Identification of Larvae and Recognition of New Synonymy for Two Chinese *Onycholyda* Sawflies (Insecta: Hymenoptera: Pamphiliidae) based on Molecular Data

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A molecular analysis based on mitochondrial COI+tRNA(Leu)+COII gene sequences has shown that unidentified larvae collected in Hunan, Hubei and Sichuan Provinces, China, belong to *Onycholyda flavicostalis* Shinohara, 2012, and *O. sichuanica* Shinohara, Naito and Huang, 1988 (Hymenoptera: Pamphiliidae), and that the latter species is conspecific with *O. fanjingshanica* Jiang, Wei and Zhu, 2004. *Onycholyda fanjingshanica* is thus synonymized with *O. sichuanica*. The first host plant records are *Rubus adenophorus* Rolfe (Rosaceae) for *O. flavicostalis* and *Rubus* spp. for *O. sichuanica*. The larvae of *O. flavicostalis* and *O. sichuanica* are briefly described and *O. flavicostalis* is newly recorded from Hubei and Sichuan Provinces.

Key Words: Hymenoptera, Pamphiliidae, *Onycholyda*, molecular analysis, larva, host plant, *Rubus*, new synonymy, China.

Introduction

Holometabolous insects exhibit conspicuous morphological differences between larval and imaginal stages and often between sexes. Identification of previously unknown larvae solely based on morphology is therefore usually impossible and conspicuous sexual dimorphism often causes publication of different names for different sexes of the same species, thus bringing confusion into classification. The only reliable conventional method for solving these problems is to obtain firm evidence for direct relationships between larvae and adults or between different sexes of adults by rearing, which is usually difficult and time-consuming. Recent developments of molecular methods make it much easier. Direct comparison of DNA molecules provides most useful tools for establishing such relationships (Caterino and Tishechkin 2006; Ahrens *et al.* 2007) and such molecular techniques have been applied also to some sawfly groups (Shinohara *et al.* 2016a, b, 2017).

Leaf-rolling sawflies of the *Onycholyda wongi* complex show large sexual dimorphism. This is one of the two species complexes of the *O. wongi* subgroup of the *O. luteicornis* group and is characterized by the roundly swollen upper frons in the female, the bidentate left mandible and the long 3rd antennomere (Shinohara 2002). It is represented by eight species mainly distributed in southern China. Of these, both sexes are known for *O. odaesana* Shinohara and

Byun, 1993, and *O. flavicostalis* Shinohara, 2012, whereas only the male is known for *O. sichuanica* Shinohara, Naito and Huang, 1988, and only the female is known for each of the five remaining species, including *O. fanjingshanica* Jiang, Wei and Zhu, 2004 (Shinohara and Wei 2012). Shinohara and Wei (2012) suggested a high possibility that *O. sichuanica* and *O. fanjingshanica* are conspecific, the two taxa being the opposite sexes of one and the same species. Of the eight species of the *O. wongi* complex, immature stages and host plants are known only for *O. odaesana* (Shinohara and Wei 2010).

In 2009–2015, Shinohara obtained adult specimens of *O. odaesana*, *O. flavicostalis*, *O. sichuanica* and *O. fanjingshanica*, as well as unidentified larvae presumably of *Onycholyda* species feeding on *Rubus* spp. (Rosaceae), including *Rubus adenophorus* Rolfe, in Hunan, Hubei and Sichuan Provinces, China. Here we present a result of our attempt to determine the specific relationships of *O. sichuanica* and *O. fanjingshanica* and to identify the unidentified larvae by using molecular methods.

Materials and Methods

Sampling. We examined 12 adult individuals of *Onycholyda* determined as *O. flavicostalis*, *O. odaesana*, *O. sichuanica* and *O. fanjingshanica* and 17 unidentified larvae presumably of *Onycholyda* collected in Hunan, Hubei and

Sichuan Provinces, China (Table 1). Two male adult specimens of *Pamphilius atricaudatus* Shinohara, 2012, were used as the outgroup. The genera *Onycholyda* and *Pamphilius* belong to the tribe Pamphiliini, which is considered monophyletic (Shinohara 2002). The intergeneric relationships within the tribe are not well resolved, though *Onycholyda* is evidently a monophyletic group with a sister relationship with *Pamphilius* or its component subgroup (Shinohara 2002). *Pamphilius atricaudatus* belongs to the *komonensis* subgroup of the *alternans* group, which is a part of the large assemblage of ten species groups and this is a possible sister group of *Onycholyda* (Shinohara 2002). *Pamphilius atricaudatus* is thus well qualified as an outgroup species for the present analysis. Shinohara identified all the adult specimens based on published papers (Shinohara *et al.* 1988; Shinohara and Byun 1993; Jiang *et al.* 2004; Shinohara and Wei 2012) and examination of the relevant type material. All the materials are kept in the National Museum of Nature and Science, Tsukuba.

DNA extraction, PCR and Sequencing. Adults and larvae collected in the field for DNA analysis were preserved in 99% ethanol and kept in a fridge. Genomic DNA was extracted from middle legs of adults or anterior part of larval abdomen using NucleoSpin Tissue (MACHEREY-NAGEL) following standard protocol. Polymerase chain

reaction (PCR) was used to amplify a sequence of about 1300 base pairs that is combination of partial cytochrome *c* oxidase subunit I (COI) gene (733 bp), complete tRNA Leucine (67–69 bp) and partial cytochrome *c* oxidase subunit II (COII) gene (461 bp) from total DNA template using two newly designed primers, HymenoF2 (5'-ACC AGG ATT TGG TAT AAT TTC TCA-3') and HymenoR1 (5'-CGA CCA GGG ATA GCA TCA AT-3'). PCR condition was: initial denaturation for 2 min at 94°C, followed by 30 cycles of 10 s at 98°C, 15 s at 60°C, and 1 min at 72°C, with a final extension for 10 min at 72°C. Reactions were aided by ExTaq HS polymerase (TaKaRa) and Tks Gflex DNA Polymerase (TaKaRa), and the products were purified with Exo-SAP-IT (Affymetrix). Sequencing reaction was performed using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies) and electrophoresed on an ABI 3500xl sequencer (Life Technologies). The obtained sequences have been deposited in the DDBJ/EMBL/GenBank database under accession numbers given in Table 1.

Phylogenetic analysis. DNA sequences were aligned using MUSCLE implemented in MEGA7 (Edgar 2004; Kumar *et al.* 2016) with the initial setting. Phylogenetic trees were obtained by maximum parsimony (MP) and maximum likelihood (ML) methods implemented in MEGA. For MP analyses, heuristic searches were conducted with 10

Table 1. Material used for molecular analysis. All collected by A. Shinohara.

sample no.	collection data		GenBank	species
765	♂	HUNAN, Mt. Yunshan, 14. IV. 2012	LC360118	<i>Onycholyda flavicostalis</i>
766	♂	HUNAN, Mt. Yunshan, 9. IV. 2012	LC360119	
767	♀	HUNAN, Mt. Yunshan, 14. IV. 2010	LC360120	
778	♀	HUNAN, Mt. Yunshan, 10. IV. 2012	LC360123	
466	larva	HUBEI, Muyu, Shennongjia, 18. V. 2010, on <i>Rubus adenophorus</i> , fixed 21. V.	LC360113	<i>Onycholyda flavicostalis**</i>
470–472	larva	HUBEI, Muyu, Shennongjia, 18. V. 2010, on <i>Rubus adenophorus</i> , fixed 22. V.	LC360114–LC360116	
587	larva	SICHUAN, Mt. Emeishan, about 32 km from Baoguosi, 14. V. 2015, on <i>Rubus</i> sp., fixed 18. V.	LC360117	
775	larva	HUBEI, Qianjiaping, Shennongjia, 18. V. 2012, on <i>Rubus adenophorus</i> , fixed 22. V. (AS120518A)	LC360121	
777	larva	HUBEI, Qianjiaping, Shennongjia, 18. V. 2012, on <i>Rubus adenophorus</i> , fixed 22. V. (AS120518B)	LC360122	
781	larva	HUBEI, Guanmenshan, Shennongjia, 23. V. 2012, on <i>Rubus adenophorus</i> , fixed 22. V. (AS120523)	LC360124	
783–785	larva	HUNAN, Mt. Yunshan, 2. V. 2009, on <i>Rubus adenophorus</i> , fixed 8. V. (AS090502C)	LC360125–LC360127	
763	♂	HUNAN, Mt. Yunshan, 10. IV. 2012	LC360134	<i>Onycholyda sichuanica</i>
764	♂	HUNAN, Mt. Yunshan, 16. IV. 2012	LC360135	
779, 780	♀	HUNAN, Mt. Yunshan, 10. IV. 2012	LC360136 LC360137	<i>Onycholyda sichuanica*</i>
480, 488	larva	HUNAN, Mt. Hupingshan, 8. VI. 2013, on <i>Rubus</i> sp., matured & fixed 11. VI.	LC360128 LC360132	<i>Onycholyda sichuanica**</i>
481, 482	larva	HUNAN, Mt. Hupingshan, 8. VI. 2013, on <i>Rubus</i> sp., matured & fixed 10. VI.	LC360129 LC360130	
486	larva	HUNAN, Mt. Hupingshan, 8. VI. 2013, on <i>Rubus</i> sp., matured & fixed 12. VI.	LC360131	
489	larva	HUNAN, Mt. Hupingshan, 8. VI. 2013, on <i>Rubus</i> sp., matured & fixed 9. VI.	LC360133	
768	♀	HUNAN, Mt. Yunshan, 10. IV. 2012	LC360138	<i>Onycholyda odaesana</i>
771	♂	HUNAN, Mt. Yunshan, 10. IV. 2012	LC360139	
772, 773	♂	HUNAN, Mt. Yunshan, 20. IV. 2010	LC360140 LC360141	
786, 787	♂	HUNAN, Mt. Yunshan, 19–20. IV. 2012	LC360142 LC360143	<i>Pamphilius atricaudatus</i>

*Originally identified as *Onycholyda fanjingshanica*. **Identified by molecular data.

random addition analyses using equal character weights and tree-bisection-reconnection (TBR) branch swapping. Prior to ML analysis, we determined an appropriate model of sequence evolution and model parameters using MEGA. As a result, T92+I+G model was selected. Based on the selected model, ML analysis was performed with heuristic searches with subtree-pruning-regrafting-extensive (SPR) branch swapping. Nodal support for the MP and ML analyses were assessed using bootstrap analyses with 1000 replications.

Observations of morphology, plant scientific names, morphological terminology and photography. Observations of morphology were made with an Olympus SZ60 stereo binocular microscope. For the scientific names of the host plant and larval morphological terminology, we followed Lu and Boufford (2003) and Viitasaari (2002), respectively. Photographs were taken with digital cameras, Canon EOS Kiss Digital X and Ricoh Caplio GX100 (Figs 2, 3). The digital images were processed and arranged with Adobe Photoshop Elements® 9 and 12 software.

Results and Discussion

Phylogenetic analysis. The MP analysis resulted in eight most parsimonious trees (144 steps, consistency index excluding uninformative sites [CI]=0.8951, retention index [RI]=0.9743). The result of ML and MP analyses were consistent with the relatively well-supported clades (Fig. 1). Both analyses classified *Onycholyda* specimens into three clades (A–C). Haplotype sources of each clade are as follows: clade A from *O. flavicostalis* and 11 undetermined larval samples, clade B from *O. sichuanica*, *O. fanjingshanica* and six undetermined larval samples, and clade C only from *O. odaesana*. Clades A and B were reconstructed as the reciprocal monophyly but the support value was rather low. Each of the clades A and B was supported by high bootstrap values (99%) and the internal distance (maximum composite likelihood model) of each of the two clades was very small (0.5% in clade A and 0.3% in clade B), which was comparable to the intraspecific distance in *O. odaesana* (0.5%, clade C). The distances between clades A and B, B and C, and C and A were 3.4–3.7%, 3.2–3.6%, and 3.2–3.7%, respectively, and the distance between *Onycholyda* spp. and outgroup was 8.8–9.5%. This strongly suggests that *O. sichuanica* and *O. fanjingshanica* (both in clade B) are conspecific and that the undetermined larval samples in clade A belong to *O. flavicostalis* and those in clade B belong to *O. sichuanica/O. fanjingshanica*.

Onycholyda flavicostalis Shinohara, 2012 (Fig. 2)

Onycholyda flavicostalis Shinohara, in Shinohara and Wei, 2012: 57.

Distribution. China (Shaanxi, Hubei, Hunan, Sichuan).

Host plants. *Rubus adenophorus* Rolfe., *Rubus* sp.

Field observations and rearing records. *Case A.* One leaf-roll on *Rubus adenophorus* containing seven gregari-

Fig. 1. The maximum likelihood tree based on 1184 bp of mitochondrial COI+tRNA(Leu)+COII gene sequence (-ln likelihood=2213.6646). Numbers on branches indicate bootstrap values for MP/ML analyses (shown only for higher nodes with >50). Each terminal label represents the sample number.

ous middle-instar larvae was found in Mt. Yunshan, Hunan Province on May 2, 2009. The leaf-roll was on the underside of the leaf and remains of the egg shell were not found. The larvae were fixed on May 8 (including sample nos. 783–785, Table 1). *Case B.* Nine leaf-rolls, one each containing one larva, were found on *R. adenophorus* at Yaolangou, Muyu, Shennongjia, Hubei Province, on May 18, 2010. All the nine larvae were fixed in ethanol on May 19 and 21 (one of them, sample no. 466). Another leaf-roll containing three larvae was also found on *R. adenophorus* at the same locality on the same day. The three larvae were fixed in ethanol on May 22 (sample nos. 470–472). For all the ten leaf-rolls examined, remains of the egg shells were not found. *Case C.* Two leaf-rolls were found on *R. adenophorus* in Qianjiaping, Shennongjia, Hubei Province on May 18, 2012. One of them (Fig. 2A) contained one larva and the other (Fig. 2B) contained two larvae. Each of the leaf-rolls was on the underside of the leaf and remains of the egg shell were not found. The larvae matured (one of them in Fig. 2C) and were fixed on May 22 (including sample nos. 775, 777). *Case D.* Two

Fig. 2. *Onycholyda flavicostalis*, larval leaf-rolls (A, B), mature larva (C, D), and eggs deposited on the leaf of *Rubus adenophorus* (E, F), all collected at Shennongjia, Hubei Province, and photographed at the site (A, B, E, F) or indoors (C, D) by A. Shinohara. —A, Leaf-roll (one larva, sample no. 775 inside), Qianjiaping, May 18, 2012; B, leaf-roll (two larvae, including sample no. 777 inside), Qianjiaping, May 18, 2012; C, mature larva (sample no. 775), May 22, 2012; D, mature larva (sample no. 781 from one of the eggs shown in E), June 6, 2012; E, leaves with two groups of eggs (arrowed), Guanmenshan, May 23, 2012; F, same, enlarged.

groups of eggs, consisting of five eggs and three eggs, respectively, were found on the same leaf of *R. adenophorus* in Guanmenshan, Shennongjia, Hubei Province on May 23,

2012. The groups of eggs were deposited on the lateral veins in a line in the basal part of the underside of a leaf (Fig. 2E, F). The eggs hatched on May 27 and the larvae were fixed on

Fig. 3. *Onycholyda sichuanica*, larval leaf-rolls (A–D), remains of egg shells (D, E) and late instar and mature larvae (G–I), all collected at Mt. Hupingshan, Hunan Province, June 8, 2013 and photographed at the site (A, C, D) or indoors (B, E–I) by A. Shinohara. —A, Leaf-roll on *Rubus* sp. (arrowed); B, leaf-roll on *Rubus* sp.; C, leaf-roll on *Rubus* sp.; D, leaf-roll on *Rubus* sp.; E, close-up of two egg shells, same as in D; F, close-up of three egg shells, same as in B; G, late instar larva, June 14; H, late instar larva, June 14; I, mature larva, June 9 (sample no. 489). Remains of egg shells arrowed in B–D.

June 6 (one of them sample no. 781). **Case E.** One leaf-roll containing one larva was found on *Rubus* sp. along a narrow unpaved car road at about 1600m in altitude about 32km from Baoguosi on Mt. Emeishan, Sichuan Province, on May 14, 2015. The larva was fixed in ethanol on May 18 (sample no. 587).

Egg. Normal for pamphiliids, yellowish white, deposited in a row along lateral veins in basal part of the leaf (Fig. 2E, F).

Larva. *Mature larva* (Fig. 2C, D). Head black, with epicranial suture and part of clypeus greenish; antenna creamy white, each antennomere more or less dark brown-

ish; labrum and mandible dark brown, palpi pale brown, and other mouthparts greenish, partly marked with blackish brown; trunk pale greenish, terminal segment largely creamy white; cervical sclerite partly blackish; thoracic legs and subanal appendage greenish white; suranal hook dark brown.

Remarks. Our molecular analysis has clearly shown that the larvae feeding on *Rubus adenophorus* from Shennongjia, Hubei Province, and Mt. Yunshan, Hunan Province, and those feeding on *Rubus* sp. from Mt. Emeishan, Sichuan Province, belong to *O. flavicostalis*, which was described from Mt. Yunshan, Hunan Province. This is the first record of the host plant and larva of *O. flavicostalis* and the first distribution record of this sawfly from Hubei and Sichuan Provinces.

Onycholyda flavicostalis and *O. odaesana* Shinohara and Byun, 1993, occur together on Mt. Yunshan in Hunan Province (Shinohara and Wei 2012) and the larvae of the two species feed on the same host plant, *R. adenophorus* (Shinohara and Wei 2010; present study). The larva of *O. flavicostalis* is a gregarious or solitary leaf-roller with a black head, whereas the larva of *O. odaesana* is solitary and has a pale brown head with three black spots. Among the non-Chinese species, the larva of *O. esakii* (Takeuchi, 1938) from Japan is a gregarious leaf-roller and has a color pattern very similar to that of *O. flavicostalis* (Shinohara and Kojima 2009). We are not able to distinguish the larvae of the two species based on a current knowledge besides the differences in distribution and the host plant species. The mature larva of *O. sichuanica* also has an entirely black head and a pale green trunk but the cervical sclerite is entirely pale in this species, unlike those of *O. flavicostalis* and *O. esakii*.

As noted in the section of field observations above, the female of *O. flavicostalis* deposits eggs in a row (up to seven eggs together) and the middle-instar larvae live gregariously in one leaf-roll, whereas the late-instar larvae may live in a small group or even solitarily. The range of variation in the larval gregariousness of this species should be confirmed by obtaining further information.

Onycholyda sichuanica Shinohara, Naito and Huang, 1988 (Fig. 3)

Onycholyda sichuanica Shinohara, Naito and Huang, 1988: 92; Xin and Wu, 2010: 10; Shinohara and Wei, 2012: 56.

Onycholyda fanjingshanica Jiang, Wei and Zhu, 2004: 44; Shinohara and Wei, 2012: 56. **N. syn.**

Distribution. China (Sichuan, Gansu, Guizhou, Guangxi, Hubei, Hunan, Anhui, Fujian).

Host plants. *Rubus* spp.

Field observations and rearing records. On June 8, 2013, Shinohara found seven leaf-rolls (four of them in Fig. 3A–D), containing ten larvae, on *Rubus* spp. at Huatai, ca. 900 m, Mt. Hupingshan, Hunan Province. Of the seven leaf-rolls, one contained three larvae (three egg shells), one contained two larvae (one of them sample no. 486, two egg shells, Fig. 3D, E), while the other five contained one larva,

respectively (sample nos. 480–482, 488, 489; for four of them, no egg shells were found, whereas five egg shells were found on the remaining one leaf, Fig. 3B, F). Of the ten larvae, one larva (sample no. 489) matured and was fixed in ethanol on June 9, two (sample nos. 481, 482) on June 10, two (sample nos. 480, 488) on June 11, one (sample no. 486) on June 12, while the remaining four were fixed in ethanol on June 14 before maturation. Six of the ten larvae were used for molecular analysis (Table 1).

All the leaf-rolls and remains of the egg shells were found on the under surface of the leaves and all the egg shells were located on the lateral veins near the base of the leaf (Fig. 3B–F). The larval abodes were irregular in shape (Fig. 3A–D).

Larva. *Late instar* (Fig. 3G, H): Head black; antenna creamy white, each antennomere more or less dark brownish; trunk pale greenish white, terminal segment largely creamy white; thoracic legs and subanal appendage white. *Mature larva* (Fig. 3I): Similar to the preceding, but antenna pale brown and trunk, including thoracic legs and subanal appendage, vivid pale green.

Remarks. Shinohara and Wei (2012) suspected that *O. sichuanica* (only males known) and *O. fanjingshanica* (only females known) are opposite sexes of the same species, because the two “species” had much in common in morphology and were obtained together in the same area on Mt. Yunshan, Hunan Province. Our analysis based on mitochondrial COI+tRNA(Leu)+COII gene sequences perfectly supported the hypothesis that the two taxa are conspecific, and we hereby propose to treat them as synonyms.

The adult specimens from Mt. Yunshan (sample nos. 763, 764, 779, 780) and larval material from Mt. Hupingshan (sample nos. 480–482, 486, 488, 489), both localities in Hunan Province, agreed in mitochondrial COI+tRNA(Leu)+COII gene sequences and we determine the larvae as *O. sichuanica*. This is the first record of the larvae and host plant of this sawfly.

The previously unknown larva of this species is well characterized by the entirely black head and entirely pale green trunk in late-instar and prepupal stages (Fig. 3G–I). It is quite similar to that of *O. flavicostalis* except for the entirely pale cervical sclerite (compare Fig. 3G–I with Fig. 2D). The larva of the Japanese *O. esakii* also has a similar color pattern, but the cervical sclerite is black (Shinohara and Kojima 2009). The observed larvae of *O. sichuanica* were solitary or living together in a small group.

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