Article

Resolving cryptic species with morphology and DNA; thrips as a potential biocontrol agent of Brazilian peppertree, with a new species and overview of *Pseudophilothrips* (Thysanoptera)

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Abstract

Molecular and morphological evidence is presented to support the description of a second species of *Pseudophilothrips* from Brazil in association with *Schinus terebinthifolius*, an invasive weedy tree in North America. *Pseudophilothrips* is here recognized as a weakly defined genus comprising 13 described species from the Americas. This genus is presumably derived from within, rather than sister-genus to, the worldwide genus *Liothrips* of leaf-feeding species.

Key words: *Pseudophilothrips gandolfoi* **sp. n.**, *Pseudophilothrips ichini*, weed biological control, mtDNA COI gene, cryptic species

Introduction

Brazilian peppertree, *Schinus terebinthifolius* (Anacardiaceae), is an invasive weed that infests natural and agricultural areas in the subtropics of many areas of the world including the USA and Australia. Originally from eastern Brazil, northern Argentina, Uruguay, and Paraguay, this species was introduced into Florida between about 1898 and 1900 (Williams *et al.*, 2007). In the southern USA it now invades both disturbed and undisturbed plant communities, ranging from pinelands, hammocks, and mangrove coastal areas (Johnson, 1994). Currently, this weed infests over 280,000ha in Florida and is one of the most difficult invasive plant species to control (Schmitz *et al.*, 1997). Moreover it has been a problem in Hawaii for many years (USFS PIER, 2010) and is becoming increasingly invasive in California (Randall, 2000). Regardless of the duration of the infestation, classical biological control constitutes one of the best options for sustained population reductions of this invasive species.

Classical weed biological control seeks specialized herbivore species from the weed's native range that will inflict damage to invasive populations while causing minimal harm to valued plants. Correct identification of both the target weed and of the potential biological control agent is of paramount importance, but traditionally has depended upon morphological determinations. However, the occurrence of hidden, or cryptic, species complexes poses unforeseen complexity and challenges. Although species with narrower host ranges may emerge from the analysis of these cryptic species complexes, the methods of distinguishing them may require a combination of both traditional morphological and novel molecular techniques (Smith *et al.*, 2008). Recent examples demonstrate the potential of this approach to improve natural enemy selections for weed biological control projects (Goolsby *et al.*, 2006; Madeira *et al.*, 2006; Tracy and Robbins, 2009). The information presented here constitutes a further example of the importance of collaboration between morphological taxonomists and molecular specialists. The complexities of natural field associations in the target's home range must be carefully evaluated and assessed before a potential control agent can be selected for importation into quarantine for study and subsequent safety testing.
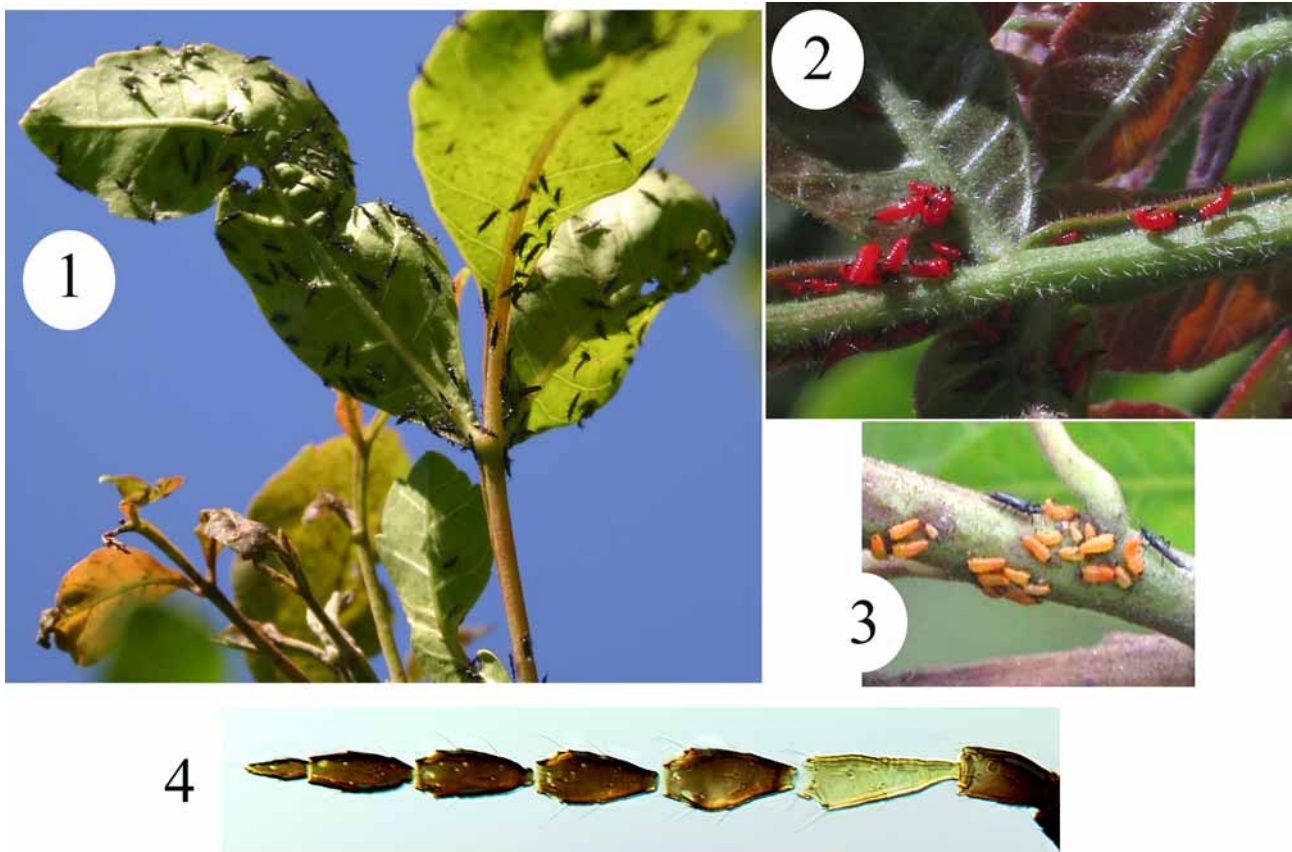
FIGURES 1–4. *Pseudophilothrips* from *Schinus terebinthifolius*. **(1)** colony of adults inducing leaf distortion. **(2)** *P. gandolfoi* red larvae. **(3)** *P. ichini* orange larvae. **(4)** *P. gandolfoi* holotype antenna.

A Brazilian thrips species, *Pseudophilothrips ichini* (Hood), feeds on the flush leaves of S. *terebinthifolius* (Fig. 1). First collected near Rio de Janeiro, Brazil, and described by Hood (1949) in the genus *Liothrips*, the species was later re-assigned by Johansen (1979) to a new genus *Pseudophilothrips*. This thrips has been the subject of several studies examining its potential as a biological control agent (Garcia, 1977; Hight *et al.*, 2002). However, while conducting comparative host range tests in Florida, Manrique *et al.* (2008) noted that structurally very similar thrips from different parts of Brazil differed greatly in their survival and adult longevity on different strains of *S. terebinthifolius*. These observations led to DNA studies on different populations of the thrips, and subsequently to a search for structural differences between the adults. Unfortunately, the thrips involved are members of a group of Thysanoptera for which taxonomic studies are particularly poorly based, with many species known only from one or few specimens with no host-plant data, and for which the generic classification is equally poorly founded.

The purpose of this paper is to describe a new species of *Pseudophilothrips* that is known to breed on *S. terebinthifolius* only in the region of Curitiba, Paraná, Brazil. In contrast, *P. ichini* is widespread on *S. terebinthifolius* in lowland areas along the east coast of Brazil between Santa Catarina and Bahia, and has also been found inland at higher altitudes in Minas Gerais. Recognition of this new species is based on DNA data as well as adult structure and larval colour. This study has necessitated a re-evaluation of the related species, and as a result the genus *Pseudophilothrips* is here recognized to comprise 13 Neotropical species that were placed previously in the worldwide genus *Liothrips*.

DNA studies

DNA extraction, amplification, and sequencing

Thrips were collected in 95% ethanol and stored at minus 20ºC until DNA extraction. DNA was extracted from single thrips using the method of Rugman-Jones *et al.* (2006). The thrips abdomen was pierced with a micro needle and placed in 200 µl lysis buffer (75 mM NaCl, 25 mM EDTA, 1% SDS) with 7 µl of Proteinase K (20 mg/ml) and incubated at 55ºC overnight. DNA was then purified from the buffer using the salting out method of Sunnucks and Hales (1996). The remaining carcass was removed and stored in 95% EtOH at 20ºC, each specimen subsequently being dehydrated, cleared in clove oil, then slide mounted in Canada balsam (Mound and Marullo 1996). Table I lists the origins of the specimens processed in this manner. In addition, sequences were also included of five other unique haplotypes from individuals putatively identified as *P. ichini* in the field and collected as part of a large scale population genetics study of that species. These latter specimens were processed by first grinding them in lysis buffer, and so carcasses were not available for subsequent morphological identification.

TABLE 1. Species and collection information for thrips used in this study. Number refers to the number of thrips genotyped at the mtDNA COI gene and identified by morphology of slide-mounted residual carcasses; *P. ichini* (H3-H11) are unique haplotypes from individuals putatively identified as *P. ichini* in the field.

Species (haplotype)	Number	Location	Latitude	Longitude	GenBank Accession No.
Pseudophilothrips perseae	5	Sacatepequez, Guatemala	14.5738	-90.6702	GU942810
Pseudophilothrips perseae	2	Sacatepequez, Guatemala	14.594	-90.6671	GU942810
Liothrips tractabilis	4	Chaco, Argentina	-26.7919	-60.4506	GU942811
Pseudophilothrips gandolfoi	2	Parana, Brazil	-25.41426	-49.25317	GU942809
Pseudophilothrips ichini (H1)	2	Minas Gerais, Brazil	-20.36911	-43.56029	GU942813
Pseudophilothrips ichini (H2)	2	Minas Gerais, Brazil	-20.36911	-43.56029	GU942814
Pseudophilothrips ichini (H3)	1	Minas Gerais, Brazil	-20.88058	-42.79039	GU942812
Pseudophilothrips ichini (H4)	1	São Paulo, Brazil	-23.90511	-46.29618	GU942818
Pseudophilothrips ichini (H5)	1	Rio de Janeiro, Brazil	-23.21139	-44.71945	GU942815
Pseudophilothrips ichini (H9)	1	Santa Catarina, Brazil	-26.08309	-48.61151	GU942816
Pseudophilothrips ichini (H11)	1	Santa Catarina, Brazil	-26.04753	-48.61627	GU942817

A portion of the mitochondrial cytochrome oxidase I gene (COI) was amplified using primers LCO1490 and HCO2198 (Folmer *et al.* 1994) and C1-J-1718 and C1-N-2191 (Simon *et al.* 1994). Amplifications were performed with 2 µl of DNA, 1.5 mM MgCl$_2$, 1X PCR buffer, 0.2 U of *Taq* polymerase (Promega), 5 pmols of each primer, and 0.2 mM dNTPs. Samples were run on an ABI 2720 thermal cycler using the following PCR profile: 94ºC for 2 min then 30 cycles of 94ºC for 15 sec, 55ºC for 15 sec, 72ºC for 1 min, then a final extension at 72ºC for 5 min. Unincorporated nucleotides and excess primers were removed from PCR products using *ExoI* and Antarctic Phosphatase (New England Biolabs) according to manufacturer protocols. PCR products were then sequenced in both forward and reverse directions using BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) and electrophoresed on an ABI 3130 genetic analyzer. Sequences were trimmed and contiged using Sequencher v. 4.8 and then aligned using Clustal W.

MEGA v.4.1 (Kumar *et al.* 2004) was used to calculate the Kimura 2-parameter distance measure (Kimura 1980) between all unique haplotypes. We then constructed a neighbor-joining tree and tested nodes with 5,000 bootstraps. All sequences have been deposited in GenBank under accession numbers GU942809-GU942818.

Genetic results

A total of 645 bp was sequenced for each individual. Sequences did not contain heterozygous peaks, indels, or stop codons suggesting they were mitochondrial in origin. The consensus neighbor-joining tree resolved the four species *P. ichini (H1 and H2)*, *P. gandolfoi* sp.n., *P. perseae*, and *L. tractabilis* (Fig. 5). Genetic divergence between these four species averaged 5.9%, (range = 4.2 – 7.7%). Genetic divergence between *P. ichini* and *P. gandolfoi* sp.n. averaged 4.4% (range 4.2-4.7%). All five haplotypes identified from the field as *P. ichini* were part of the *P. ichini* clade. Haplotypes within the *P. ichini* clade had an average divergence of 0.5% (range 0.2 – 1.1%) among them.
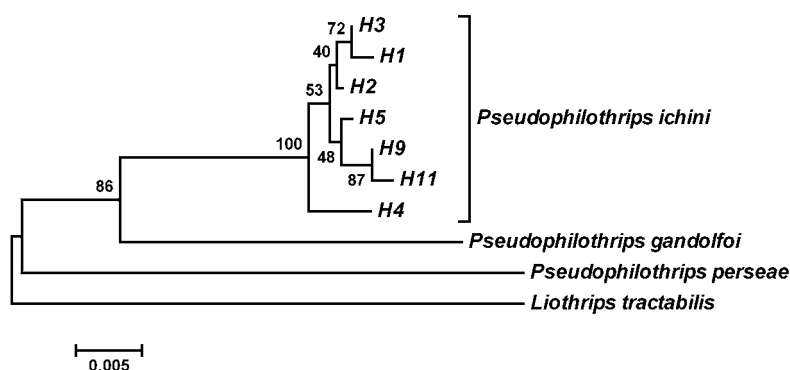

FIGURE 5. Relationships of thrips inferred using the neighbor-joining method and Kimura 2-parameter pairwise distances of mitochondrial COI sequences. Bootstrap values are shown next to the branches.

Morphology studies

Pseudophilothrips gandolfoi sp.n.

Male macroptera. Body blackish brown with red hypodermal pigment; antennal segment III sharply yellow (Fig. 4), IV with variable paler marking, II with apex pale; fore tibiae and fore tarsi also with small paler areas, hind tibiae and tarsi as dark as body; fore wing shaded around sub-basal setae with shaded area extending medially at least half-way along wing; all major setae dark, including those on tergite IX. Antennae 8-segmented, VIII slightly constricted at base, III with one slender sensorium, IV with three sensoria. Head elongate (Fig. 8), fore ocellus projecting over bases of antennae, cheeks weakly constricted medially, strongly constricted near base; maxillary stylets retracted almost to level of postocular setae, less than 0.2 of head width apart medially; postocular setae about as long as dorsal eye length, apices blunt to sub-acute; mid-dorsal setae usually no longer than diameter of an ocellus (one paratype male with one of these setae stout but short). Pronotum (Fig. 7) with all 5 pairs of major setae developed but variable in length; anteromarginal and midlateral setae sometimes longer than anteroangulars; posteroangular setae sometimes as long as median length of pronotum, usually shorter; epimeral setal pair II varying from no longer than width of base of a major seta to stout and 0.5 as long as epimeral setal pair I. Fore tarsus with no tooth. Metascutum with median setae small (Fig. 7), usually no sculpture present between bases of these setae. Prosternal basantra not developed, mesopresternum transverse but slightly narrowed medially; metathoracic sternopleural sutures scarcely developed. Fore wing sub-basal setae long with apices blunt; duplicated cilia varying in number 13–18. Pelta triangular with wavy margins (Fig. 9); tergite IX with three pairs of major setae all almost as long as tube.

Measurements (male holotype in microns). Body length 3000. Head, length 300; median width across cheeks 200; postocular setae 100. Pronotum, length 155; median width 320; major setae am 50, aa 50, ml 55, epim 125 (40), pa 110. Metanotal median setae 35. Fore wing, length 1150; sub-basal setae 80, 90, 100. Tergite IX setae, S1 280; S2 250; S3 300. Tube length 310. Antennal segments III–VIII length 100, 85, 80, 75, 70, 40.

FIGURES 6–9. *Pseudophilothrips* species. (**6**) *P. ichini* pronotum and metanotum. (**7**) *P. gandolfoi* pronotum and metanotum. (**8**) *P. gandolfoi* holotype head and pronotum. (**9**) *P. gandolfoi* holotype pelta and tergites II–III.

Female macroptera. Similar to male but often larger with longer setae.

Measurements (large female paratype in microns). Body length 3350. Head, length 330; median width across cheeks 220; postocular setae 170. Pronotum, length 180; median width 380; major setae am 80, aa 50, ml 100, epim 190 (60), pa 130. Metanotal median setae 40. Fore wing, length 1350; sub-basal setae 110, 140, 130. Tergite IX setae, S1 320; S2 350; S3 300. Tube length 320. Antennal segments III–VIII length 110, 90, 85, 80, 80, 45.

Larvae. Body colour deep crimson red (Fig. 2), in contrast to the orange larvae of *P. ichini* (Fig. 3).

Material studied. Holotype male, **BRAZIL**, Parana, Curitiba, from *Schinus terebinthifolius*, iv.2008 (G.Wheeler), in United States National Museum of Natural History, Washington.

Paratypes: 4 females 6 males collected with holotype [and 3 red larvae]; 11 females 21 males reared on same host plant under quarantine in Florida from specimens collected at Curitiba (including 2 males from which DNA was obtained).

Paratypes deposited in Departamento Entomologia, ESALQ/USP, Piracicaba; USNM, Washington; Entomology Department, University of California, Riverside; Natural History Museum, London; Senckenberg Museum, Frankfurt; Australian National Insect Collection, Canberra.

Comments. There seems to be only one non-varying structural character by which adults of this new species can be distinguished from *P. ichini*. In all the available specimens the metanotal median setae are shorter than the fore wing sub-basal setae S1 and slender, whereas in *ichini* the metanotal setae are usually stout (Fig. 6) and always longer than the fore wing sub-basal setae S1. In addition, both sexes of *ichini* from all sites usually have the mid-dorsal pair of setae on the head stout and about half as long as the major postocular setae, although almost 5% of the available specimens have one or both of the mid-dorsal setae minute and thus share this character state with the new species. Antennal segments III and IV are generally less slender in the new species than in *ichini*, but vary in both species in relation to body size. Other characters are also too variable to provide easy discrimination between the two species.

In both species, the lengths of the major pronotal setae are particularly variable, both within and between the sexes, thus obscuring any inter-specific differences. Moreover, bilateral asymmetry in the lengths of major setae, including those on the pronotum, is common in both species. This variation has clear implications for distinguishing species in this genus when they are known only from small samples. In contrast, the difference in colour between the larvae of the two species, orange in *ichini* but deep crimson red in *gandolfoi*, is very distinctive.

The systematics of *Pseudophilothrips*

The family Phlaeothripidae, to which this new species belongs, comprises two sub-families, but the supra-generic classification of the largest of these, the Phlaeothripinae, is not satisfactory. Currently, most of the leaf-feeding species in this sub-family are referred to the "*Liothrips*-lineage", a poorly defined group (Mound & Marullo, 1996) that includes several hundred species in about 100 genera, of which 40 are associated with leaf galls (Mound, 1994). The genus *Liothrips* itself includes 250 species worldwide, but many of these are known only from one or very few specimens (Mound & Pereyra, 2008). As a result, there are few well-established host-plant associations for the species, and almost no studies on the structural variation that might be expected within and between populations. For the purposes of the present study, original specimens of most of the species of *Liothrips* described from North and South America have been re-examined during visits to the major museum collections in Frankfurt, London, Washington and California. As a result several members of *Liothrips* are here transferred to *Pseudophilothrips*, and the definition of this genus is re-examined.

Johansen (1979) erected *Pseudophilothrips* for a single new species, *P. moundi*, based on eight females and four males taken from an unidentified tree in Vera Cruz State, Mexico. Shortly after this, the same author (1981) described a second species in this genus, *P. amabilis*, based on a single female from northern Mexico, and he also included in the genus the species *Liothrips ichini* Hood. The only character used to define *Pseudophilothrips* was the presence on the head of four long postocular setae, in contrast to the normal condition amongst Phlaeothripidae of only two such setae. Despite this, the only specimen of *P. amabilis* was illustrated as having one of the four postocular setae very short, and Johansen (1981) also stated that *P. ichini*

sometimes has the inner pair of these setae short. The genus was thus not clearly distinguished from the worldwide genus *Liothrips*. Mound & Marullo (1996) recognized this problem and, in transferring a fourth species into *Pseudophilothrips*, they proposed distinguishing the genus on the following character states:

1. Males with major setae pair S2 on tergite IX as long as pair S1;
2. Head long with slightly concave cheeks;
3. Head sometimes with two pairs of postocular setae
4. Pronotal epimera sometimes with two pairs of major setae.

Character state 1 is the most constant of these, although even this is variable within one species, *P. didymopanicis* (see below). The second character state cannot be considered valid; to a large extent it correlates with body size, and it recurs in various species of *Liothrips* around the world. The third and fourth character states are variable, both within and between species, but they do not occur in any of the numerous Old World species of *Liothrips*. Thus *Pseudophilothrips* apparently represents a discrete New World, mainly South American, lineage. Despite this, there is no evidence that it is sister-group to the larger genus, and presumably it was derived from within *Liothrips*. The following 13 species are here included in this genus.

Pseudophilothrips amabilis Johansen, 1981. This species is known only from the description based on a single female from Mexico.

Pseudophilothrips adisi (zur Strassen, 1978) **comb.n.** Described from Brazil in *Liothrips*, and known as a pest of *Paullinia cupana* (Sapindaceae), this species possesses character states 1 and 4 above, but there is only one pair of postocular setae, and the head is not elongate. One male in the type series has one of the two metanotal setae unusually stout, although the left seta of the pair is short as in the rest of the type specimens.

Pseudophilothrips avocadis (Hood, 1935) **comb.n.** Described in *Liothrips* and known from Panama and Costa Rica in association with *Persea americana* (Lauraceae), this species possesses character state 1 above, and has a long head, but has only a single pair of postocular and epimeral setae.

Pseudophilothrips didymopanicis (Del Claro & Mound, 1996) **comb.n.** Described in *Liothrips* from Brazil on *Didymopanax* (Araliaceae), this species possesses character states 1 and 3 above, although both are variable (even bilaterally asymmetric) among the available specimens. There is only one pair of setae on the prothoracic epimera, and the head is not elongate.

Pseudophilothrips fugitivus (Johansen, 1983). Described in *Phrasterothrips* from 20 specimens taken on an unknown tree, this Mexican species was later transferred to *Pseudophilothrips* by Mound & Marullo (1996). It possesses character states 1 and 4, but the long head is reported to have only one pair of postocular setae.

Pseudophilothrips gandolfoi **sp.n**. Known only from Curitiba in southern Brazil on *Schinus terebinthifolius*.

Pseudophilothrips ichini (Hood, 1949). Described in *Liothrips*, this species was referred to *Pseudophilothrips* by Johansen (1981). The original specimens came from Rio de Janeiro, but the species is widespread in eastern Brazil on *Schinus terebinthifolius* (Anacardiaceae).

Pseudophilothrips moundi Johansen, 1979. Described from 12 specimens from an unknown tree, this Mexican species is reported as having character states 1, 2 and 3, but not 4.

Pseudophilothrips obscuricornis (Priesner, 1921) **comb.n.** Described as a variety of *Liothrips seticollis*, this species was based on a single male from Paraguay that exhibits character state 1 above. This slide-mounted specimen, with only one antenna, has been re-examined on loan from the Museum für Naturkunde, Berlin. Antennal segment III is yellow, but segments IV–V are yellowish-brown. The metanotal median setae are small, and the setae on tergite IX are rather paler than the setae on tergites VII–VIII. This species is interpreted here as the same as a species that has been collected fairly commonly (by Estevão A. Silva) on the leaves of *Banisteriopsis malifolia* [Malpighiaceae] at Uberlândia, M.G., Brazil. In these specimens, the metanotal setae are larger than in the male from Paraguay, but vary in size, and the setae on tergite IX also vary from light to dark brown.

Pseudophilothrips perseae (Watson, 1923) **comb.n.** Described in *Liothrips* and known only from Guatemala and Honduras in association with *Persea* (Lauraceae), this species is very similar to *P. avocadis*, but has the antennae more extensively yellow.

Pseudophilothrips retanai Soto, 2000. Known only from two males taken in a trap in Costa Rica, this species has character states 1, 3 and 4 above, but was described as having antennal segments III–V pale yellow, and segment VI yellow at the base.

Pseudophilothrips seticollis (Karny, 1912) **comb.n.** Described in *Liothrips*, and known only from the few original specimens from Paraguay, this species possesses character state 1, but antennal segment III is yellow, IV shaded at only at the apex, V is yellow in the basal fifth, and VI is yellow at the base.

Pseudophilothrips varicornis (Hood, 1912) **comb.n.** Described in *Liothrips*, and studied from California, Mexico, Bahamas, Hawaii, and Tahiti, this species is associated with the leaves of various Malvaceae. It possesses character states 1 and usually 4, but the second pair of epimeral setae varies in development in both sexes.

Considering these 13 species, five of them are known to have antennal segments III and IV more or less equally yellow: *amabilis*, *didymopanicis*, *perseae*, *retanai* and *seticollis*. In contrast the other eight species have these two antennal segments differing sharply in colour; III is yellow, but IV largely dark brown. Unfortunately, *obscuricornis* is variable and intermediate between these two groups, and presumably is closely related to *didymopanicis*. Two of the eight species have the major setae on tergite IX distinctively pale, often almost white (*avocadis*, *varicornis*), whereas the other six species are considered to have these setae about as dark as the setae on the other tergites. One of these, *adisi*, has the head unusually short, less than 1.1–1.2 times as long as the maximum width, whereas the head is considerably longer (1.4 times) among the remaining five species. The type species of the genus, *moundi*, is illustrated (Johansen, 1981) as having postocular setae considerably shorter than the dorsal length of a compound eye, whereas these setae are long and slender in the remaining four species. The Mexican species *fugitivus* is reported to lack a second pair of major postocular setae, and to have the median metanotal setae long and stout. From the original description, it seems possible that this species may represent a northern race of *ichini*, but this requires further study.

In conclusion, it is clear from both the morphological and the molecular character states, that the concept of species in this genus is particularly difficult to define. Biological studies on the forms associated with *Schinus* suggest that there is likely to be a high degree of host specificity among *Pseudophilothrips* species, but that this may not always be closely correlated with structural or colour differences. The phenomenon, and taxonomic problem, of sibling species is probably widespread amongst leaf-feeding Phlaeothripidae of the *Liothrips* group, as is also clear from combined morphological and molecular studies on Australian leaf-galling thrips (McLeish *et al.*, 2006). This problem at species level is further reflected in the difficulties of assessing systematic relationships. The genus *Liothrips* occurs world wide, and the many species included exhibit a considerable range of body form. Morphological studies have failed to recognize useful segregates within this complex, with the exception of the group discussed in this paper. It is to be hoped that future molecular studies may provide data less susceptible to homoplasy than character states like setal lengths, and head length/width ratios. A similar problem exists with the species allocated to *Teuchothrips*, a particularly poorly defined genus used primarily for Australian species in the *Liothrips* complex (Mound, 2008).

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