the antennal bases (Hood 1931; Moulton 1933; Bailey 1957; Mound & Tree 2016).

Species differentiation based on male and larval specimens is even more problematic. The males of *L. piercei* and *L. nigripennis* have simple sensory cones on antennal segments III and IV, and males of *L. theobromae* have simple sensory cones on antennal segments III and IV (Mound 1999). Males of both *L. pictus* and *L. furcatus* have not been recorded (Mound 1999).

Published immature morphology of *Leucothrips* is limited to 3 species. Priesner (1923) described and illustrated the dorso-lateral abdominal setae of *L. theobromae* as gradually distally expanded and

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increasing in length from abdominal segment VII to IX. In contrast, the lateral abdominal setae on *L. piercei* terminate in minute knobs (Vance 1974) and *L. nigripennis* possesses slender capitate setae posterolaterally on segments VII to IX (Mound 1999).

Specific differences may exist in host plant use. The plant *Theobroma cacao* L. (Malvaceae) is a host for *L. theobromae* (Priesner 1923), *Capsicum annum* L. (Solanaceae) for *L. piercei* (Zamar et al. 2014), and *Pteris cretica* L. (Pteridaceae) for *L. nigripennis* (Mound 1999). *Leucothrips pictus* seem to be associated with leaves of forest trees and *L. nigripennis* with ferns (Mound 1999). Adults of *L. furcatus* have been reported from; *Erythrina* sp. L. (Fabaceae; Hood 1931); *Codiaeum variegatum* var. *pictum* (L.) Rumph. ex A. Juss. (Euphorbiaceae) (Halbert 1996), *Albizia julibrissin* Durazz. (Fabaceae) (Diffie & Srinivasan 2010), and *Lablab purpureus* (L.) Sweet (Fabaceae) (Etienne et al. 2015). Undetermined species of *Leucothrips* were reported from the leaves of *Cochlospermum vitifolium* (Willd.) Spreng. (Bixaceae), and *Ricinus* species (Euphorbiaceae) (Mound & Marullo 1996). Also, records of undetermined species of *Leucothrips* intercepted from *Sechium edule* (Jacq.) Swartz (Cucurbitaceae) during US quarantine inspections were found using the United States Department of Agriculture, Plant Protection Quarantine, Agricultural Quarantine Activity System, PestID database queried from January 1, 1985 to January 1, 2016.

The final project proposal report of the Standards and Trade Development Facility (STDF 2009) presented to the World Trade Organization identified *C. variegatum* as 1 of 4 key ornamental live plant crops in Costa Rica that posed a phytosanitary risk for the USA based on frequent quarantine pest interceptions from 2007 to 2009. The regular interception of unidentifiable larval *Leucothrips* with *C. variegatum* was cited as a high priority problem by growers due to economic loss from fumigation costs at the US port of entry and reduced plant quality after fumigation. Proposed goals developed by the STDF (2009) were to minimize the phytosanitary risk and to maintain access to the USA market for *C. variegatum*. The strategy to achieve these goals included better management practices at the farm level and additional research to determine if *Leucothrips* were an invasive threat to USA agriculture. The objective of this study was to establish the identity of *Leucothrips* populations on *C. variegatum* in Costa Rica.

Materials and Methods

Larval and adult thrips were collected from new growth leaves of: *C. variegatum* cultivar 'Petra', *Sechium edule*, and *T. cacao* in Costa Rica; and, from *C. variegatum* cultivar 'Petra' in Florida, USA (Table

1). The thrips were captured individually with a small artist brush and transferred to 70% ethyl or isopropyl alcohol. In the laboratory the thrips were separated into 2 groups, thrips in group 1 were slide mounted and those in group 2 were transferred to >95% ethyl alcohol for molecular analysis. Slides were prepared with Canada balsam media using modified methods from Mound and Marullo (1996) or Hoyer's media. The slides were cured in an oven at approximately 40 °C. After curing, the cover slips of the Hoyer slides were sealed with clear nail polish. All specimens were examined with a compound microscope (DM LB2; Leica, Wetzlar, Germany) under phase contrast at 100×, 200×, and 400× magnification, and were deposited at the Miami Plant Inspection Station (MPIS, Florida). Images were taken with Helicon Focus 6.1.0. software (HeliconSoft Ltd., Kharkiv, Ukraine), and adjusted for visual clarity with Photoshop® Elements 10 (Adobe Systems, San Jose, California).

The slide-prepared specimens (Table 1) were morphologically compared with *L. theobromae*, 1 ♀ paratype, (US National Museum of Natural History [USNM], Beltsville Maryland) collected from *T. cacao* in Paramaraibo, Suriname; *L. furcatus*, 2 ♀ ♀ paratypes, (USNM), collected from *Erythrina* sp. in Guadeloupe (12 Mar 1915); *L. furcatus*, 1 ♀ (Florida State Collection of Arthropods [FSCA], Gainesville, Florida), collected from *C. variegatum*, Fort Lauderdale, Florida (1959); and *L. theobromae*, 3 ♀ ♀, (MPIS), collected from *T. cacao*, Quevedo, Ecuador (31 Oct 2008).

Whole genomic DNA was extracted from representative specimens (Table 1), using the non-destructive EDNA HiSp-Ex™ tissue kit (Fisher Biotec, Wembly, Australia) with the following modifications to the manufacturer protocol. Individual specimens were immersed in a 60 µL mix of the proprietary solutions 1A (48 µL) and 1B (12 µL) in a microcentrifuge tube and incubated at 95 °C for 30 min. Subsequent to incubation, 15 µL of proprietary solution 2 was added. The contents of the tube were mixed by gentle vortexing, and, taking care to avoid touching the specimen, 60 µL of the DNA template was transferred to a new microcentrifuge tube, and stored at -10 °C. Isopropyl alcohol (70%) was added to the original tube containing the specimen carcass. The extracted voucher specimens were prepared and curated as previously described.

Polymerase chain reaction (PCR) was initially used to amplify part of the mitochondrial cytochrome oxidase c subunit 1 gene (*COI*) and the complete internal transcribed spacer 2 gene (*ITS2*) of the nuclear ribosomal cistron (rRNA). A section of *COI* was amplified using the mtD-7.2F and mtD-9.2R primers of Brunner et al. (2002). PCR was performed in 25 µL reactions containing 2 µL DNA template, 1× Thermopol Buffer (New England Biolabs, Ipswich, Massachusetts), 2.5 µL dNTP/dUTP mix (Thermo Scientific [#R0251], Waltham, Massachu-

Table 1. Collection data for adult (♀ or ♂) and larval (LV) *Leucothrips* specimens sequenced and examined morphologically in this study. Large numbers of additional specimens were subject to morphological examination only (**bold**).

Locality (geographic coordinates, decimal degrees)	Plant	Specimens	Date collected
Florida, USA			
(25.814693°N, 80.288156°W)	<i>Codiaeum variegatum</i>	PR12-771 (♀), PR12-772 (♂), PR12-773 (LV), 17 × ♀, 2 × ♂	22 Aug 2012
(25.823387°N, 80.292098°W)	<i>Codiaeum variegatum</i>	18 × LV	04 Jul 2016
Costa Rica			
(10.29530°N, 83.74634°W)	<i>Codiaeum variegatum</i>	PR12-776 (♀), PR12-777 (♂), 18 × ♀, 2 × ♂	24 Aug 2011
(10.56542°N, 83.65580°W)	<i>Ricinus</i> sp.	PR12-778 (♀), PR12-779 (♂), PR12-780 (LV), 11 × ♀, 2 × ♂	23 Feb 2012
(9.88566°N, 83.83165°W)	<i>Sechium edule</i>	PR12-781 (♀), PR12-782 (♀), PR12-965 (LV), 19 × ♀	17 Jan 2012
(9.97080°N, 83.64235°W)	<i>Sechium edule</i>	158 × ♀	10 Jan 2013
(9.87138°N, 83.80222°W)	<i>Sechium edule</i>	155 (♀), 156 (LV), 157 (LV), 24 × LV	17 Jun 2016
(9.87598°N, 83.65580°W)	<i>Theobroma cacao</i>	PR12-783 (♀), PR12-784 (♀), 6 × ♀	27 Jul 2012

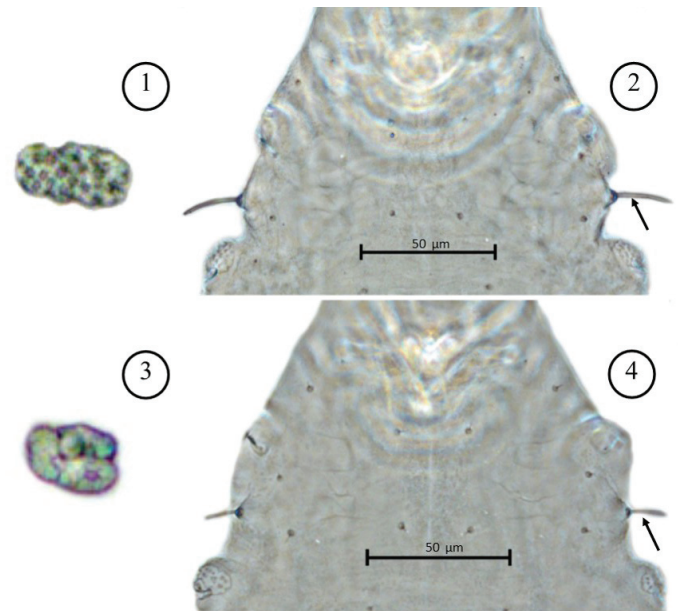
setts), 1 mM of $MgCl_2$, 10 μg BSA (New England BioLabs, Ipswich, Massachusetts), 0.4 μM of each primer, and 1.5 U *Taq* polymerase (New England BioLabs, Ipswich, Massachusetts). Following initial denaturing at 94 °C for 3 min, amplification was performed on a Mastercycler[®] ep gradient S thermocycler (Eppendorf North America Inc., New York, New York) employing 38 cycles of 94 °C for 30 s, 47 °C for 1 min, and 68 °C for 1 min 30 s. Reactions then were held at 68 °C for a further 3 min to ensure complete extension of all amplicons. *ITS2* was amplified using the *ITS2*-forward and CS250 primers and protocol described in Rugman-Jones et al. (2006) with a single modification incorporating 0.4 μM dUTP instead of 0.2 μM dTTP in case of carryover contamination (Hartley & Rashtchian 1993). Based on our comparison of *COI* and *ITS2* sequences, we subsequently used the primers 28sF3633 and 28b to amplify a section of the conserved 28S large subunit rRNA, following Rugman-Jones et al. (2010a), with the same dUTP modification detailed above. The success of the PCR was confirmed by standard gel electrophoresis, and amplicons were purified using Wizard PCR Preps (Promega, Madison, WI) or ExoSAP-IT (Affymetrix, Santa Clara, CA), prior to direct sequencing in both directions at the Institute for Integrative Genome Biology, University of California, Riverside, California.

Sequences were compiled and trimmed (to remove primers) using Sequencher[®] 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). Flanking 5.8S and 28S regions of the *ITS2* were identified using the annotate tool in *ITS2* database (Keller et al. 2009, Ankenbrand et al. 2015), and removed. Sequence sets were aligned in MAFFT version 7.293 (Katoh & Standley 2013) using the G-INS-1 strategy and all sequences were deposited in GenBank (Benson et al. 2008); accession numbers KY679041–KY679088. *COI* sequences were translated using the EMBOSS-Transeq website (Rice et al. 2000; Goujon et al. 2010) to confirm the absence of nuclear pseudogenes (Song et al. 2008), and then collapsed into haplotypes using DnaSP v5.10.01 (Librado & Rozas 2009). The number and nature of polymorphic sites in the *COI* dataset was characterized using DnaSP, and pairwise divergence between the different haplotypes was estimated by calculating Kimura 2-parameter distances (K2P) using MEGA version 6 (Tamura et al. 2013). K2P was used to construct an unweighted pair group method with arithmetic mean (UPGMA) tree and branch support was estimated using a bootstrap procedure with 1000 replicates. Sequences of the 2 rRNA genes (*ITS2* and 28S) were not subject to formal analysis, but instead, the aligned dataset of each was examined by eye, for evidence of differentiation.

Results

No morphological differences were detected between the collected *Leucothrips* adults and the paratypes of *L. furcatus*. The collected adults, before maceration, did not have a hypodermal crimson spot between the antennal bases as observed in the paratype of *L. theobromae*. However, 2 distinct 2nd instar morphotypes were observed in larval specimens. The 2nd instar morphotype-A, collected from *S. edule* in Costa Rica had between 7 to 15 circular pores within each spiracular area of abdominal tergite II (Fig. 1) and the pronotal setae pair VI were ~19 to 24 μm in length (Fig. 2). The 2nd instar morphotype-B that were collected from *C. variegatum* and *T. cacao* possessed 3 to 5 pores within each spiracular area of abdominal tergite II (Fig. 3) and the pronotal setae pair VI were ~10 to 14 μm in length (Fig. 4).

Aligned sequences of a 434 base pair (bp) section of *COI* from 16 *Leucothrips* specimens (GenBank accessions KY679057–KY679072) harbored 74 polymorphic sites and collapsed into 7 haplotypes. The



Figs. 1–4. Second instar larva of *Leucothrips* species: (1) morphotype-A abdominal tergite II spiracle at 1000 \times magnification; (2) morphotype-A prothorax; arrow indicates setae D6; (3) morphotype-B abdominal tergite II spiracle at 1000 \times magnification; (4) morphotype-B prothorax; arrow indicates setae D6; scale = 50 μm .

Costa Rican specimens from *S. edule* harbored a single haplotype, which in the UPGMA analysis formed a clade (Clade A), with 100% support, that differed by approximately 18% from a second fully supported clade containing all remaining haplotypes (Clade B; K2P ranged from 0.180–0.186; Fig. 5). Within Clade B, maximum divergence among the 6 haplotypes was 1.6% (K2P = 0.016; Fig. 5). The majority of nucleotide substitutions were synonymous, but changes at 4 positions (47–48, 254, and 368) resulted in 3 changes to the encoded amino acid chain, 2 of which (47–48, and 254) were diagnostic of the Costa Rican specimens from *S. edule*.

The same division was evident in the sequences of both regions of rRNA. There were multiple, consistent differences in a 505 bp aligned matrix of *ITS2* sequences (GenBank accessions KY679073–KY679088), between Costa Rican specimens from *S. edule* and those from the other hosts or localities (Fig. 6). Similarly, across a 786 bp section of the highly conserved 28S, there were consistent substitutions at 4 nucleotide positions; 109, 180, 287, and 558 (GenBank accessions KY679041–KY679056).

Discussion

Based on adult morphology, specimens of *Leucothrips* from populations in Costa Rica and Florida could not be differentiated. However, differences in larval morphology, and the DNA sequences of 3 separate genes (*COI*, *ITS2*, and 28S), divided these *Leucothrips* specimens into 2 concordant groups. One group (Clade B; Fig. 5) consisted of specimens collected from *C. variegatum* in both Florida and Costa Rica, and also Costa Rican specimens from *T. cacao* and *Ricinus* sp. Low genetic divergence (*COI* <1.6%) and high morphological affinity (adult and 2nd instar larva) among the specimens in this group provide strong evidence that they constitute a single species. In contrast, despite the absence of adult morphological differences, the second group (Clade A; Fig. 5), consisting only of specimens collected from *S. edule* in Costa Rica, was clearly genetically divergent (*COI* ~18%). The

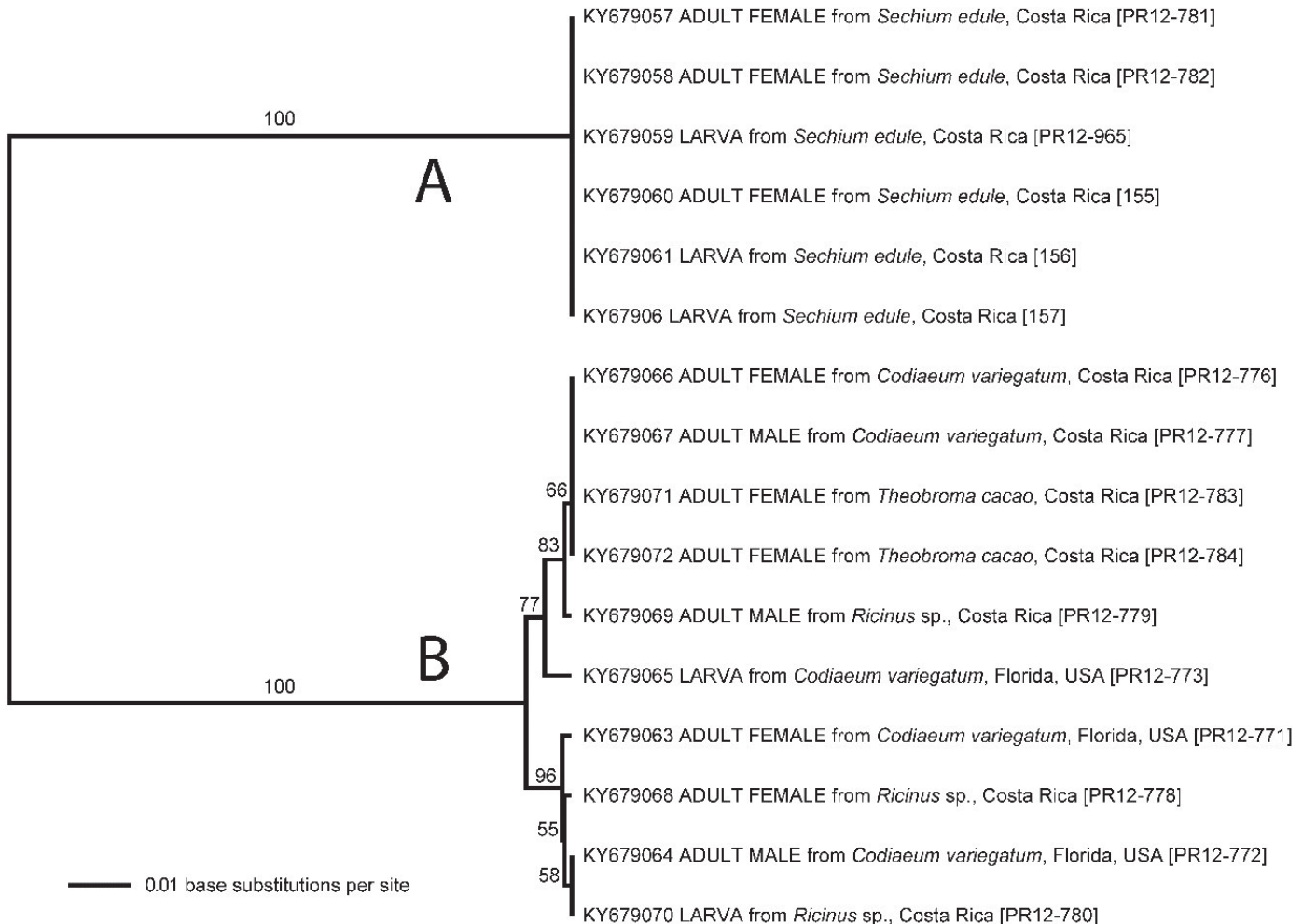


Fig. 5. An unweighted pair group method with arithmetic mean (UPGMA) tree of Kimura 2-parameter distances (K2P) among the cytochrome oxidase c subunit 1 gene (*COI*) sequences of *Leucothrips* collections that are indistinguishable based on adult morphology. The optimal tree with the sum of branch length = 0.19814793 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale. Analyses were conducted in MEGA version 6.

2nd instar larva of Clade A also were morphologically different from those of Clade B, further indicating that the former likely represents another, cryptic species.

This is not the first time that such cryptic diversity has been uncovered in Thysanoptera, with the aid of DNA sequence data. For example, previous molecular studies of important thrip (Thysanoptera: Thripidae) pest species such as *Frankliniella occidentalis* Pergande (Rugman-Jones et al. 2010b), *Scirtothrips dorsalis* Hood (Dickey et al. 2015), and *Thrips tabaci* Lindeman (Brunner et al. 2004; Jacobson et al. 2016), have all revealed evidence of cryptic species within those taxa. In each of these species, reexamination of adult morphology in light of DNA evidence, has failed to reveal any differential characters (Rugman-Jones et al. 2010b; Dickey et al. 2015; Brunner et al. 2004). However, an earlier morphological and biological study of *T. tabaci*, conducted before the invention of DNA barcoding, noted a difference in larval morphology between, what were therein proposed to be biotypes; the “*tabaci* type” and the “*communis* type” (Zawirska 1976). The types differed not only in larval morphology (absence of abdominal tergite IX posteromarginal teeth in *tabaci* vs. presence in *communis*), but also in reproductive strategy (arrhenotoky in *tabaci* vs. thelytoky in *communis*), and behavior (specialized feeding in *tabaci* vs. polyphagy

in *communis*). These differences were later corroborated by the finding of deep genetic divergence between the types (Brunner et al. 2004). Similarly, we also found a morphological difference between the larvae of our 2 *Leucothrips* genetic types. Furthermore, and again similar to *T. tabaci*, we also found potential differences in both reproductive strategy and feeding behavior between the *Leucothrips* types, although this is based on a relatively limited sample. There was a complete absence of males in the genetic type that was collected only from *S. edule*, suggesting that this species may be thelytokous and monophagous. Conversely we were able to find males in the genetic type collected from *C. variegatum* and *Ricinus* sp.

From our limited dataset, it appears that only 1 of the 2 types is established in Florida, however the specific identity of the 2 *Leucothrips* types remains ambiguous. Female adult morphology of both types matches that of *L. furcatus*. Interestingly, there is a complete absence of males in the type series of *L. furcatus* (Hood 1931), and, therefore, it may be inferred that the specimens from *S. edule*, that contained no males, are in fact the true *L. furcatus*. Indeed a much broader sampling of plant hosts and locations, accompanied by morphological and molecular study, is warranted before any taxonomic decisions are made.

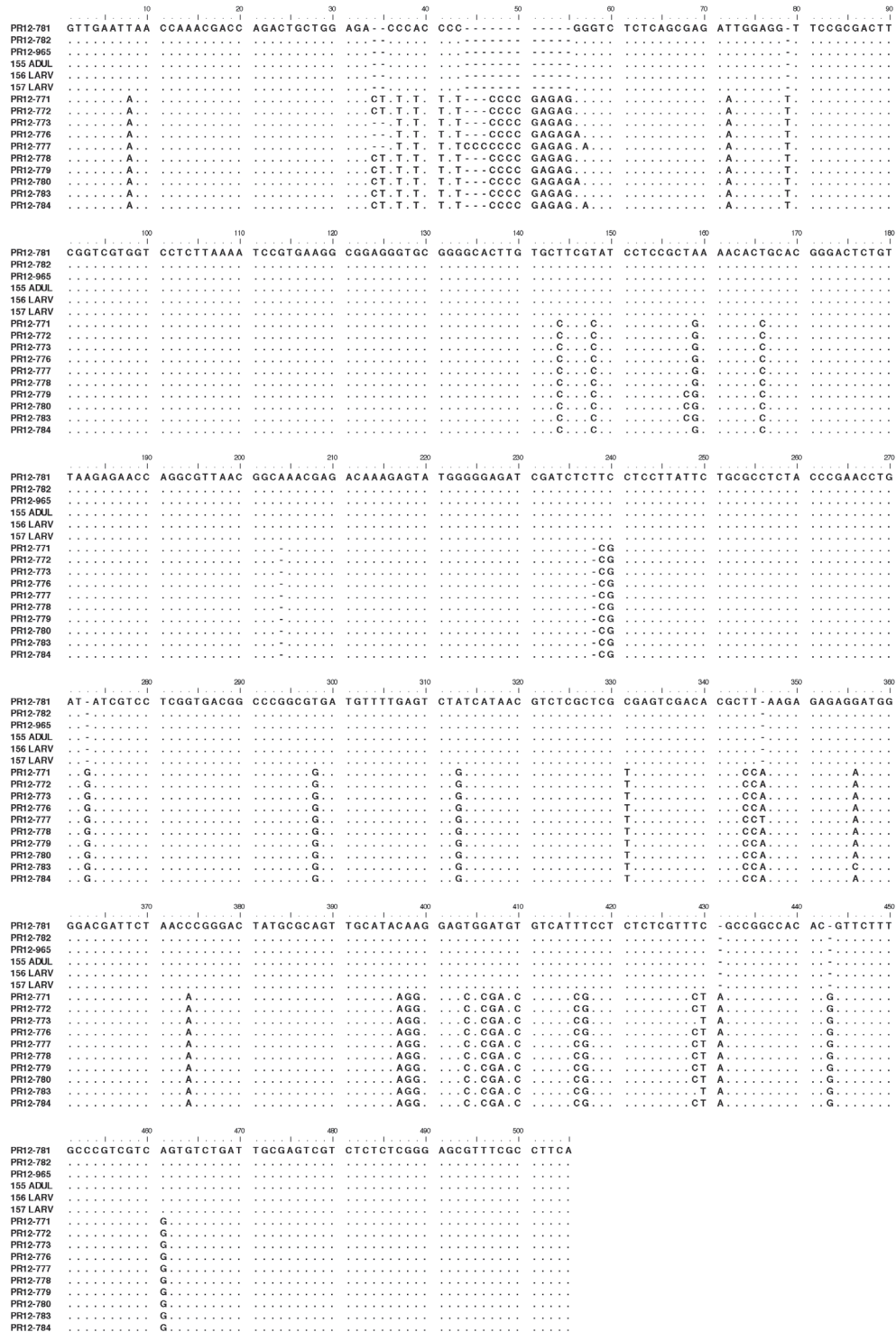


Fig. 6. Divergence in the internal transcribed spacer 2 (*ITS2*) DNA sequence of *Leucothrips* specimens from *Secchium edule* in Costa Rica relative to those from other hosts. Sequences were aligned with MAFFT v7.293 using the G-INS-1 strategy.

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