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Molecular Systematics of Tephritidae (Insecta: Diptera): Testing Phylogenetic Position of Korean *Acidiella* spp. (Trypetini) Using Mitochondrial 16S rDNA Sequences

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Phylogenetic relationships of Korean *Acidiella* species were tested using mitochondrial 16S ribosomal RNA gene sequences. We used 16 published sequences as outgroup, and 10 new sequences for nine Korean *Acidiella* species as ingroup. The number of aligned sites was 1,281 bp, but 1,135 bp were used for the analysis after excluding sites with missing data or gaps. Among these 1,135 sites, 464 sites were variable and 340 were informative for parsimony analysis. Phylogenetic information was extracted from this data set using neighbor-joining, maximum likelihood and maximum parsimony methods and compared to a morphology-based phylogenetic hypothesis. Our molecular data suggest that: (1) the tribe Trypetini appears to be monophyletic even when the nine additional *Acidiella* species are added to our previous phylogenetic analysis; (2) all the Korean *Acidiella* species belong to the *Trypeta* group, but the genus *Acidiella* is not supported as monophyletic; (3) the close relationship of *A. circumvaga*, *A. issikii*, and *A. sapporensis* is supported; (4) the close relationship of *A. pachypogon* and two additional new *Acidiella* species is strongly supported; and (5) the possible presence of two or more cryptic species among the specimens previously identified as *A. obscuripennis* is suggested. Sequence data from the mitochondrial 16S rDNA allowed us to better understand the systematic status of Korean *Acidiella* species. They indicated that the current concept about the genus *Acidiella* is insufficient and needs to be refined further. This study also showed a few interesting relationships, that had not been recognized by morphological study alone. Based on this study, we were able to plan further experiments to analyze relationships within the *Trypeta* Group.

The Trypetini was recently redefined based on both morphology (Han, 1992, 1999) and molecules (Han, 2000; Han and McPheron, 1997, 1999). In these studies, the phylogenetic position of the Trypetini within Tephritidae was inferred and a number of subtribal groups within the Trypetini were recognized. However, only a fraction of higher taxa were sampled for the molecular analysis and many higher taxa remain to be included.

Within the Trypetini, Han (1999) defined the monophyletic *Trypeta* group of 12 genera using male genitalic characters. Han (2000) later tested the monophyly of the *Trypeta* group using molecular data. Other than supporting the group's monophyly, however, these studies neither resolved relationships within the *Trypeta* group nor provided evidence for the monophyly of some of the included genera. Therefore, more rigorous morphological and molecular studies were needed.

The present study aimed at elucidating the phylogenetic relationships of the Korean *Acidiella* species within the *Trypeta* group, in conjunction with the previously accumulated morphological and molecular data. Monophyly of *Acidiella* was previously suggested based on a single synapomorphy: posterior surstylar lobe reduced and anterior surstylar lobe expanded and bent inward (Han, 1992, 1999). A total of 39 *Acidiella* species are currently known in the world (Han, 1992, 1999; Norrbom et al. 1999), but some of the species have not been examined for their genitalic structure. Instead, these species were included here simply because their wing patterns are similar to other better known *Acidiella* species. Furthermore a few examined species that did not have the morphological synapomorphy were also tentatively included in *Acidiella* based on their similar wing patterns. These wing patterns might have resulted from convergent evolution, but no adequate generic placements for such species are currently available. Therefore, it is possible

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that the demarcation of *Acidiella* will be modified a great deal as more investigation progresses.

In Korea, a total of seven *Acidiella* species have been recognized (Han and Kwon, 2000). Among these species, only *A. maculata* and *A. obscuripennis* have the generic synapomorphy, and the remaining species were tentatively placed in *Acidiella* because of their similar wing patterns. There are also two additional new species which closely resemble *A. pachypogon*. Except for *A. maculata*, we were able to obtain fresh specimens of all the species, from which 16S mitochondrial ribosomal DNA sequences were obtained. We conducted a combined analysis of this data and the previous dataset from Han (2000).

Materials and Methods

We sequenced almost the entire 16S rDNA (1,223-1,232 base pairs) for a total of 10 specimens of the nine species (Table 1). Two *A. circumvaga* sequences were included because they showed substantial variation in their wing patterns and were initially thought to represent two different species. Either a whole body or a single leg was used for each DNA extraction. In both cases, voucher specimens (wing or remaining body part) were deposited in the Department of Life Science, Yonsei University, Korea.

Nucleic acid extractions followed a standard protocol optimized for individual freshly frozen flies (Sheppard et al., 1992). Pinned or alcohol-preserved specimens were extracted by the modified protocol described in Han and McPheron (1997). Some samples were also prepared using DNeasy tissue kit (Quagen Inc.).

The region to be analyzed was amplified using standard PCR approaches (e.g., Kocher et al., 1989). Double-stranded amplification product (40 amplification cycles of 93℃(1 min), 45℃(1 min), 72℃(2 min)) was purified by isolating the desired band after 2% agarose gel electrophoresis in 1X TAE buffer. Preparation of gel-purified template followed the freeze and thaw method of Palumbi et al. (1991). This product was re-amplified asymmetrically using one of the PCR primers or an internal primer as a limiting primer (1:25-100 ratio). Single-stranded DNA was purified and concentrated using the isopropanol precipitation method (Palumbi et al. 1991). Single-stranded DNA was sequenced by the dideoxy, chain-termination method (Sanger et al., 1977) using Sequenase version 2.0 (Amersham Co.).

The following primers were used to conduct PCR and sequencing reactions for the 16S rDNA: LR-N-13398 (5′-CGCCTGTTTATCAAAAACAT-3′) and LR-J-12883 (5′-CTCCGGTTTGAACTCAGATC-3′) (primers A and B, respectively, of Xiong and Kocher (1991)); LR-J-13021 (5′-ACGCTGTTATCCCTAAAGTA-3′); LR-N-13182 (5′-TTAAAAGACGAGAAGACCCTA-3′); LR-J-13323 (5′-ACTAATGATTATGCTACCTT-3′); LR-J-13677 (5′-AGCTTATCCCATAAAATATT-3′); LR-N-13770 (5′-AGAAATGAAATGTTATTCGT-3′); and TV-N-14112 (5′-AGCATTTCATTTACATTGAA-3′). For each relatively fresh specimen (e.g., less than 2 year old pinned or alcohol specimen kept in ambient temperature), two gel-purified templates were made using the primers LR-J-12883/LR-N-13398 and the primers LR-J-13323/TV-N-14112, respectively. For specimens with a high degree of DNA degradation, shorter segments were amplified for gel-purification. The DNA fragment used in this study represents the portion bracketed by the primers LR-J-12883 and TV-N-14112.

Table 1. Collection and voucher information of the Korean tephritid flies sequenced.

Acidiella angustifascia (Hering). Gangwon-do, Injae-gun, Girin-myeon, Gangseon-ri, Mt. Jeombongsan. 7.VIII.1997, H.-Y. Han et al. (wing slide; AF46785).
A. circumvaga (Ito) - 1. Gangwon-do, Wonju-si, Panbu-myeon, Mt. Baegunsan 900-1087m, 23.VI.1998, H.-Y. Han & D.-S. Choi (wing slide; AF467676).
A. circumvaga (Ito) - 2. Gangwon-do, Wonju-si, Gwirae-myeon, Cheoneun Temple to Mt. Sibjabong. 7.VI.1998. D.-S. Choi & S.-K. Kim (body without right hind leg and left wing; right wing slide; AF467677).
A. issikii (Shiraki). Gangwon-do, Wonju-si, Panbu-myeon, Mt. Baegunsan 900-1087m, 21.VI.1998. H.-Y. Han & D.-S. Choi (wing slide; AF467678).
A. pachypogon (Ito). Gangwon-do, Injae-gun, Girin-myeon, Gangseon-ri, Mt. Jeombongsan. 20.VI.1998. H.-Y. Han et al. (wing slide; AF467682).
A. sapporensis (Shiraki). Gangwon-do, Hongcheon-gun, Nae-myeon, Mt. Gachilbong. 25.V.1996. H.-Y. Han & H.-W. Byun (wing slide; AF467681).
A. n. sp.-1. Gangwon-do, Wonju-si, Gwirae-myeon, Cheoneun Temple to Mt. Sibjabong. 7.VI.1998. D.-S. Choi & S.-K. Kim (wing slide; AF467683).
A. n. sp.-2. Gangwon-do, Wonju-si, Panbu-myeon, Mt. Baegunsan 900-1087m, 5.VII.1998. H.-Y. Han et al. (left wing mounted on card point; AF467684).
A. sp.-3 near *obscuripennis* Chen. Gangwon-do, Injae-gun, Girin-myeon, north valley of Mt. Gyebangsan. 16.VI.1998, female. H.-Y. Han & K.-E. Ro (body without right hind leg and left wing; right wing slide; AF467680).
A. sp.-4 near *obscuripennis* Chen. Gangwon-do, Hongcheon-gun, Nae-myeon, Mt. Gachilbong. 25.V.1996, male. H.-Y. Han & H.-W. Byun (wing slide; AF467679).

Status of the voucher specimens and GenBank accession numbers are indicated in parentheses. Voucher information for the other species used in this study is found in Han (2000).

Alignment of the sequences was conducted using CLUSTAL X software (version 1.81, 2000; Thompson et al., 1997), but refinement of the alignment was made manually to increase sequence similarity using BioEdit software (Hall, 1999). Sites with missing data or gaps were excluded from the analyses to minimize artifacts of the alignment. Neighbor-joining (NJ) analysis was performed using MEGA version 2.1 (Kumar et al., 2001). The two-parameter model of nucleotide substitution (Kimura, 1980) was selected for the analysis based on Nei's (1991) guideline for choosing the most appropriate distance measure. The reliability of clustering pattern in trees was tested by the standard error test for the internal branches of NJ trees (Rzhetsky and Nei, 1992) and bootstrapping (Felsenstein, 1985) (2,000 replications). Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed with PAUP software (version 4.0b8; Swofford, 2000), using the heuristic search procedure. ML analysis

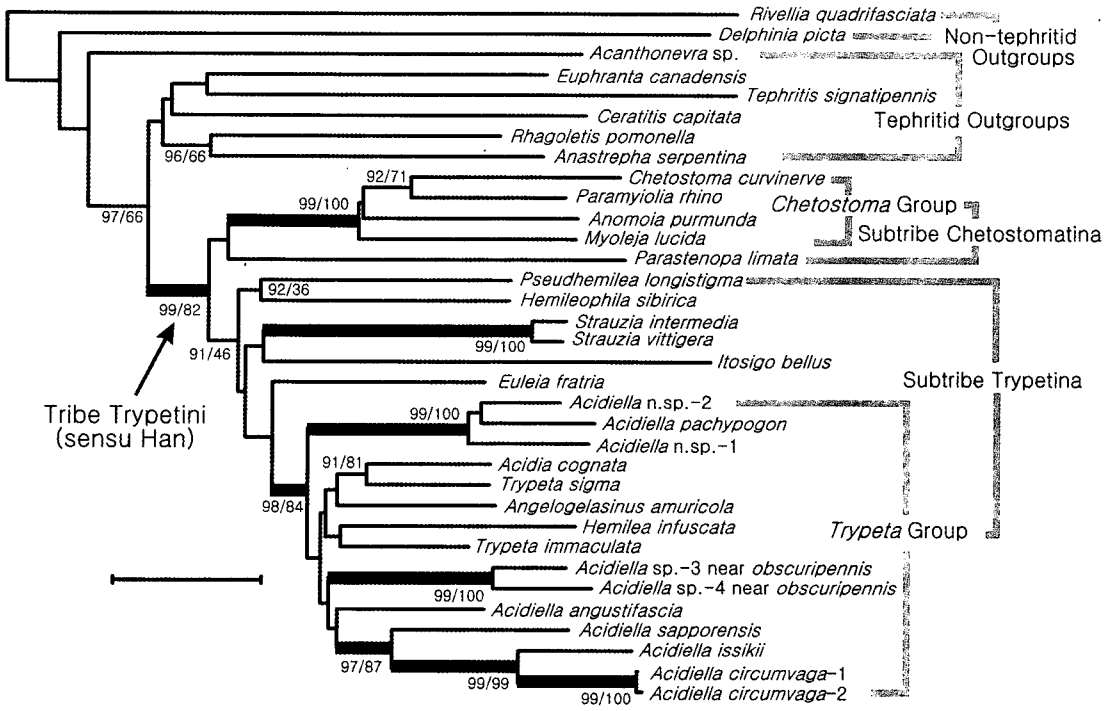

Fig. 1. Trypetine relationships inferred from neighbor-joining tree based on Kimura two parameter distances using mitochondrial 16S rDNA sequences (1,135 bp after gaps and sites with missing data removed). The first number on each branch is the Pc value from the standard error test (only values higher than 90% are shown), and the second number is the Pb from the bootstrap test (2,000 replications). Scale bar = 0.02 (Kimura 2-parameter distance).

employed a heuristic search, with the tree bisection-reconnection (TBR) branch swapping method and the start tree derived by stepwise addition. Bootstrapping of the MP analysis (500 replications) and ML analysis (100 replications) was also conducted under the heuristic search procedure, with a maxtree setting of 200 trees. All characters were treated as unordered and equally weighted for MP.

Sequence data including alignment generated in this study have been deposited in GenBank (Accession Nos. AF467676~AF467685). The alignment is also available as a Nexus file from the authors upon request.

Results

For 34 sequences used in this study, the number of aligned sites was 1,281 bp, but 1,135 bp were used for analysis after excluding sites with missing data or gaps. Among these 1,135 sites, 464 sites were variable and 340 were informative for parsimony analysis. The average proportion of A:T:C:G was 38.1:44.5:5.7:11.7 with a narrow standard error around means, but the base composition varied substantially in different portions within the sequences. Variability of different portions of the sequences varied substantially, and is also roughly correlated with the A+T content of the region (Han and McPheron, 1999).

The inferred phylogenetic trees based on NJ (Fig. 1), ML (Fig. 2A) and MP (Fig. 2B) were essentially same for the branches with higher statistical support. The NJ and ML topologies were more similar than the MP topology. The poor resolution in the MP tree was consistent with our prior experience with 16S rDNA, which showed severe rate heterogeneity, especially in base substitution (over 80% A+T) (Han and McPheron, 1999; Han, 2000). Such rate heterogeneity was taken into account with both NJ and ML methods, but our interpretation of the results was largely based on the NJ tree, for which more rigorous statistical testing of the interior branches were possible. Based on our previous analysis with the same 16S rDNA region, we considered Pc values (P values from standard error test) greater than 95% as statistically significant and Pb values (P values from bootstrap test) greater than 70% as generally informative. In the following discussion, we compared the molecular topologies with morphological hypotheses based on the previous taxonomic studies of the genus *Acidiella.*

Discussion

Trypetini is supported as a monophyletic group even when the nine additional *Acidiella* species are added to our previous phylogenetic analysis (Han, 2000). The two subtribes within the Trypetini, however, are only

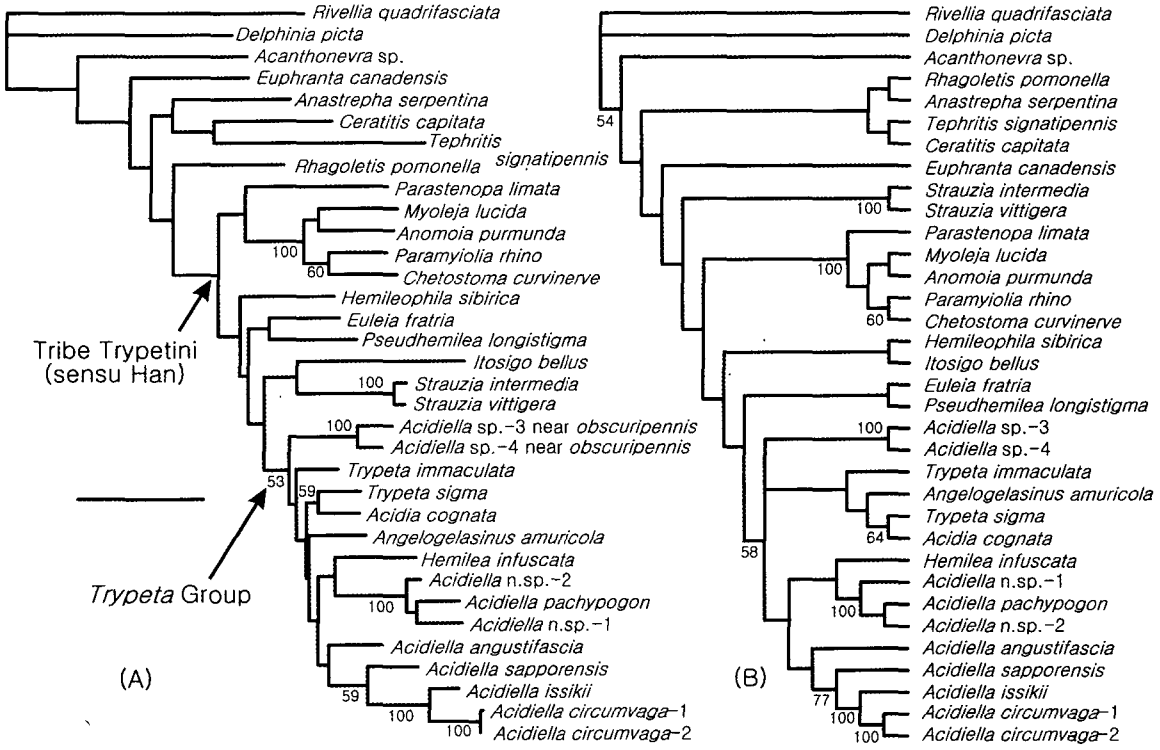

Fig. 2. Trypetine relationships inferred from (A) maximum likelihood tree based on HKY85 model, and (B) strict consensus tree of five most parsimonious trees. Statistics for each most parsimonious tree. L; 1573, CI; 0.4126, RI; 0.4487, RC; 0.1851. Bootstrap support is indicated on the nodes (only values greater than 50% are presented). Scale bar = 0.05.

supported topologically in both NJ and ML trees, but not in the MP tree.

All the Korean *Acidiella* species belong to the *Trypeta* group, whose monophyly is strongly supported. The group's monophyly is supported in all three analyses, but with the strongest statistical support in the NJ tree. Within the *Trypeta* group, however, *Acidiella* is not supported as monophyletic. Instead, the molecular results indicate that a serious revision of the taxonomic status of this genus is needed in the future. Fig. 3 shows wing patterns of the included species mapped on the unrooted NJ tree of the *Trypeta* group. If the molecular data are more reliable, the overall similarity of the wing patterns is not particularly useful in inferring their generic placement.

The close relationship of *A. circumvaga*, *A. issikii*, and *A. sapporensis* is strongly supported. *A. circumvaga* and *A. issikii* are morphologically very similar and have almost identical wing patterns (Fig. 3), and, for that reason, they were once suspected to be variants of a single species (Korneyev, 1998). Han and Kwon (2000) recently showed that they were separate species based on a series of specimens collected sympatrically over a few years. Both species were observed forming mating leks on the leaves of various broad leaf trees near and on the peaks of mountains over 1,000 m high in Korea. *A. sapporensis*, however, has been observed on rocks along streams in deep woods. Our study shows that *A. sapporensis* is closely related to the two former species. More rigorous morphological analysis of these *Acidiella* species is in progress to determine if they share morphological synapomorphies (Han, in preparation). Since *A. issikii* is the type species of the genus *Pseudacidia*, currently regarded a synonym of *Acidiella*, resurrection of this genus may be considered as more comparative data are accumulated.

The close relationship of *A. pachypogon*, *A.* n.sp.-1, and *A.* n.sp.-2 is strongly supported. The latter two new species resemble each other very closely, but their females can be clearly separated by the color of the oviscape. Our DNA samples were from female specimens. The males presumably conspecific with these females have sexually dimorphic wing patterns and can be roughly sorted into two species. However, since no specimens in copula were collected, we still have not been able to associate which males and females are conspecific. For this reason, we have not yet formally described them as new species. Females of these two species are relatively common along mountain trails in deep woods, but only a small number of males have been collected so far. We believe that nucleotide sequence data will provide critical evidence to associate the males and females correctly (Han and Ro, in preparation). *A. pachypogon*

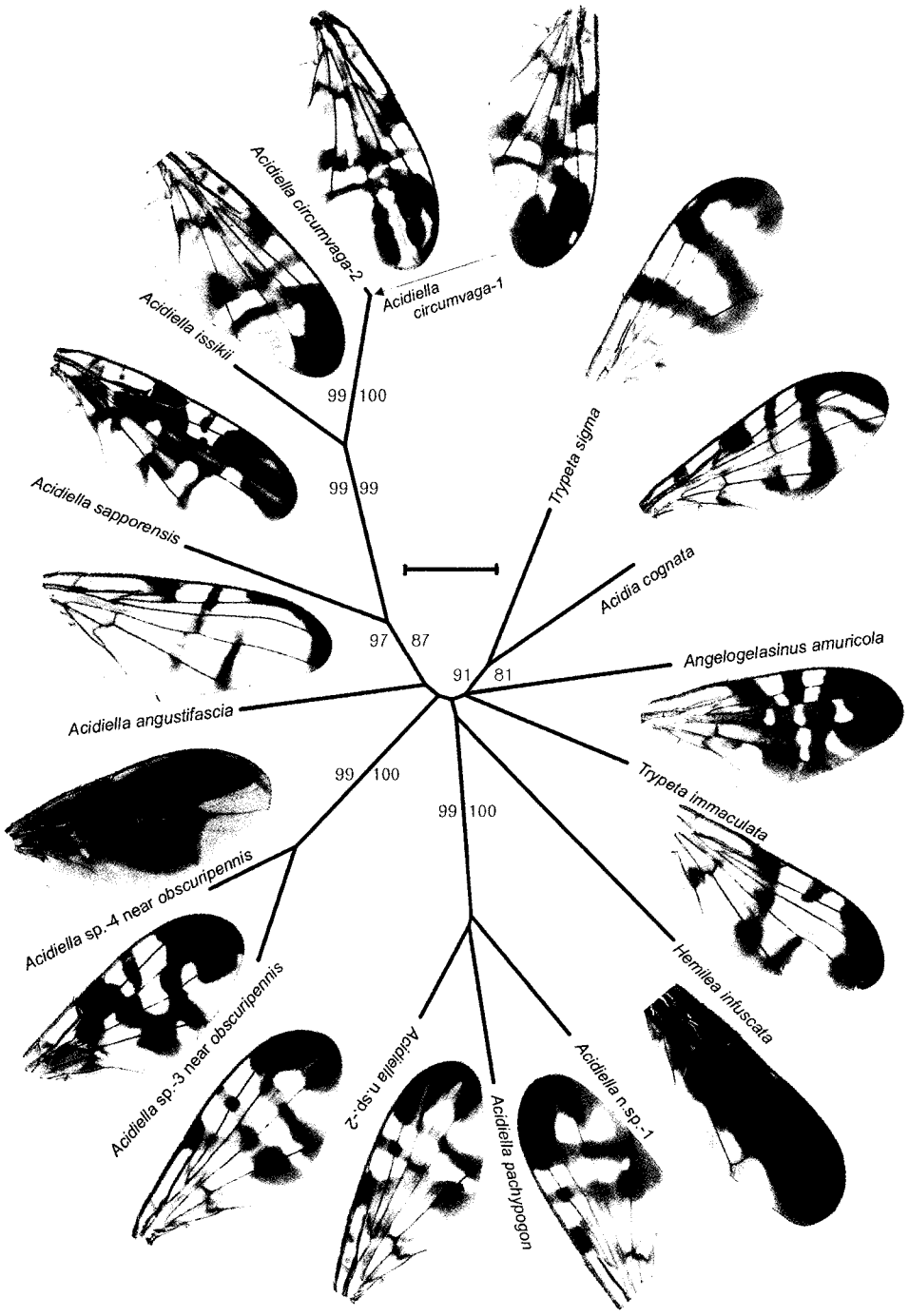

Fig. 3. Phylogenetic relationships of 15 Korean flies of the *Trypeta* group inferred from neighbor-joining tree based on Kimura two parameter distances using mitochondrial 16S rDNA sequences. The first number on each branch is the Pc value from the standard error test (only values higher than 90% are shown), and the second number is the Pb from the bootstrap test (2,000 replications). The wing patterns for the included taxa are shown. Scale bar = 0.01 (Kimura 2-parameter distance).

is also similar to these new species, but both sexes can be easily distinguished by having several strong genal setae.

Acidiella obscuripennis has a very distinct sexual dimorphism in its wing pattern (almost completely dark in male and typical *Acidiella* pattern in female - as in Fig. 3). We first thought that the *A.* sp.-3 and -4 were both sexes of *A. obscuripennis*, but their genetic

distance seems to indicate separate specific status (Figs. 2, 3). Since there is a rather wide variation in wing pattern among the collected females, a combined morphological and molecular study might distinguish two or more cryptic species.

The sequence data from the mitochondrial 16S rDNA allowed us to better understand the systematic status of Korean *Acidiella* species. They indicated that the current concept about the genus *Acidiella* is insufficient and needs to be refined further. They also showed a few interesting relationships that had not been recognized by morphological study alone. Based on this study, we were able to plan further experiments to analyze relationships within the *Trypeta* group.

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