ABSTRACT

Title of Document: PHYLOGENOMICS, LIFE-HISTORY EVOLUTION AND TAXONOMY OF LEAF-MINING MOTHS (LEPIDOPTERA: GRACILLARIOIDEA)

Akito Yuji Kawahara, Ph.D., 2010

Directed By: Professor Charles Mitter,
Department of Entomology

Phytophagous insects dominate the terrestrial earth. While many are external plant feeders, a large diversity of insects specialize on feeding internally within plants. This study constructs one of the first phylogenies of the diverse leaf-mining moth superfamily Gracillarioidea, and examines broad patterns of life history evolution.

This dissertation begins with a short introduction (Chapter 1), before a molecular phylogenetic analysis of the Gracillarioidea utilizing over 14,800 nucleotides (Chapter 2). Results indicate that 1) Douglasiidae probably does not belong in Gracillarioidea; 2) the phylogenetic position of Bucculatricidae in Gracillarioidea is generally weak, but strong when non-synonymous changes are analyzed alone; 3) deep divergences in the superfamily are difficult to resolve even with 21 genes; and 4) four strongly supported clades, roughly corresponding to Kumata's classifications were recovered in the Gracillariidae.

Chapter 3 is a preliminary examination of life-history evolution in Gracillariidae, focusing on the "top down" effects from parasitoids that may have shaped the life histories of gracillariids. Results include: 1) larval traits (larval habit, cocoon ornamentation) is conserved on phylogeny, but traits associated with hosts are less so; 2) that host shifts in gracillariids are more common among closely related plants, and that closely related insects feed on closely related hosts; 3) blotch mining is the ancestral condition of mine form in Gracillariidae; 4) tentiform blotch mining, a modification of the simple blotch mine, may be an evolutionary innovation against parasitoids. The final three chapters focus on the taxonomy, life-history, and morphology of several gracillariids, including the description of three new species. The central theme is *Phyllocnistis*, a diverse, yet poorly studied serpentine mining gracillariid genus.

PHYLOGENOMICS, LIFE-HISTORY EVOLUTION AND TAXONOMY OF LEAF-MINING MOTHS (LEPIDOPTERA: GRACILLARIOIDEA)

By

Akito Yuji Kawahara

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Advisory Committee:
Professor Charles Mitter, Chair
Dr. Jerome C. Regier
Dr. Michael P. Cummings
Dr. Donald R. Davis
Dr. Conrad C. Labandeira
Dr. David L. Wagner

Foreword

Three of the six chapters in this dissertation were previous published:

Chapter 4: De Prins, J. and A. Y. Kawahara. 2009. On the taxonomic history of *Phyllocnistis* Zeller 1848 (Lepidoptera: Gracillariidae). *Nota Lepidopterologica* 32(2): 113-121.

Chapter 5: Kawahara, A. Y., Nishida, K., and D. R. Davis. 2009. Systematics, host plants, and life histories of three new *Phyllocnistis* species from the highlands of Costa Rica (Lepidoptera, Gracillariidae, Phyllocnistinae). *Zookeys* 27: 7-30.

Chapter 6: Kawahara, A. Y., Sohn, J.-C., De Prins, J., and S. Cho. 2010. Five species of Gracillariidae (Lepidoptera) new to Korea. *Entomological Research* 40: 131-135.

The student, Akito Kawahara, made substantial contributions to all aspects of these publications, justifying their inclusion in this dissertation.

Dedication

To my family, for supporting my interest in lepidopterology for over 30 years.

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Chapter 1: Introduction

Insect herbivores and their host plants dominate terrestrial biomes and may constitute nearly half of the earth's biodiversity (excluding microorganisms, Strong et al., 1984). As herbivores and pollinators, Lepidoptera are one of the primary insect groups responsible for the radiation of flowering plants (Powell et al., 1998; Scoble, 1992). Since the pioneering work of Ehrlich and Raven (1964) on the co-evolution of butterflies and their hosts, there has been great interest in trying to detect and understand macroevolutionary patterns in insect-plant associations (e.g., Farrell, 1998b; 2001; Kergoat et al., 2005; Mitter et al., 1988; Percy et al., 2004; Sequeira and Farrell, 2001). Most macroevolutionary studies on herbivorous insects have focused on external plant feeders (e.g., Ehrlich and Raven, 1964; Janz and Nylin, 1998; McKenna et al., 2009), and few have examined patterns of life history evolution for internal herbivores such as leaf miners.

Moths in the superfamily Gracillarioidea constitute the primary group of plant mining Lepidoptera. Gracillariidae, the most diverse family in the superfamily, feed on a wide range of different host plant families, and the larva typically consumes the soft tissue between the outer leaf surfaces (Davis, 1987). Physical and spatial features of their mines differ markedly across taxa within the family (Hering, 1951; Vári, 1961), and the variation provides a unique opportunity to utilize phylogeny to test ecological and evolutionary hypotheses that led to broad host use and diverse larval habits.

The current accepted classification of the Gracillarioidea, set by Davis and Robinson (1998), recognizes putative morphological characters for the superfamily and four families within: Bucculatricidae, Douglasiidae, Gracillariidae, and Roeslerstammiidae. However, monophyly of these families, and relationships among and within them, has not been adequately tested. A molecular phylogenetic analysis sampling across the Gracillarioidea lays the foundation necessary to conduct studies on the Gracillariidae, the most diverse family that exhibits the greatest variation in life history traits.

Gracillariidae currently includes approximately 2,000 species in 100 genera (De Prins and De Prins, 2010), but a huge fraction of its diversity still remains undescribed, especially from Central and South America. Many gracillariid species are economically important (Abu-Yaman, 1966; Heppner and Dixon, 1995; Shapiro et al., 2008) and new, undescribed gracillariid pests are regularly being discovered from tropical agricultural plantations (Davis and Wagner, in prep.). Despite such large numbers of unknown species and the need to describe them, little progress is being made on the taxonomy of Neotropical Gracillariidae. While constructing a molecular phylogeny of the Gracillarioidea and examining life history evolution are the primary goals for this dissertation, a portion is devoted to morphological descriptions, life-history observations, and revealing the complex taxonomic history of a diverse, poorly studied genus, *Phyllocnistis*.

This dissertation begins with a test of the phylogenetic hypotheses of Gracillarioidea (Chapter 2). The goal for the chapter is to present one of the first phylogenies of the superfamily based on molecular data. Next, the emphasis is on applying phylogeny to uncover some of the broad patterns of life-history evolution in Gracillariidae (Chapter 3). Gracillariids have a plethora of unique life-history traits, and numerous untested hypotheses on life history evolution in the family exist. I take an exploratory approach and examine life history patterns with an exemplar sampling of 68 gracillariid species. The last three chapters focus on adding more observational and descriptive data to the accumulating knowledge of gracillariids life histories. Generation of novel morphological, taxonomic, and life-history data allows the application of powerful methods to synthesize the different sources of information. I conducted three separate studies, each examining a different aspect of gracillariid systematics: the taxonomic history of one of the most diverse, and morphologically challenging genera, *Phyllocnistis* (Chapter 4), life-history studies of three new Neotropical *Phyllocnistis* species (Chapter 5), and a morphological description of several new Korean gracillariids (Chapter 6).

CHAPTER 2

Molecular phylogeny of leaf-mining moths (Lepidoptera: Gracillarioidea): Initial evidence from 21 nuclear protein-coding genes

Abstract

Gracillarioidea (approximately 2,000 described species) is the most diverse group of leaf-mining moths, with many economically important agricultural pests. While the majority of species are leaf miners, the superfamily shows a diversity of other life-history strategies, such as fruit mining, stem mining, leaf rolling, boring, and galling. Despite their economic importance and wealth of life-history strategies, relationships among gracillarioid families and subfamilies remain uncertain. Fifty-seven taxa, including twelve outgroups, were initially sequenced for ten nuclear protein-coding genes (8,436 bp). An additional 11 genes (6,375 bp) were sequenced for 27 taxa and combined with the original ten to create a data set of 14,811 bp. The concatenated, all taxa, all-gene data set and three other data sets of different taxa and gene sampling design were analyzed with maximum-likelihood, and statistical significance of non-monophyly examined with the Approximately Unbiased (AU) test. Partially or fully augmenting a data set with more characters tended to increase bootstrap support for particular deep nodes, and this increase was dramatic when non-synonymous changes were analyzed alone. Supporting a recent study, we find strong evidence for the exclusion of Douglasiidae from Gracillarioidea, as monophyly of the superfamily was statistically rejected in eight of nine analyses ($P \leq 0.009$). Our results strongly support the monophyly of Gracillariidae, Lithocolletinae + *Leucanthiza*, and the *Acrocercops* and *Parectopa* groups. There was

strong support for the 'G.B.R.Y.' clade, a group comprising of the Gracillariidae + Bucculatricidae + Roeslerstammiidae + Yponomeutidae, when analyzed with non-synonymous changes only, but this group was frequently split when synonymous and non-synonymous changes were analyzed together. Base compositional heterogeneity at the third nucleotide position may explain the spurious position of Bucculatricidae when synonymous changes are included. The limited resolution among the major lineages within the Gracillarioidea reinforces the idea that estimating deep relationships in Lepidoptera can be very challenging.

Introduction

Gracillarioidea, one of the largest groups of plant mining Lepidoptera, includes over 2,000 described species (Davis and Robinson, 1998; De Prins and De Prins, 2010). Most Gracillarioidea create serpentine or blotch mines in plant leaves, and some have caused substantial agricultural and economic damage as introduced pests (Gilbert et al., 2005; Heppner, 1993; Shapiro et al., 2008). Gracillarioids, while primarily leaf miners, show a diversity of other life-history strategies, such as fruit mining, stem mining, leaf rolling, boring, and galling (Davis, 1987; De Prins and De Prins, 2010). Gracillariid larvae are also known to undergo spectacular ontogenetic changes in feeding behavior, and the number of larval instars can vary from 4 to 11 depending on species (Davis, 1987). The larva may transition from a sap feeding form (with a flattened head, sap-feeding mouthparts), to a dramatically different, tissue-feeding form that resembles a typical lepidopteran larva (with a cylindrical body, a round head, chewing mouthparts and a functional spinneret), and some are also known to have a transitional quiescent

instar in which the larva does not feed (Davis, 1987; Kumata, 1978; Wagner et al., 2000). Numerous hypotheses exist on the evolution of gracillarioid life histories. For example, it has been thought that the most ancestral lineages within Gracillarioidea are bark miners, while the more derived groups are mine in leaves (Kuznetzov and Stekol'nikov, 1987). Davis (1987) postulated that the most ancestral lineages within Gracillariidae, the most diverse family within Gracillarioidea, produce folded or rolled leaves while derived lineages mine in leaves. Gracillarioid phylogeny will offer the initial framework to test and examine the evolution of many life-history strategies.

Despite the economic and ecological importance of Gracillarioidea, monophyly of the superfamily remains putative. The current accepted classification by Davis and Robinson (1998) includes four families, Bucculatricidae, Douglasiidae, Gracillariidae, and Roeslerstammiidae, but others have previously included only the Bucculatricidae and Gracillariidae (Gerasimov, 1948), Bucculatricidae, Gracillariidae, and Lyonetiidae (Heppner, 1984; Zimmerman, 1978), or Bucculatricidae, Gracillariidae and Roeslerstammiidae (Robinson, 1988). Recent molecular studies on the higher phylogeny of Lepidoptera have included several Gracillarioidea, and strongly support a close relationship of Gracillarioidea to Yponomeutoidea (Mutanen et al., 2010; Regier et al., 2009). Phylogenetic studies within Gracillarioidea have focused at the genus level or below (e.g., *Epicephala* [Kawakita and Kato, 2009; Kawakita et al., 2004]; *Phyllonorycter* [Lopez-Vaamonde et al., 2003; 2006], *Acrocercops transecta* species-group [Ohshima, 2008; 2010]), and there have been no broad analyses of relationships among families, subfamilies and genera.

Of particular difficulty in the systematics of Gracillarioidea has been the Bucculatricidae and Douglasiidae. The Bucculatricidae includes approximately 250 species, mostly in the genus *Bucculatrix,* that are morphologically very similar (Braun, 1963; Heppner, 1991). Douglasiidae includes only about 25 species, which are leaf miners and stem borrers (Common, 1990; Gaedike, 1974; Gaedike, 1990). They were putatively included in the Gracillarioidea based on nine morphological features that they share with Gracillariidae and Roeslerstammiidae, including two from the larva, two from the pupa, and five from the adult (Davis and Robinson, 1998). These afore-mentioned two families also have striking unique morphological features, such as the presence of a broad antennal scape (Bucculatricidae) and ocelli (Douglasiidae) (Davis and Robinson, 1998). A recent study directed at the broader relationships of Lepidoptera included fourteen Gracillarioidea species, and suggested that the Gracillarioidea may not include the Bucculatricidae or Douglasiidae (Mutanen et al., 2010).

The purpose of this paper is to utilize multiple nuclear genes to tackle the problem of gracillarioid phylogeny. Fifty-seven taxa, including exemplars representing the major lineages of Gracillarioidea plus outgroups, were sampled. Because recent phylogenetic analyses of ditrysian Lepidoptera based on 6,157 bp (Mutanen et al., 2010), and 6,759 bp (Regier et al., 2009) have revealed the difficulty of resolving deep splits within Ditrysia, we first sequenced ten genes (8,436 bp) for 57 taxa, and then an additional 11 genes (6,375 bp) for 27 taxa representing the major lineages of Gracillarioidea (21 genes total, 14,811 bp). This approach was taken as it has been shown that deep node resolution can

sometimes be increased with greater gene sampling for only a subset of exemplar taxa (Cho et al., 2010; Cummings and Meyer, 2005; Graybeal, 1998; Mitchell et al., 2000; Wiens, 2003; Wiens, 2005).

However, with few taxa come additional problems, mainly pertaining to phylogeny estimation. Sampling only a few taxa but more characters can lead to artifacts such as long-branch attraction in the case for parsimony (Felsenstein, 1978), and while probabilistic methods tend to do better, they still can be subject to such artifacts under particular conditions (Philippe et al., 2005). Following Cho et al. (2010), we examined whether sampling design has an effect on estimated relationships of Gracillarioidea. We constructed four different data sets, which we have termed data sets A – D: (A) 10 genes (8,436 bp) and 27 taxa; (B) 21 genes (14,811 bp) and 27 taxa (11 genes added to data set A); (C) 10 genes and 57 taxa (30 taxa added to data set A), and (D) an all-sequence, all-taxa data set formed by combining data sets B and C, and containing a large block of missing data (Fig. 2.1). We also examined the effect of including and excluding synonymous change, as base compositional bias can result in misleading relationships when synonymous substitutions are present (Foster and Hickey, 1999; Lockhart et al., 1994).

Methods

Taxon sampling

Forty-five species of Gracillarioidea were included in the present study. Taxa were chosen to represent the major lineages as defined by the classification of Davis and

Robinson (1998). Whenever possible, we included the type species or genus. Twelve outgroups were chosen based on the availability of sequence data and their phylogenetic proximity to Gracillarioidea in recent molecular phylogenetic studies of ditrysian Lepidoptera (Cho et al., 2010; Regier et al., 2009). GenBank sequence numbers for each species are listed in Table 2.6.

Gene sampling

Ten genes, totalling 8,436 bp, were initially sequenced for 27 taxa (data set A). An additional 11 genes, totaling 6,375 bp, were then sequenced and added to create data set B (27 taxa, 21 genes; 14811 bp). The eleven additional genes are a subset of 68 gene regions developed for Arthropoda, specifically, those with the highest rates of non-synonymous change (Regier et al., 2008b), and were chosen specifically for estimating a "backbone" phylogeny of Lepidoptera (see http://www.leptree.net/). We also created data set C (57 taxa, 10 genes) and combined data sets B and C to create data set D (57 taxa, 27 gene). Gene and amplicon names, their lengths, and their inclusion into data sets A-D are listed in Table 2.1, and GenBank accession numbers for each gene is listed in Table 2.6.

For nearly all genes, nucleic acid sequences were generated from mRNAs amplified with RT-PCR following the laboratory protocols, primer sequences, and amplification strategies of (Regier, 2008). For elongation factor-1 alpha (Cho et al., 1995) and Histone 3 (Ogden and Whiting, 2003), we followed methods outlined in Kawakita et al. (2006; 2004) and Ogden and Whiting (2003), respectively. Sequences were first checked for contamination and sample-switching error, before being assembled, edited,

and concatenated with the software Geneious 4.6.4 (Drummond et al., 2009). The final data set was aligned using MAFFT 6.703 (Katoh, 2009a), implementing the E-INS-i function. The entire edited sequence data set is deposited as a Nexus file in TreeBASE (http://www.treebase.org), study accession number xxx.

Phylogenetic analysis

Phylogenetic analyses were conducted with maximum likelihood (ML) as implemented in GARLI 1.0 (Genetic Algorithm for Rapid Likelihood Inference, Zwickl, 2006) and GARLI-PART 0.97 (Zwickl, unpublished). All settings were kept as default except where indicated below. We used jModelTest (Posada, 2008) to determine the best substitution model for data set, which in each case was chosen as the General-Time-Reversible (GTR) model (Lanave et al., 1984; Tavaré, 1986), with among-site rate heterogeneity modeled according to a gamma (Γ) distribution (Yang, 1994) while allowing for a proportion of invariable sites (I) (Gu et al., 1995). Two thousand ML and bootstrap tree searches were conducted for analyses that applied a nuclear substitution model. We also applied the Goldman and Yang (1994) codon model, running four ML searches with 1 to 4 rate categories for each data set, and then choosing the appropriate parameters based on the tree with the highest likelihood score. We ran 100 ML tree searches and 100 bootstrap replicates for all codon model analyses. To expedite tree searches, we used Grid computing (Cummings and Huskamp, 2005) through The Lattice Project (Bazinet and Cummings, 2009). For consistency in the characterization of results, we will refer to bootstrap support of 70-79% as "moderate" and support ≥ 80% as

"strong." We use the arbitrary cutoff of 80% bootstrap support as a measure to compare the number of nodes with strong support across individual genes.

Base compositional heterogeneity

Base compositional bias can lead to independent lineages incorrectly grouping together (Foster and Hickey, 1999; Lockhart et al., 1994). While models for phylogenetic analysis assume compositional homogeneity, strong compositional heterogeneity is common at sites capable of undergoing synonymous substitution (Regier et al., 2008a; Regier et al., 2008b; Regier et al., 2009). For this reason, we examined four different character partitions, with and without synonymous change: (a) "nt123": all nucleotides and all changes; (b) "codon": all nucleotides and changes, but implementing a codon model to down-weight the synonymous sites; (c) "degen1" (Regier et al., 2010; Zwick, 2010): all synonymous changes degenerated, an extension of the RY coding scheme of Phillips et al. (2004); and (d) "partitioned": all nucleotides, synonymous and non-synonymous sites treated with different model parameters, which correspond to the partitions, "noLRall1 + nt2" and "LRall1 + nt3" of Regier et al. (2010).

To further investigate the potential influence of compositional heterogeneity, we conducted chi-square tests of among-taxon heterogeneity on data set B. We chose data set B because it includes the largest number of characters (14,811 bp) with the lowest percentage of missing data (13.96%) out of the four data sets. Chi-square tests were conducted on a character set undergoing mostly synonymous change, nt3, and one undergoing mostly non-synonymous change, degen1. We conducted the test for various

groups in the Gracillariidae and outgroups on both the entire character set, and after eliminating invariable sites in the degen1 data set. To gauge the possible effect of compositional heterogeneity on phylogeny inference, we compared Neghibor-Joining trees using two different distances: ML distances based on the GTR model, which can be influenced by compositional heterogeneity; and Euclidean distances calculated on the proportions of the four nucleotide states treated as independent characters, which will reflect only compositional heterogeneity. Euclidean distances were generated using a Perl script that was written with modification of the MBE Toolbox (Cai et al., 2005), and the calculations conducted with PAUP* 4b10 (Swofford, 2002).

Testing alternative hypotheses

Morphological evidence supports the monophyly of Gracillarioidea, Gracillariidae, Gracillariinae (Davis and Robinson, 1998), Gracillariinae + Lithocolletinae (Kuznetzov and Stekol'nikov, 1987), and Oecophyllembiinae + Phyllocnistinae (Kumata, 1998), but some of these proposed higher-level groups were not recovered. To ascertain whether these differences between morphological and molecular inferences were "real," i.e. not attributable to sampling error in the molecular data, we used the Approximately Unbiased (AU) test of Shimodaira (2002). With that test, we determined whether the best tree possible under the constraint of monophyly of the morphology-based group is a significantly worse fit to the molecular data than the best tree without that constraint. For each combination of one character set and one group of uncertain monophyly, we performed an ML analysis under the constraint of monophyly for the group in question, and an unconstrained analysis. Each analysis applied the same

number of ML runs determined to be appropriate for that character set as described above. Site likelihoods were estimated with PAUP* (Swofford, 2002) and the CONSEL package (Shimodaira and Hasegawa, 2001). In CONSEL, the AU test statistic of Shimodaira (2002) was used to determine the difference in fit to data of the constrained and unconstrained trees.

Results

Relationships of Gracillarioidea and Gracillariidae

All analyses resulted in a paraphyletic Gracillarioidea, and monophyly of the superfamily can be confidently rejected at $P \leq 0.009$ by the Approximately Unbiased Test in eight of nine analyses (Table 2.2). Support for the monophyly of Gracillariidae was high for nt123, codon and partitioned analyses, and also for degen1 with 27 taxa (Table 2.3). In general, nt123, codon and partitioned results were similar in topology and branch support, while degen1 results differed in topology and generally provided lower branch support, except that support for some deep relationships was strikingly high. For data set B, degen1 resulted in a monophyletic 'G.B.R.Y.' clade (Gracillariidae + Bucculatricidae + Roeslerstammiidae + Yponomeutidae), with strong support (BP = 90%), while this group was typically not recovered in nt123, codon and partitioned ML trees. Instead, the latter three methods resulted in the Bucculatricidae diverging before all taxa except the designated outgroup, Tineidae (e.g., Figs. 2.5, 2.6), and support for monophyly of the G.B.R.Y. clade, for data sets A – D was weak (BP ≤ 62%; Table 2.3).

Within Gracillariidae, monophyly clearly cannot be conclusively rejected for the sister-group relationship of the Oecophyllembiinae + Phyllocnistinae, as $P > 0.1$ under the Approximately Unbiased test in all cases (Table 2.2). Monophyly of Gracillariinae + Lithocolletinae is rejected by nt123 and codon model analyses ($P < 0.05$), but not for degen1 results from data set C and D (degen1, $P = 0.471$ and 0.138). Monophyly of Lithocolletinae (including *Leucanthiza*) was strongly supported in trees generated from nt123, codon, and partitioned analyses (Table 2.3). Monophyly of the Gracillariinae is rejected significantly by data sets C and D, but not by data set B. Within Gracillariinae, postulated relationships such as Kumata's (1982; 1988) *Acrocercops* and *Parectopa* groups were monophyletic with strong support in all analyses conducted. The *Gracillaria* group was monophyletic, but strongly supported only in analyses of the degen1 data set. Morphology also corroborates the monophyly of several of these groups: at least two morphological synapomorphies support Gracillariidae (Robinson, 1988); hindwing venation and larval chaetotaxy characterizes the Lithocolletinae; unique features of the male eighth abdominal segment define the *Acrocercops* and *Gracillaria* groups (Kumata, 1982; Kumata et al., 1988); and all species in the *Parectopa* group share an antrum that opens at the 7th sternum, an unusual character state for female Lepidoptera (Toshio Kumata pers. comm.).

Agreement and conflict among individual genes

There were no strongly supported groups that conflicted with each other across genes, and few nodes above the subfamily level were moderately or strongly supported by any one gene alone. Nodes strongly supported by only one gene were: CAD (BP =

83% for Gracillariidae, BP = 96% for the *Acrocercops* group), and Period (BP = 82% for Oecophyllembiinae + Phyllocnistinae; Table 2.5).

Gene versus taxon sampling

The addition of ~6.4 kb of sequence data to data set A increased bootstrap values for some deep nodes, most notably when analyzed with degen1. For instance, bootstrap support rose 16% (from 74% to 90%) for the G.B.R.Y. clade. An increase was also seen when we analyzed the complementary 11 gene, 27 taxa data set (data set B minus A), which had a BP = 84% for that clade. Bootstrap support for the *Acrocercops* group decreased 10-20% when 11 genes supplemented the original ten. This effect however, is probably due to the fact that *Acrocercops brongniardella* is missing 8,966 (60.1%) of the 14,811 characters. Indeed, when 30 additional taxa (including four additional *Acrocercops* group species) were added to data set B, bootstrap values rose above 97% for the *Acrocercops* group (data set D, Table 2.3).

The addition of 30 taxa (sampled for 10 genes) to data set A did not have a very strong effect on bootstrap support values for most nodes that could be compared. However, two nodes, the G.B.R.Y. clade and Gracillariidae, had strikingly higher bootstrap values under degen1 coding with fewer taxa (data set A) than more taxa (data set C), rising from < 50% to 74% and from 68% to 100% respectively.

Base compositional heterogeneity

Results of the chi-square tests for compositional heterogeneity are shown in Table 2.4. Homogeneity could not be rejected for any groups in the degen1 character set. When invariable sites were removed, only the Gracillariidae became significantly heterogenous. In contrast, nt3 showed highly significant heterogeneity across all taxa and the five taxon subsets. As a gauge of the possible misleading signal produced by compositional heterogeneity, we calculated Neighbor-Joining trees on distances reflecting only composition for nt123 and nt3. In these trees, Bucculatricidae is clustered with five other taxa that are together separated by long internal branches from the Tineidae and the remaining species in the tree (Fig. 2.4).

Degen1 ML trees from data sets A, B, and D recovered a monophyletic G.B.R.Y. clade (Figs. 2, 3A, 3B). As an alternative means to filter synonymous signal, we also created a noLRall1 + nt2 data set and calculated branch support, following the same methods outlined for nt123. This data set, which removes all nt3 sites and all nt1 sites that contain at least one sequence that codes for either argenine or leucine, also provided strong support for the G.B.R.Y. clade (BP = 88%, results not shown). These results support our previous findings (e.g., Regier et al., 2009) that filtering synonymous signal (and thereby compositional heterogeneity) can result in robust phylogenetic inference at deep levels.

Discussion

Phylogenetic relationships of Gracillarioidea

Our results provide one of the first molecular estimates of relationships within Gracillarioidea. Some previous hypotheses about those relationships were confirmed, as well as several novel ones. We focus our discussion on the degen1 ML tree for data set D (Fig. 2.2) unless otherwise noted. Gracillarioidea was paraphyletic in all analyses conducted, a result that is not in agreement with Davis and Robinson (1998). Davis and Robinson included Douglasiidae in Gracillarioidea, but monophyly of the superfamily so defined was rejected significantly in eight of nine AU tests (Table 2.2). Recently, Mutanen et al. (2010) reached the same conclusions based on fewer genes and taxa. In their analyses, Gracillarioidea were never monophyletic, and Douglasiidae was consistently placed in Apoditrysia. Mutanen et al. (2010) also had difficulty in placing the Bucculatricidae, which, in their analyses, was paraphyletic with respect to *Tritymba* (Plutellidae), and this group (Bucculatricidae + *Tritymba*) was sister to the Gracillariidae with weak (< 50%) ML bootstrap support. The close relationship of Yponomeutidae to Gracillarioidea (excluding Douglasiidae) is also consistent with previous molecular studies (Cho et al., 2010; Mutanen et al., 2010; Regier et al., 2009). These reports suggest, at least tentatively, that the putative morphological apomorphies proposed for Gracillarioidea by Davis and Robinson (1998) may be homoplasies. In order to restore monophyly of the superfamily, we would need to exclude Douglasiidae from Gracillarioidea and include Yponomeutidae. However, more convincing resolution of inter-family relationships is desirable before any formal taxonomic changes are made.

Monophyly of Gracillariidae was strongly supported in nearly all analyses, but relationships among subfamilies were not strongly resolved. The grouping of Oecophyllembinae + Phyllocnistinae, which share unique serpentine mine morphology (Davis, 1994) and a highly specialized spinning instar (Davis, 1987), was supported weakly or not at all in our multi-gene analyses. However, this pairing could not be rejected by any of the nine AU tests (Table 2.2), and was strongly supported (BP = 82%) by the only individual gene, Period, that provided strong evidence for or against that grouping (Table 2.5). The sister group relationship of Gracillariinae to Lithocolletinae proposed by Kuznetzov and Stekolnikov (1987) was rejected by seven AU tests (Table 2.2). Our results strongly support the inclusion of *Leucanthiza* in Lithocolletinae, suggesting that that this genus should be transferred here from the Gracillariinae. Monophyly of Gracillariinae (both with and without *Leucanthiza*) was rejected by the AU test in more than half of the data sets, suggesting that this subfamily needs to be redefined. However, we did identify two genus-level groups with strong support within Gracillariinae, the *Acrocercops* and *Parectopa* groups, closely corroborating prior morphological hypotheses (Kumata, 1982; 1988).

Phylogenetic contribution of adding genes versus taxa

Our results are consistent with Cho et al. (2010) and support the general observation that partial augmentation of gene sampling can improve estimates of deep relationships. When analysis is restricted to 27 species, full-augmentation to 21 genes also increased bootstrap support for some deep nodes, a result consistent with other empirical studies (e.g., Cummings et al., 1995; 1999; Mitchell et al., 2000; Otto et al.,

1996; Poe and Swofford, 1999; Regier et al., 2008b; Rokas et al., 2003; Zwick et al., submitted). While partial or full augmentation of genes improved branch support for deep nodes, especially for degen1, many nodes below the family level were still challenging even with > 14 kb of sequence data.

Increasing taxon sampling from 27 to 57 did not have a major impact on branch support for higher groups, except when non-synonymous sites were analyzed alone (degen1). Under degen1 coding, support for deep nodes dropped sharply when 30 taxa were added. A similar result was observed when comparing more genes (data set B) to more taxa (data set C). Bootstrap support for the G.B.R.Y. clade and the Gracillariidae was dramatically higher for data set B than for data set C. The difference appears to be due to the combination of both greater gene sampling and lesser taxon sampling, but the difference was greater when more genes were sequenced (Table 2.3).

The large block of missing data in data set D, amounting to roughly a fourth of the total possible sequence for a complete matrix of these dimensions, does not appear to induce the phylogenetic artifacts of missing data (Lemmon et al., 2009). The partially augmented data set D pulls the Bucculatricidae, a problematic group in the present study, into the G.B.R.Y. clade, from which it is left out in the ML tree from non-augmented data set C (Figs. 2, 3C). Previous support for a close relationship of Bucculatricidae to the Gracillariidae, from morphology (Gerasimov, 1948; Heppner, 1984; Kuznetzov and Stekol'nikov, 1987; Robinson, 1988; Zimmerman, 1978) and molecules (Mutanen et al.,

2010), allows us to favor the topology from the partially augmented data set D over the non-augmented data set C.

While our study is concordant with the results of Cho et al. (2010) and the simulation results of Wiens (2003; 2006), it is plausible that our results are biased as our sampling design was restricted to blocks of pre-determined number of genes and taxa. It would ideally be best to test these conclusions with different empirical data sets and with different blocks of genes within our present data set.

Base compositional heterogeneity

Compositional heterogeneity may account for the difference in placement of *Bucculatrix* (Bucculatricidae) between the nt123 and degen1 trees. Because strong non-synonymous signal supports the monophyly of the G.B.R.Y. clade, synonymous signal, mostly at nt3, must be accountable for the less decisive placement of Bucculatricidae in nearly all nt123 trees.

Strong compositional bias can incorrectly group unrelated taxa together (Foster and Hickey, 1999), or equivalently, widely separate a taxon with strong bias from its true relatives. In nearly all analyses that included synonymous signal, *Bucculatrix* was placed along a long internal branch between the Tineidae and the remaining taxa. Non-synonymous signal as reflected in both degen1 and noLRall1 + nt2 resulted in a monophyletic G.B.R.Y. clade, for which support from some analyses was very robust. Only weak signal remains for this clade when synonymous sites are added (ML bootstrap

consensus trees in all but two cases provided 50-60% branch support for this clade). We speculate that analyses that include synonymous signal, regardless of whether they down-weight or model parameters for synonymous and non-synonymous changes separately, do not effectively correct for the strong compositional heterogeneity found at nt3. Instead, synonymous signal appears to be obscuring true underlying phylogenetic signal of non-synonymous characters.

A comparison of the ML topology with the Neighbor-Joining GTR ML distance, and Euclidean compositional distance trees for nt123 and nt3 suggests that the uncertain placement of Bucculatricidae in the nt123 data set is largely due to nt3 (Fig. 2.4). In the compositional distance trees, six taxa (*Bucculatrix sp., Atteva punctella*, *Eumetriochroa hederae*, *Hemerophila felis*, *Phyllocnistis citrella*, and *P. magnoliaeela*) fall between the Tineidae and the remaining taxa along a long internal branch. In the nt123 ML tree, in contrast, all taxa but *Bucculatrix* move to parts of the ML nt123 tree that are generally well supported and expected based on morphology (e.g., *Eumetriochroa* with *Phyllocnistis*, and *Atteva* with *Eucalantica*).

Results of the ML nt3 analysis are very different, providing further evidence that compositional heterogeneity can affect trees based on nt3 alone. Despite providing about 90% of the total character change, the nt3 character set alone yields bootstrap support > 50% for only 6 nodes as compared to the full data set (nt123; 14 supported nodes), fewer even than the degen1 character set (12 supported nodes). Some unexpected relationships

are found, such as *Bucculatrix* + *Eumetriochroa*, which break up well-supported groups, in this case the monophyletic Gracillariidae (Fig. 2.4F).

Conclusions

Our results demonstrate the difficulty of resolving deep level relationships in Lepidoptera. The phylogeny obtained in this study largely corroborates the results of Mutanen et al. (2010), in that 1) the Douglasiidae do not appear to belong in the Gracillarioidea and 2) that the Bucculatricidae are difficult to place when both non-synonymous and synonymous characters are analyzed together. While Mutanen et al. (2010) did not propose a solution to the "bucculatricid problem" in their ML analysis, we believe the problem with Bucculatricidae (and possibly other lepidopterans that are difficult to place) is that base compositional heterogeneity at nt3 may be obscuring true underlying phylogenetic signal. Based on the tests for compositional heterogeneity and stronger bootstrap values obtained when synonymous changes are excluded, we tentatively conclude that the Bucculatricidae is closely related to Gracillarioidea + Roeslerstammiidae + Yponomeutidae. Since the majority of phylogenetic models assume compositional homogeneity, molecular phylogenetic studies, especially those focusing on deep-level questions, would do well to systematically examine the effect of synonymous versus non-synonymous change.

Table 2.1. Representation of genes and their amplicon names in each of the four data sets. Shaded boxes indicate genes that were included in the particular data set.

				Data set			
Gene	**Amplicon name**	**Length (bp)**	**Reference**	**A**	**B**	**C**	**D**
				10g x 27t	21g x 27t	10g x 57t	21g x 27t
40fin2_3	phosphogluconate dehydrogenase	750	Regier (2008)				
42fin1_2	putative GTP-binding protein	840	Regier (2008)				
109fin1_2	gelsolin	552	Regier (2008)				
192fin1_2	glutamyl- & prolyl-tRNA sybphetase	402	Regier (2008)				
197fin1_2	triosephosphate isomerase	444	Regier (2008)				
262fin1_2	proteasome subunit	501	Regier (2008)				
265fin2_3	histidyl-tRNA sybphetase	447	Regier (2008)				
268fin1_2	AMP deaminase	768	Regier (2008)				
3007fin1_2	glucose phosphosphate dehydrogenase	621	Regier (2008)				
3017fin1_2	tetrahydrofolate sybphase	594	Regier (2008)				
3070fin4_5	alanyl-tRNA sybphetase	705	Regier (2008)				
8028fin1_2	"nucleolar cysteine-rich protein"	324	Regier (2008)				
8091fin1_2	glucose phosphate isomerase	666	Regier (2008)				
acc2_4	acetyl-coA carboxylase	501	Regier (2008)				
CAD	pyrimidine biosynthesis	2913	Moulton and Wiegmann (2003)				
DDC	dopa-decarboxylase	708	Fang et al. (1997)				
EF1-alpha	Elongation-factor 1-alpha	519	Cho et al. (1995)				
enolase	--	1134	Farrell et al. (2001)				
histone 3	--	273	Ogden and Whiting (2003)				
period	--	747	Regier et al. (1998)				

Table 2.2. Results of Approximately Unbiased (AU) significance tests (Shimodaira 2002) for non-monophyly of predicted clades. Codon = codon model analysis, degen = degeneracy1 data set, nt123 = all nucleotide data set. Groups that were significant at $\alpha = 0.05$ are shown in bold.

	***P* values: data sets B/C/D**		
Predicted clade	nt123	codon	degen
Gracillarioidea	**0.003/0.009/0.001**	**0.006/0.006/0.009**	**<0.001**/0.104/**<0.001**
(Gracillariinae+Litho)	**0.039/0.001/0.002**	**0.016/0.021/0.007**	**0.029**/0.471/0.138
Gracillariinae	0.169/**<0.001/0.001**	0.079/**0.001**/**<0.001**	0.311/**0.001/<0.001**
Gracillariinae (-*Leucanthiza*)	--/**0.003/0.001**	--/**0.02/<0.001**	--/0.121/**0.028**
Oecophyllembiinae + Phyllocnistinae	0.910/0.425/0.115	0.963/0.327/0.647	0.564/0.364/0.318

Table 2.3. Bootstrap support values across data sets for selected clades. Square brackets indicate support values for clades that were not present in the ML tree. "G.B.R.Y. clade" refers to Gracillariidae + Bucculatricidae + Roeslerstammiidae + Yponomeutidae.

Data set	Analysis	Gracillariidae + Bucculatricidae + Roeselerstammidae + Yponomeutidae	Gracillariidae	Lithocolletinae + *Leucanthiza*	*Acrocercops* group	*Gracillaria* group	*Parectopa* group	Phyllocnistinae + Oecophyllembinae + *Marmara* + *Dendrorycter*
A	nt123	<50	99	N/A	87	N/A	100	N/A
	codon	<50	99	N/A	86	N/A	100	N/A
	degen	74	100	N/A	99	N/A	100	N/A
	partitioned	<50	99	N/A	89	N/A	100	N/A
B	nt123	[58]	98	N/A	77	N/A	100	N/A
	codon	[54]	100	N/A	73	N/A	100	N/A
	degen	90	100	N/A	82	N/A	100	N/A
	partitioned	[62]	100	N/A	74	N/A	100	N/A
C	nt123	<50	94	96	95	68	100	<50
	codon	<50	96	96	99	<50	100	<50
	degen	<50	68	68	99	89	100	<50
	partitioned	<50	99	100	97	77	100	<50
D	nt123	[54]	95	97	97	63	100	52
	codon	[53]	88	89	98	65	100	<50
	degen	57	68	67	99	89	100	<50
	partitioned	[59]	99	100	97	67	100	51

Table 2.4. Results of Chi-square tests of nucleotide compositional homogeneity. The number of sampled species in each group is in brackets.

	P value for character set		
Taxon (number of species)	degen1	degen1 (var. sites only)	nt3
All (27)	>0.999	0.191	<0.001
Gracillariidae (11)	0.982	0.005	<0.001
Oecophyllembiinae + Phyllocnistinae (3)	0.935	0.663	<0.001
Bucculatricidae + Tineidae (3)	0.820	0.828	<0.001
Bucculatricidae + Outgroups + Klimeschia – Tineidae (10)	>0.999	0.539	<0.001
Outgroups + Klimeschia – Tineidae (9)	>0.999	0.517	<0.001
Total number of characters	14811	2420	4937

Table 2.5. Single gene bootstrap values for all nodes in the nt123 tree of data set B. Dark shaded boxes are nodes with > 80% bootstrap support, lightly shaded boxes are nodes with > 50%, but < 80%. "ALL" refers to the concatenated (all-gene) data set B.

	ALL	CAD	DDC	ENO	PER	wg	ACC	EF1a	H3	40fin	42fin	109fin	192fin	197fin	262fin	265fin	268fin	3007fin	3017fin	3070fin	8028fin	8091fin
Total nodes	**22**	**22**	**10**	**20**	**13**	**19**	**21**	**9**	**10**	**17**	**17**	**15**	**17**	**19**	**20**	**16**	**12**	**15**	**15**	**20**	**15**	**21**
Tineidae	100	100	100	100	100	100	100	-	-	100	100	100	100	100	100	100	100	-	-	100	99	89
Tortricidae	100	100	48	100	91	53	68	81	24	-	100	56	84	87	78	72	70	99	-	97	95	11
Yponomeutidae	71	47	5	2	-	11	0	-	-	7	27	6	27	0	17	93	-	2	-	7	2	3
G.B.R.Y. clade	36	46	0	0	0	0	0	0	0	4	0	0	-	0	0	0	0	0	0	0	0	0
Gracillariidae	98	83	0	0	28	0	0	0	0	0	2	0	0	0	0	5	4	0	0	0	0	0
Roeslerstammiidae	100	100	-	100	-	-	68	-	-	98	100	98	-	23	79	93	-	99	33	100	90	73
Phyllocnistinae	100	100	-	100	100	96	64	-	-	100	-	92	86	100	98	100	-	-	99	100	95	100
Lithocolletinae	100	100	-	98	-	87	9	-	-	99	82	99	94	77	80	95	-	100	20	96	34	90
Caga+Cbim+Phyl	58	10	-	15	-	2	1	-	-	67	6	3	8	51	1	43	-	10	0	16	0	0
Phyllocnistinae + Oecophyllembiinae	78	3	-	2	82	45	1	-	-	0	-	-	6	9	19	7	-	-	0	7	24	1
Acrocercops group	77	96	-	-	-	56	0	-	0	-	-	-	-	-	-	-	-	2	-	27	-	0
Parectopa group	100	100	-	90	-	-	51	4	4	-	-	66	95	-	49	16	93	-	-	97	-	-
Caga+Cbim+Cdel+Phyl	46	0	-	3	-	2	0	-	-	4	7	0	1	0		14	-	2	0	0	0	0
Abrg+Ehdr+Phcn+Pmgl+ Sput	58	0	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	0
Cole+Eeu	88	7	7	15	10	2	2	51	2	7	16	-	9	1	15	-	51	20	3	12	-	25
Cole+Eeu+Emon	45	32	0	24	2	0	0	0	0	0	2	-	6	0	0	-	0	0	0	0	-	4
Cole+Eeu+Emon+Ktr	31	5	-	0	0	0	0	0	0	0	0	-	0	0	0	-	8	0	0	0	-	2
Hfel+Ursp	36	13	21	2	3	0	16	-	-	0	0	4	4	0	0	1	20	-	16	-	21	15
Eeu+Cole+Emon+Ktr+Hfel +Ursp	12	1	-	0	0	0	0	-	-	0	2	-	0	0	0	-	0	-	0	-	-	0
Agel+Rstm+Atpu2+Ysp	30	36	-	0	-	-	0	-	-	0	0	0	-	0	0	0	-	0	0	3	0	0
Arga+Pida2+Alsp	70	15	46	7	0	0	8	0	2	-	1	0	0	0	0	17	7	0	-	42	0	2
Eeu+Cole+Emon+Ktr+Hfel +Ursp+Arga+Pida2+Alsp	32	41	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	9	5	0	0

Table 2.6. The sampled 45 ingroup and 12 outgroup taxa with AToLep voucher identification numbers, and GenBank numbers. GenBank numbers to be inserted in the publication that stems from this work.

Family	Subfamily	Species	Code name	Accession No.	CAD	DDC	EF1-a	enolase	H3	period	wingless	ACC	40fin	42fin	109fin	192fin	197fin	262fin	265fin	268fin	3007fin	3017fin	3070fin	8028fin	8091fin
Bucculatricidae	-	*Bucculatrix sp.*	Bucc	DRD-05-0270																					
Bucculatricidae	-	*Bucculatrix staintonella*	Bsta	TH-08-6083																					
Douglasiidae	-	*Klimeschia transversella*	Ktr	DRD-01-0017																					
Douglasiidae	-	*Tinagma gaedikei*	Tgak	TH-08-6082																					
Gracillariidae	Gracillariinae	*Acrocercops brongniardella*	Abrg	AYK-08-8215																					
Gracillariidae	Gracillariinae	*Amblyptila sp n.*	Atila	AK-07-070																					
Gracillariidae	Gracillariinae	*Aristaea sp n.*	Aris	GRACI054-07																					
Gracillariidae	Gracillariinae	*Callisto denticulella*	Cdel	AYK-08-8214																					
Gracillariidae	Gracillariinae	*Caloptilia bimaculatella*	Cbim	DRD-05-0248																					
Gracillariidae	Gracillariinae	*Caloptilia sapporella*	Csap	JCS-08-1033																					
Gracillariidae	Gracillariinae	*Conopomorpha cramerella*	Ccrm	AYK-08-8231																					
Gracillariidae	Gracillariinae	*Dendrorycter marmaroides*	Dend	GRACI103-07																					
Gracillariidae	Gracillariinae	*Deoptilia heptadeta*	Deoa	GRACI108-07																					
Gracillariidae	Gracillariinae	*Dialectica sp.*	Dtia	AK-07-011																					
Gracillariidae	Gracillariinae	*Epicephala relictella*	Epic	JCS-06-0172																					
Gracillariidae	Gracillariinae	*Eucalybites aureola*	Euau	GRACI102-07																					
Gracillariidae	Gracillariinae	*Gibbovalva quadrifasciata*	Gibb	GRACI105-07																					
Gracillariidae	Gracillariinae	*Leucanthiza amphicarpeaefoliella*	Leuz	DRD-01-0064																					
Gracillariidae	Gracillariinae	*Leucospilapteryx venustella*	Lven	TH-08-6105																					
Gracillariidae	Gracillariinae	*Leurocephala sp n.*	Gran	DRD-07-4001																					
Gracillariidae	Gracillariinae	*Liocrobyla lobata*	Lioc	GRACI104-07																					
Gracillariidae	Gracillariinae	*Marmara serotinella*	Msll	DRD-05-0265																					
Gracillariidae	Gracillariinae	*Micrurapteryx salicifoliella*	Msai	TH-08-6098																					
Gracillariidae	Gracillariinae	*Neurobathra strigifinitella*	Neur	DRD-01-0129																					
Gracillariidae	Gracillariinae	*Parectopa robiniella*	Prbn	DRD-01-0009																					
Gracillariidae	Gracillariinae	*Calopitila obliquatella*	Pvob	SWC-06-0265																					
Gracillariidae	Gracillariinae	*Spulerina dissotoma*	Sput	AYK-04-5630																					
Gracillariidae	Gracillariinae	*Stomphastis sp.*	Stss	AK-07-025																					
Gracillariidae	Gracillariinae	*Conopomorpha sp.*	Cono	AK-07-071																					
Gracillariidae	Lithocolletinae	*Cameraria gautheriella*	Caga	DRD-01-0113v																					
Gracillariidae	Lithocolletinae	*Cameraria guttifinitella*	Cgut	TH-08-6116																					
Gracillariidae	Lithocolletinae	*Cameraria sp.*	Came087	AK-07-087																					

Gracillariidae	Lithocolletinae	*Chrysaster ostensackenella*	Cyosk	DRD-05-0247																					
Gracillariidae	Lithocolletinae	*Cremastobombycia sp n.*	Crby	KN-06-0004																					
Gracillariidae	Lithocolletinae	*Hyloconis wisteriae*	Hwis	GRACI111-07																					
Gracillariidae	Lithocolletinae	*Lithocolletinae gen. n.*	Akgn126	AK-07-126																					
Gracillariidae	Lithocolletinae	*Neolithocolletis hikomonticola*	Nlik	GRACI110-07																					
Gracillariidae	Lithocolletinae	*Phyllonorycter insignitella*	Phzz	GRACI147-07																					
Gracillariidae	Lithocolletinae	*Phyllonorycter n.sp. (malus)*	Phyl	TPF-89-1114																					
Gracillariidae	Lithocolletinae	*Porphyrosela2 (gen. n.) sp. n.*	Porp121	AK-07-121																					
Gracillariidae	Oecophyllembiinae	*Eumetriochroa hederae*	Ehdr	AK-08-8111																					
Gracillariidae	Phyllocnistinae	*Phyllocnistis citrella*	Phcn	JBA-06-0025																					
Gracillariidae	Phyllocnistinae	*Phyllocnistis magnoliella*	Pmgl	DRD-05-0244																					
Roeslerstamiidae	-	*Agriothera elaeocarpophaga*	Agel	DRD-01-0143																					
Roeslerstamiidae	-	*Roeslerstammia pronubella*	Rstm	DRD-01-0142																					
OUTGROUPS																									
Aluctidae	Alucitinae	*Alucita sp.*	Alsp	JBA-05-0004																					
Choreutidae	Choreutinae	*Hemerophila felis*	Hfel	DRD-01-0218																					
Cosmopterigidae	Antequerinae	*Euclemensia bassettella*	Cole	JWB-05-0044																					
Ethmiidae	Ethmiinae	*Ethmia eupostica*	Eeu	MJM-96-0277																					
Pterophoridae	Pterophorinae	*Emmelina monodactyla*	Emon	CWM-05-1000																					
Tineidae	Tineinae	*Tinea columbariella*	Tco2	EN-91-0006																					
Tineidae	Tineinae	*Tineola bisselliella*	Tbi3	JG-96-0009																					
Tortricidae	Tortricinae	*Argyrotaenia alisellana*	Arga	CWM-94-0262																					
Tortricidae	Sparganothini	*Platynota ideausalis*	Pida2	JWB-05-0029																					
Urodidae	-	*Urodus decens*	Ursp	DA-03-3349																					
Yponomeutidae	Attevinae	*Atteva punctella*	Atpu2	JWB-05-0041																					
Yponomeutidae	Plutellinae	*Eucalantica sp. n.*	Ysp	DA-03-3364																					

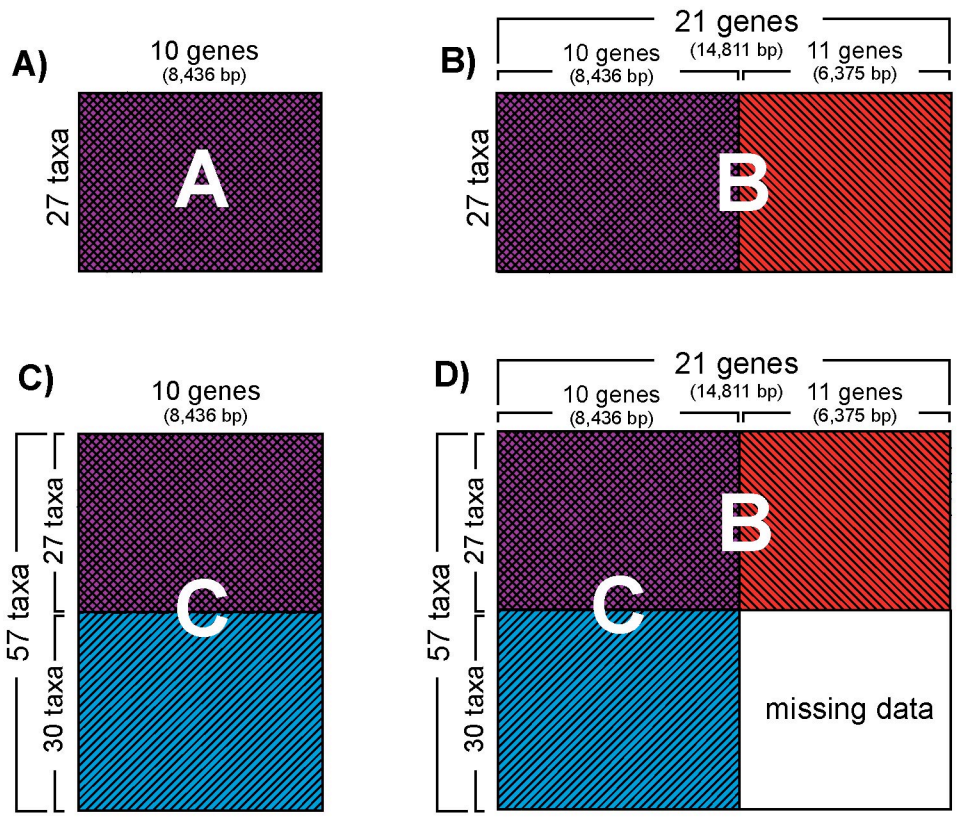


Fig. 2.1. Four data sets with different sampling strategies. **A.** 27 taxa and 10 genes, **B.** 27 taxa and 21 genes, **C.** 57 taxa and 10 genes, **D.** combination of B and C into a single data set with a large block of missing data.

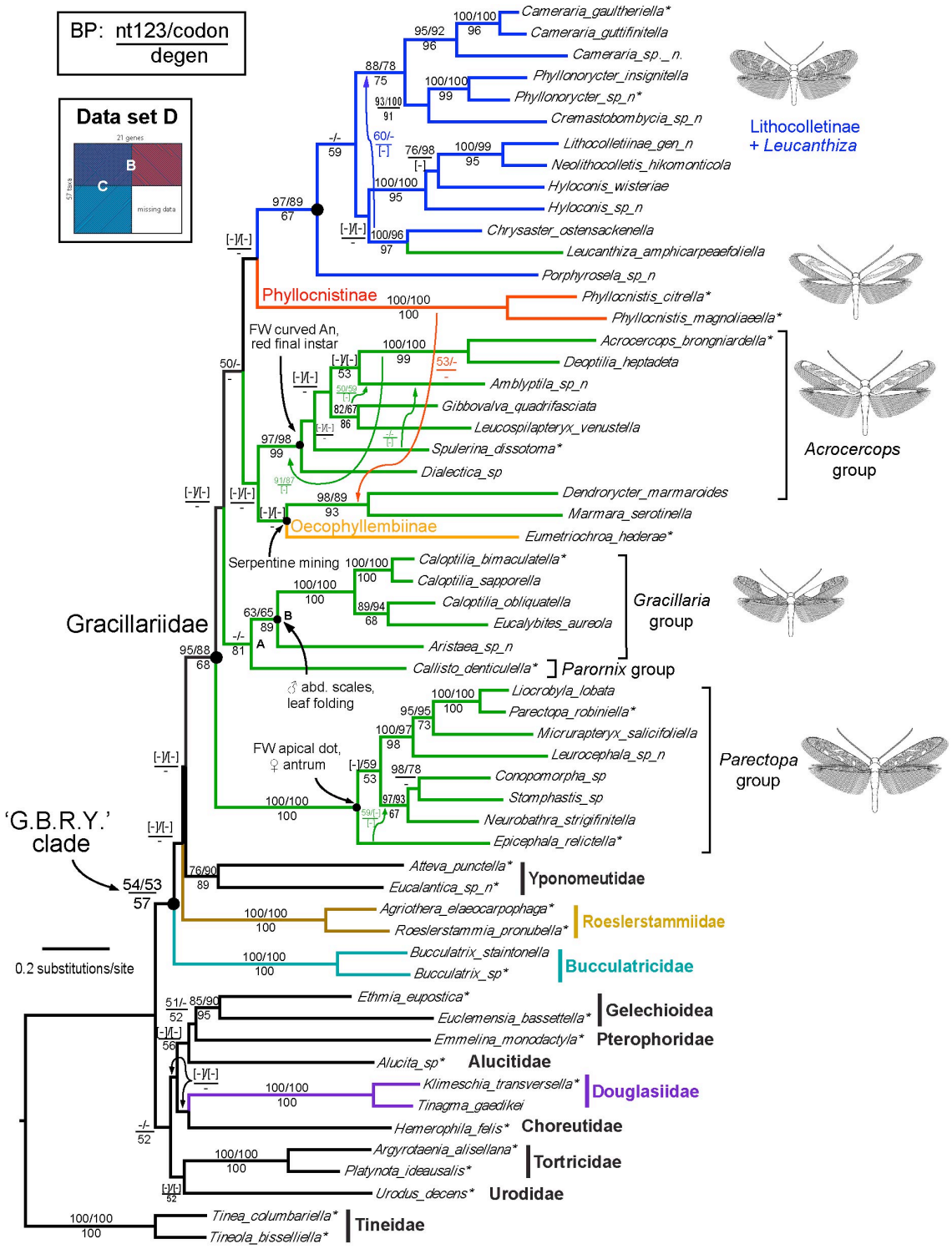


Fig. 2.2. Maximum likelihood degen1 tree of data set D. Taxa sequenced for 21 genes are indicated with asterisks. Hyphens indicate support values < 50%, square brackets indicate relationships that were not present in the ML tree of that analysis. Square brackets are only shown for nodes where there is a relationships > 50% in one of the analyses that conflict with the degen1 ML tree.

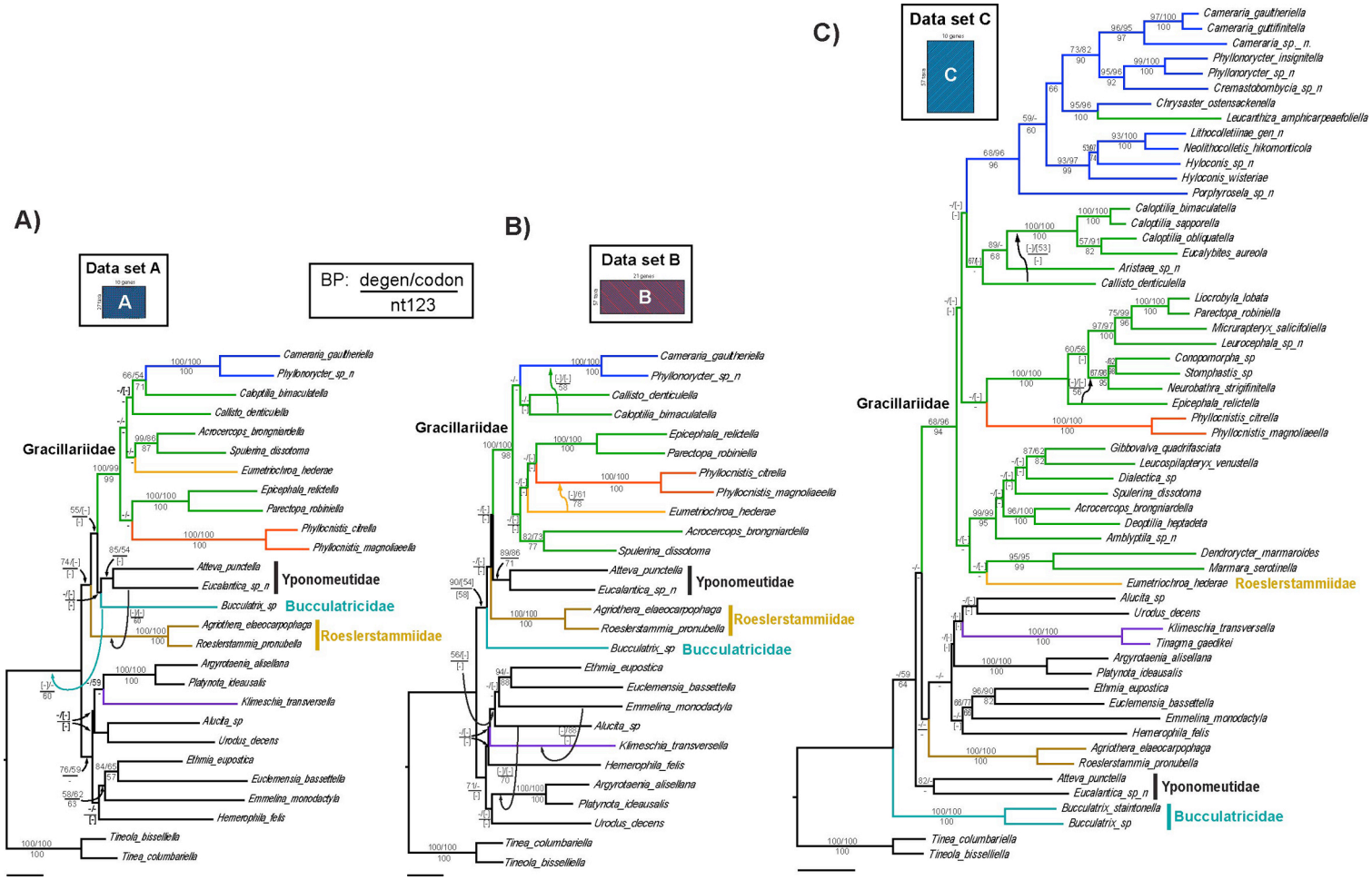


Fig. 2.3. ML trees based on non-synonymous sites (degen1) of datasets A-C. Bucculatricidae + Gracillariidae + Roeslerstammiidae + Yponomeutidae (G.B.R.Y. clade) are monophyletic for data sets A and B. Scale bar = 0.02

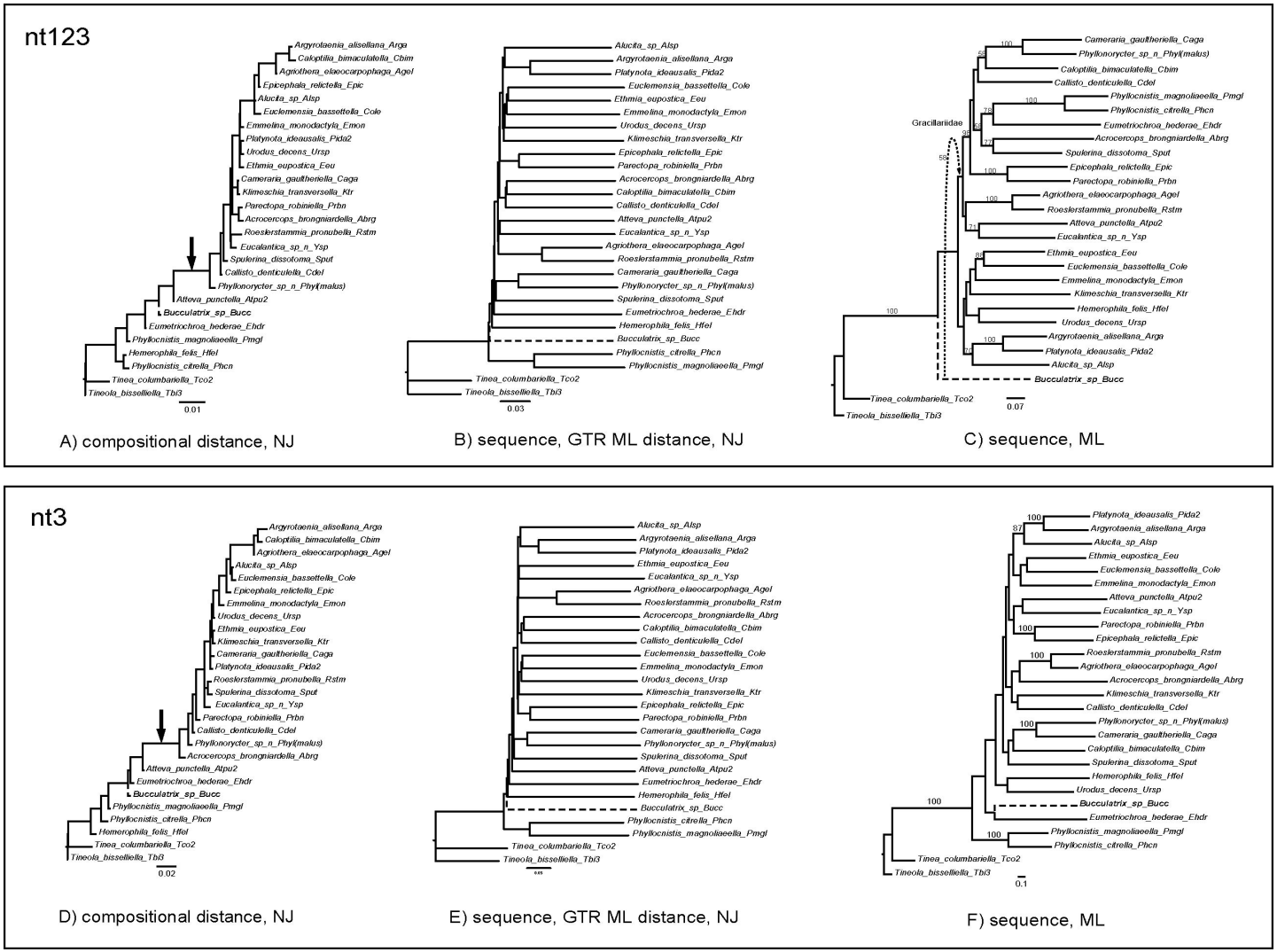


Fig. 2.4. Comparison of Euclidean compositional distance (NJ), GTR ML distance (NJ), and ML trees for nt123 and nt3. Arrows indicate a long internal branch in the Euclidean compositional distance trees.

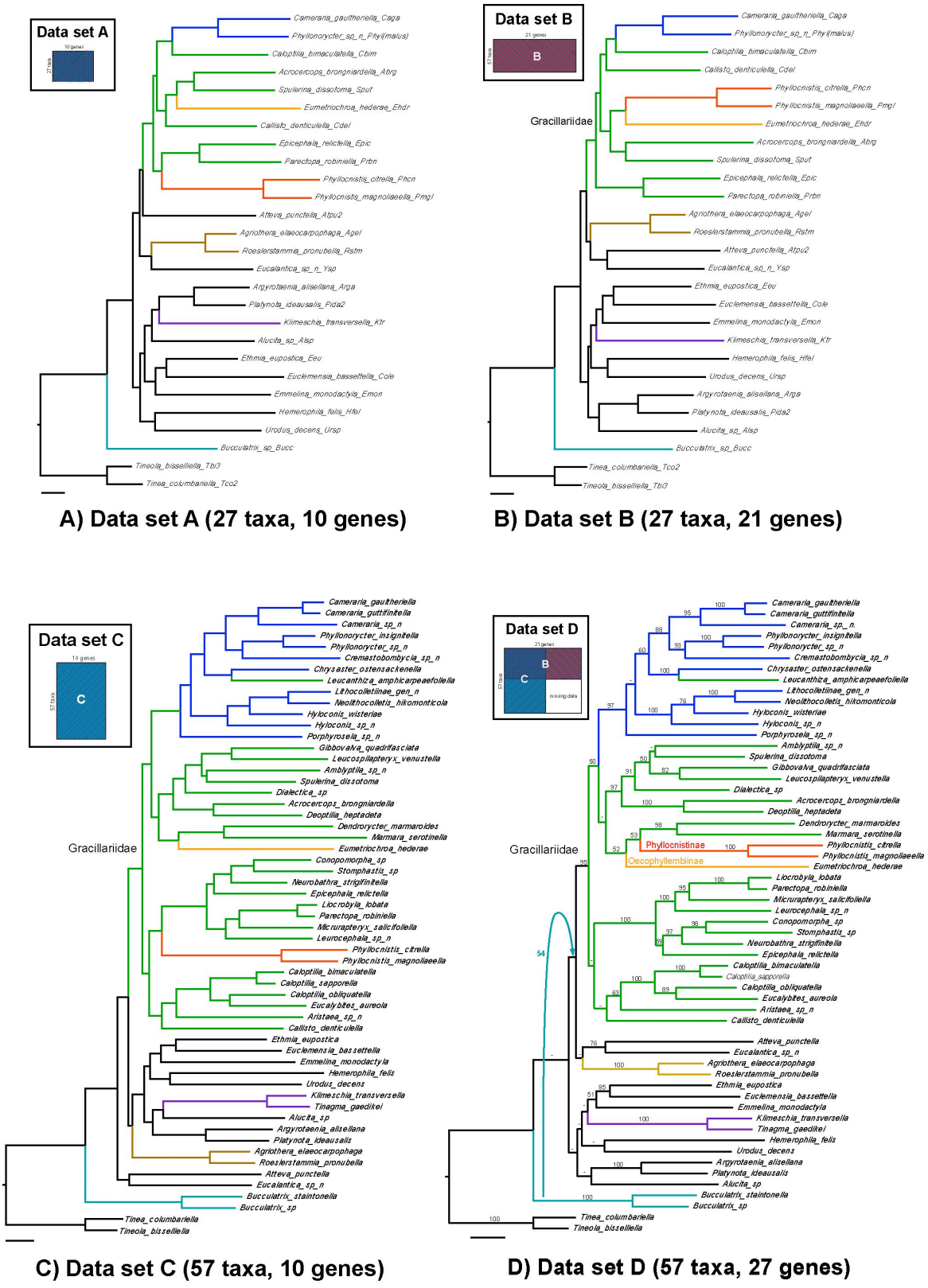


Fig. 2.5. Maximum likelihood nt123 trees of data sets A-D. Scale bar = 0.07 substitutions/site.

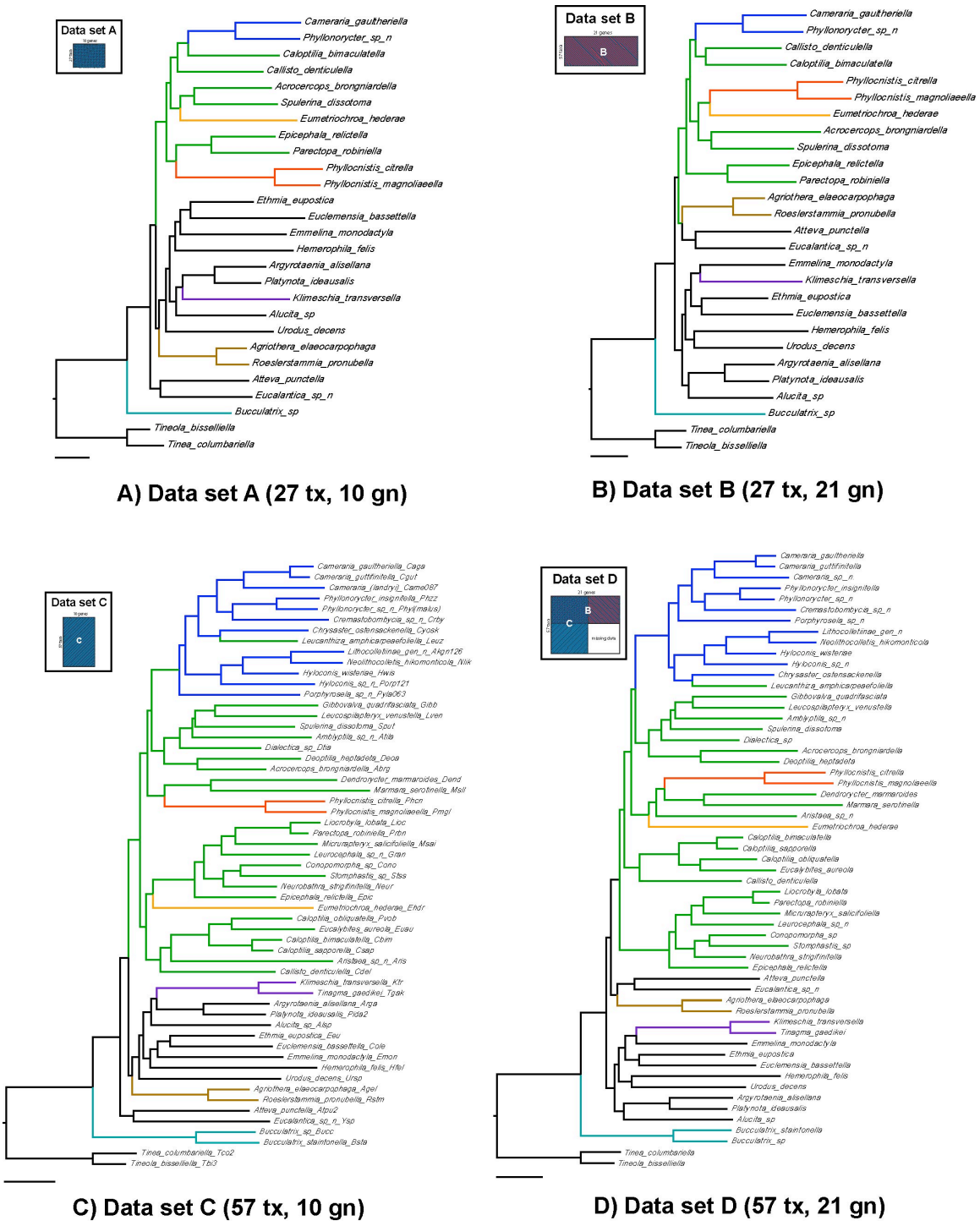


Fig. 2.6. Maximum likelihood codon-model trees of data sets A-D. Scale bar = 0.03 substitutions/site.

CHAPTER 3

Larval habits, host use, and life-history evolution in leaf-mining moths (Lepidoptera: Gracillariidae): An initial exploration

Introduction

Ecological opportunity, such as an adoption of a new "adaptive zone", is thought to be fundamental in accelerating diversification rates (Simpson, 1953). Key innovations, such as the ability to overcome plant chemical defenses (Ehrlich and Raven, 1964), or the development of new morphological or behavioral traits, may allow a lineage to enter a new adaptive zone (Futuyma, 1991). The adaptive zone concept has played a central role in evolutionary biology for more than half a century, and is thought to explain many broad diversification patterns in insects (Berenbaum, 1983; Mitter et al., 1988; Winkler and Mitter, 2008). Theoretical advances, coupled with the recent availability of molecular sequence data and phylogenetic dating methods, have made it increasingly easier to study the evolutionary mechanisms that led to adaptive radiations.

In phytophagous insects, host chemistry is often attributed as the main factor leading to radiations (Berenbaum, 1983; Ehrlich and Raven, 1964; Feeny, 1975; 1976; Scriber and Slansky, 1981; Zangerl and Berenbaum, 1993). In their seminal paper, Ehrlich and Raven (1964) described the "escape-and radiation" scenario, where insects and their hosts are in an arms race and each side develops new innovations to counter the opponent's strategy. An insect species that has successfully colonized a host and

overcame its chemical defense could enter a new adaptive zone and shift to closely related groups of plants that may also have similar defenses, and trigger a rapid radiation. Ehrlich and Raven argued that such processes led to the general pattern that closely related insects feed on closely related plants. Several empirical studies of external feeding phytophagous insects have corroborated that pattern (e.g., Farrell, 1998a; Farrell, 2001; Janz and Nylin, 1998).

While host chemistry has been viewed as one of the primary factors influencing the evolution of phytophagous insects, other aspects of host-plant variation, such as host growth form, are also thought to play an important role in the evolution of insect-plant interactions (Janz and Nylin, 1998; Powell, 1980). Plants of different growth forms dominate different habitats and typically bear different chemical defenses (Janz and Nylin, 1998). Feeny (1976) postulated that herbs have diverse "qualitative" toxins that require numerous specialized adaptations by the herbivore, while trees are characterized by relatively few widespread "quantitative" defenses such as the presence of tannins, a generalized digestion-reducing agent. If Feeny's postulate is correct, we would expect more host shifts among trees than herbs, as it would be easier for the herbivore to switch hosts in a group of relatively homogenous plants. This trend has been observed in butterflies (Janz and Nylin, 1998), but few other empirical studies have examined whether host shifts are more common in trees than herbs (but see Lopez-Vaamonde et al., 2003; Menken et al., 2009).

Although most attention has focused on aspects of the host plant, strong pressures from predators or parasitoids could also be important mediators of evolution and diversification patterns in phytophagous insects (Singer and Stireman, 2005). Such "top-down" effects on phytophage diversification might be especially pronounced for internal feeders. Endophages, especially leaf miners, often experience strikingly high (> 80%) mortality from parasitoids (Askew, 1980; Askew and Shaw, 1979; Hawkins et al., 1997; Kato, 1984), and therefore should experience strong diversifying selection to prevent parasitoid attack (Djemai et al., 2000; Kato, 1985). Lepidopteran leaf miners are thought to be approximately 100 Mya old (Labandeira et al., 1994) and parasitoids specializing on leaf miners may date back to more than 50 Mya (Labandeira, 2002; Murphy et al., 2008; Zaldívar-Riverón et al., 2008). If parasitoid lineages have been applying pressure throughout leaf miner evolution, the development of morphological, behavioral, and physiological innovations by both parasitoids and hosts may have led to arms races in certain lineages. For instance, it has been postulated that tentiform miners have progressively deepened their mine depths in order to counter the increasing longer parasitoid ovipositor (Brandl and Vidal, 1987). Furthermore, leaf miners that have developed innovations against parasitoids might be expected to be more diverse than their sister-groups that lack the trait. While these hypotheses are plausible, there have as yet been few rigorous analyses of the evolution and evolutionary consequences of endo-phytophage life history evolution, including the relative importance of "top-down" versus "bottom up" influences.

In this study we present an exploratory study of patterns of life history evolution and their possible effects on diversification in an exceptionally species-rich group of internal-feeding Lepidoptera, the family Gracillariidae. Gracillariid leaf-mining moths are an excellent group for testing hypotheses on life-history evolution of internal feeders because of their host specificity, diversity, and many specialized life history traits. Unlike most internal feeding microlepidoptera, the Gracillariidae are every diverse, and life history records for gracillariids are extraordinarily well documented (De Prins and De Prins, 2010). Specialized life history innovations include, among many others, switches between external and internal feeding (Davis, 1987), changes in mine form (Davis and Robinson, 1998), and larval hypermetamorphic development (De Gryse, 1916; Fitzgerald and Simeone, 1971; Kumata, 1978; Wagner et al., 2000). The disproportionate number of particular mine forms in gracillariids may signify an innovation that freed these moths at least in part from parasitoids.

We focus first on four potential on anti-parasitoid defense strategies that may explain the unequal diversity of particular gracillariid groups. These are: (1) complex serpentine mines that can increase parasitoid search time, and in turn, increase miner survival (Ayabe et al., 2008; Kato, 1984; Kato, 1985); (2) tentiform mines that prevent parasitoid ovipositors from reaching the leaf miner (Brandl and Vidal, 1987); (3) decorative bubbles on the cocoon that may act as a barrier against parasitoids (Wagner et al., 2000) and (4) the presence of dense frass that may attract parasitoids (Heinrich, 1976). As a contrast to these traits reflecting "top-down" evolutionary pressures, we examine phylogenetic patterns in leaf miner traits reflecting "bottom-up," host-plant-

related evolutionary pressures, namely, host plant phylogeny and growth form. While the timing of parasitism attack may be important in determining mine morphology, we did not examine parasitoid timing in this initial study.

As a first step in assessing the potential significance of all these traits in gracillariid evolution, we examined their phylogenetic histories using a new, expanded molecular phylogeny of Gracillariidae. The taxon sample, while representing less than 10% of gracillariid species diversity, is chosen to represent most of the obvious morphological and life history variation across the family. The overall aim of the study is to provide an overview and catalog of evolutionary hypotheses about life history traits related to host plant use in gracillariids, as well as an initial assessment as to which of these are the most promising for further study and at what scale of evolutionary divergence.

Methods

Taxon and gene sampling

Eighty-six species, expanded from the preliminary taxon set of Chapter 2, were included in the present study. Taxa were chosen based on a broad sampling of genera and the goal to capture the greatest life-history variation from the limited number of samples available. We included multiple species from genera that were known to be diverse, such as *Caloptila, Cameraria,* and *Phyllonorycter*. Table 3.3 lists the percentage of known species in each genus and the number of species sampled for each.

Eight protein-coding nuclear genes, totalling 7,626 bp, were chosen from a set of 26 genes that are currently being sampled to establish a backbone molecular phylogeny of Lepidoptera (see http://www.leptree.net). Gene names and the total length of the sequence included in this study are: CAD (2,886 bp), the 1.7sF–4sR region of DDC (708 bp), enolase (1,135 bp), acc2_4 (501 bp), 109fin1_2 (561 bp), 265fin2_3 (447 nt), 268fin1_2 (768 bp), and 3007fin1_2 (620 bp). GenBank numbers for each sequence is listed in Table 3.3.

Sequencing, alignment, and validation

PCR primers, amplification strategies, and laboratory protocols followed Regier (2008). Nucleic acid sequences were generated from mRNAs amplified with RT-PCR. Sequences were gel-isolated, purified, and nested amplifications conducted whenever necessary. Sequences were first checked for error, before being assembled, edited, and concatenated with the software Geneious 4.8.4 (Drummond et al., 2009). The final data set was aligned using MAFFT 6.717 (Katoh, 2009b), implementing the E-INS-i function (mafft –genafpair maxiterate 1000). The entire aligned sequence data set is deposited as a Nexus file in TreeBASE (http://www.treebase.org), study accession number xxx. Because seven extracts were made from larvae, and three from the pupae (Table 3.4), we conducted NCBI-BLASTn and tBLASTx searches (Altschul et al., 1997) in the nr database on all sequences to assure there were no contaminants. We discounted matches with other Lepidoptera, but recorded the hits that had the highest percentage identity with parasitoid sequences.

Phylogenetic analysis

Phylogenetic analyses were conducted with maximum likelihood (ML) and Bayesian Inference. We first used jModelTest (Posada, 2008) to determine the best nucleotide substitution model for the aligned data set and also for a data set that excluded synonymous change (degen1, Regier et al., 2010; Zwick, 2010). We conducted degen1 analyses because previous studies have revealed stronger signal for some deep-level nodes when only non-synonymous changes are included (e.g., Cho et al., 2010; Regier et al., 2010; Zwick et al., submitted).

The ML analysis was conducted with GARLI 1.0 (Genetic Algorithm for Rapid Likelihood Inference, Zwickl, 2006), and the Bayesian analysis with MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). For the ML analysis, we conducted 1000 ML tree searches and 2000 bootstrap replicates, utilizing the parallel nature of grid computing (Cummings and Huskamp, 2005) through The Lattice Project (Bazinet and Cummings, 2009). Bayesian analyses were conducted locally with two parallel runs of four chains each with a temperature of 0.15, employing default priors and a random starting tree. Trees were sampled every 1000 generations for 10^7 generations. Convergence of the two runs was assessed by examining whether the standard deviation of split frequencies fell below the 0.01 threshold (Ronquist and Huelsenbeck, 2003), and by checking the stability of clade splits with the "Cumulative" option in AWTY online (Wilgenbusch et al., 2004). Seventy percent of the post-burnin trees were discarded, and the remaining trees used to calculate the Bayesian consensus. Since Bayesian posterior probability values can be excessively high (Cummings et al., 2003; Suzuki et al., 2002), we

interpreted the Bayesian posterior probabilities on groups supported only if a value of 1.0 was achieved. Because ML and Bayesian analyses resulted in near identical results, we conducted PTP tests (Faith and Cranston, 1991) of life history traits on the ML tree.

Life history coding and ancestral state reconstruction

Data on the life history traits described in subsequent sections were compiled from the literature (Davis and Wagner, 2005; De Prins and De Prins, 2005; Kumata, 1961; 1963; 1977; 1978; 1982; 1985; 1993; 1998; Kumata et al., 1988; Wagner et al., 2000), online resources (De Prins and Steeman, 2010; Edmunds, 2009; Harrison, 2010; Suzuki, 2010) and also personal observations. Because erroneous host plant records are known to exist, we tried to be conservative and excluded anecdotal host plant records. For each moth species, plant records were included only if there was more than one report of the moth feeding on the host plant family. However, if there was only a single known host record, then the record was included. Life history data were scored only for the species that were sequenced. Life histories and their character state codings used in this study are listed in Table 3.

All life history characters were coded as standard, unordered, binary or multistate characters and optimized with both parsimony and ML ancestral state mapping in Mesquite ver 2.72 (Maddison and Maddison, 2009). For parsimony mapping, we applied accelerated (ACCTRAN) and delayed (DELTRAN) optimization, and the Mk1 model (Lewis, 2001) for the ML ancestral state analysis.

Because outgroup choice for Gracillariidae is problematic, we scored life history characters only within gracillariids. The present study placed Roeslerstammiidae + Yponomeutidae as the sister-group to gracillariids, but with very weak support (Fig. 3.5). Bucculatricidae may also be closely related to the Gracillariidae, as shown by additional molecular data (Chapter 2) and shared morphological structures (Davis and Robinson, 1998; Kuznetzov and Stekol'nikov, 1987). Since relationships among these families remain unclear, the ancestral condition of Gracillariidae, inferred here only from observations within that family, will be further explored in a future study with greater ingroup and outgroup sampling.

Phylogenetic conservatism of life history traits

We assessed the degree of phylogenetic conservatism of each life history character over different scales of comparison using the permutation tail probability (PTP) test of Faith and Cranston (1991). For each character, PTP tests were carried out for Gracillariidae as a whole, and separately for four strongly supported sub-clades thereof (bootstrap values > 98% and a posterior probabilities = 1.0). Outgroups were omitted from these tests. In the PTP test, the observed character states are repeatedly and randomly redistributed across taxa to generate an expected frequency distribution of the minimum number of trait shifts under the null hypothesis that the observed distribution of states is independent of the phylogeny. The number of changes inferred from the observed data is then compared to the null distribution. These calculations were carried out using PAUP* 4b10 (Swofford, 2002). We also calculated the retention index (RI, Farris, 1989a; Farris, 1989b) for each trait to assess the level of homoplasy.

Larval feeding habit, bubble ornamentation, and frass deposition

To test the postulate that the less common types of gracillariid larval feeding habits exhibited in later instars – serpentine mining, tentiform mining, leaf rolling and leaf galling – could be later stages in "arms races" with parasitoids, we created and reconstructed the evolution of a "larval habit" character with five states: "blotch miner", "serpentine miner", "tentiform miner", "galler" and "roller". Because nearly all miners build a serpentine mine during their first several instars, we restricted our categories to reflect the habit of the final instar. We also coded the presence and absence of "bubbles" on the cocoon, and the presence of dense frass in the final instar mine, both of which may be related to parasitoid pressure. Bubbles are created from the abdomen of the larva and individually placed on the outer surface of the cocoon. They are filled with air and trace amounts of an unknown whitish or yellowish substance. They are wrapped with silk and individually positioned (Wagner et al., 2000). Because bubble density differs among gracillariid species (Wagner et al., 2000), we scored bubble density into two states, sparse (< 10 bubbles) and dense (≥ 10 bubbles). We coded frass as "dense" if more than a quarter of the width of the mine was covered in thick, dark frass. We scored as many mines possible for each species. When characterization of a particular trait was difficult, we scored the trait for that species as ambiguous.

Host plant use and host growth form

To examine the rate of host plant shifts, we first compiled a list of known hosts for the gracillariid species sampled in the present study. Hosts were scored at two taxonomic levels, order and subclass; to determine the level at which host associations might be most strongly conserved. Host plant records were compiled from the Global Taxonomic Database of Gracillariidae (De Prins and De Prins, 2010), and arranged according to current classification of the Angiosperm Phylogeny Group (APG_III, 2009; Chase and Reveal, 2009). Moth species typically only had one host family, but those that had more than one were coded as having two or more. Host growth form was determined via the Flowering Plants Gateway website (Watson and Dallwitz, 1992 onwards), and moth taxa scored as feeding on "herbs", "shrubs", "trees", or "vines".

Results

Parasitoids and sequence validation

All amplified sequences were first compared to sequences of the same locus in the NCBI GenBank database. Sequences generated from adult moth extracts did not result in any BLAST hits that suggest contamination from parasitoids. However, four sequences, one from *Parornix angicella* and three from *Telamoptilia* sp. nov. recorded GenBank sequence similarity scores that were closest to chalcidoid sequences (Table 3.4). Based on BLAST searches and suspiciously long branches for these taxa, we concluded that these larvae were probably parasitized. Thus, they were excluded from the final data set.

Gracillariid phylogeny

The appropriate substitution model for the fully aligned dataset was determined as the general-time-reversible substitution model (Lanave et al., 1984; Tavaré, 1986), with among-site-rate-heterogeneity modeled according to a gamma (Γ) distribution (Yang, 1994) while allowing for a proportion of invariable sites (I) (Gu et al., 1995). Our nt123 and degen1 results led to very similar topologies, but our discussion focuses on the nt123 data set because it yielded stronger phylogenetic signal within Gracillariidae. The ML tree, with branch lengths and outgroups, is available as a supplementary file (included here in this dissertation as Fig. 3.5).

Our results were very similar to those based on the nt123 data set with more genes but fewer taxa (Chapter 2). The Gracillariidae, Lithocolletinae + *Leucanthiza*, and three groups within Gracillariinae that roughly correspond to Kumata's (1988) *Acrocercops*, *Gracillaria*, and *Parectopa* groups were monophyletic with strong support (> 98% bootstrap, posterior probability = 1, Fig. 3.1). We refer to these four well-supported groups as the "core gracillariid clades" throughout the remainder of this chapter.

Ancestral state reconstruction

Both parsimony and ML mapping suggest that the ancestral larval feeding condition in gracillariids is blotch mining (Fig. 3.1). Serpentine mining, on the other hand, appears to be a secondary trait that appeared in the Oecophyllembiinae, Phyllocnistinae, and *Dendrorycter* + *Marmara*. The most parsimonious scenario is two

independent origins of serpentine mining, but support for nodes separating the two groups was weak.

Tentiform mining was restricted to a single well-supported clade, *Cremastobombycia* + *Phyllonorycter*. Final instar leaf rolling was only found in the monophyletic clade that included the *Gracillaria* group, *Aristaea, Callisto, Macarostola*, and *Parornix*. A transition to gall feeding was found in a single species nested within this clade, *Caloptilia murtfeldtella* (Fig. 3.1). Bubble ornamentation is present in three groups in Gracillariinae: in the ancestral lineages within the *Acrocercops* group; *Dendrorycter* + *Marmara*, and the ancestor of the *Parectopa* group, despite a secondary loss in *Micrurapteryx* and *Parectopa* (Fig. 3.2A). Dense frass was absent in the *Acrocercops* group and *Liocrobyla* + *Micrurapteryx* + *Parectopa* (Fig. 3.2B).

Host plants and growth form in the lower parts of the gracillariid tree were equivocal. However, there was a strong tendency for the four core gracillariid groups to feed on fabids. In Lithocolletinae + *Leucanthiza*, the ancestral association was Fabales. Many host switches were observed, especially among rosids, but there were occasional associations with distantly related to non-eurosid plants, such as Magnoliaceae and Ranunculaceae (Fig. 3.3).

Phylogenetic conservatism of life history traits

All characters pertaining to mine form and habit were more phylogenetically conserved, as measured by the Retention Index, than any of the characters pertaining to

properties of the host plant (Table 3.2). However, nearly all traits showed a significant correlation with the phylogeny across the Gracillariidae, the only exception being host growth form. Mine form/habit characters were almost invariably also significantly correlated with phylogeny within gracillariid subclades, the only exception being the presence/absence of "bubbles" within the *Acrocercops* group. In contrast, the only half (8/16) of the PTP tests for phylogenetic conservatism for host-plant-related traits within gracillariid subclades were significant (Table 3.2).

Gracillariidae favored rosid hosts (69.5%, 41 of 59), especially the fabids (78.1%, 32 of 41). A total of 56 host records were on core eudicots, while only three gracillariids utilized non-core eudicot groups. The PTP test showed significant phylogenetic clustering ($P = 0.001$) of gracillariid species according to host plant order. Host shifts across the Gracillariidae were most frequent among host plants of the same order or subclass, as inferred from the PTP results. Parsimony optimization of larval habits on the ML tree indicates that there were probably five changes in larval habits during the evolutionary history of the Gracillariidae. In contrast, there were 28 shifts to different host orders.

Discussion

Evolution of leaf-mining and related habits in gracillariids - anti-parasitoid innovations?

Both parsimony and ML reconstructions point with high confidence to blotch mining as the ancestral form of leaf-mining in gracillariids. There were two separate

subsequent shifts, to tentiform mining in *Phyllonorycter* + *Cremastobombycia*, and to leaf rolling and galling in the *Gracillaria* group. These results contradict the prediction that leaf rolling was ancestral to internal feeding in gracillariids (Davis, 1987); rather, external feeding (but inside a shelter) appears to be a derived condition. The subsequent transition from leaf rolling to galling inferred here parallels findings in willow-feeding sawflies (Nyman et al., 1998; Nyman et al., 2000) and in thrips that induce galls on *Acacia* (Crespi and Worobey, 1998). While the evidence is still limited, it may be that leaf rolling is an evolutionary transitional state that facilitates the shift to galling from external feeding.

Given the high mortality that leaf miners often face from parasitoids (Askew, 1980; Askew and Shaw, 1979; Godfray et al., 1999; Hawkins et al., 1997; Kato, 1984), and the long historic association between parasitoids and their leaf-mining hosts (Murphy et al., 2008; Zaldívar-Riverón et al., 2008), we would expect strong selection favoring mine innovations that limit parasitoid attack. Our results identify several evolutionary transitions within subgroups of gracillariids that might be interpreted as such innovations. One is serpentine mining in the later instars. Studies of leaf-mining agromyzid flies show that mine forms with complex networked serpentine forms can increase parasitoid search time and miner survival (Ayabe et al., 2008; Kato, 1984; Kato, 1985). Our study revealed one or two independent origins of serpentine mining, as support for nodes separating the two origins was weak. With greater gene sampling but slightly less taxon sampling (Chapter 2), all serpentine miners are often grouped together, but again with low support. Thus, the number of origins of late-instar serpentine mining remains unclear.

A second possible anti-parasitoid innovation is the production of tentiform blotch mines, unique among gracillariids to *Phyllonorycter* + *Cremastobombycia*, in which the leaf epidermis is folded with internal silk to produce a convex arch. The result is a mine with greatly increased internal height within the leaf mine (Hering, 1951). Our results, in according with the prediction of Hering (1951), show that the tentiform leaf mine is a modification of an ancestral blotch mining habit (Fig. 3.1). Brandl and Vidal (1987) hypothesized that the greater depth of tentiform mines may prevent parasitoids with short ovipositors from reaching their hosts, and in consequence, result in an evolutionary arms-race between the miner and parasitoid, where the depth of the mine increases over time in response to the increasing length of the parasitoid ovipositor. If so, derived lineages of the *Phyllonorcyter* + *Cremastobombycia* clade may have developed deeper tentiform mines. Unfortunately, we could not test this hypothesis with our limited taxon sampling.

A third possible defensive innovation is exhibited by the many gracillariid larvae, including most sampled members of the *Acrocercops* and *Parectopa* groups, which decorate the outer surface of their cocoon with hardened bubbles (Davis et al., 1991; Davis and Wagner, 2005; Kumata, 1978; Needham et al., 1928; Wagner et al., 2000). Such bubble decorations, particularly when dense, may provide a physical barrier that distances the pupa from the ovipositor of parasitoids (Wagner et al., 2000), or contain chemicals that repel parasitoids (D. Davis, pers. comm.). While we cannot formally test whether this trait is an anti-predatory defense, parsimony mapping reveals at least three independent origins of bubble making behavior, and at least two secondary losses (Fig.

3.2A). Interestingly, taxa known to have dense bubble ornamentation (*Dendrorycter, Marmara*, and *Neurobathra*), were distantly related. This suggests that dense bubble ornamentation has evolved near the base of the Gracillariidae (specifically the *Acrocercops* group) and subsequently lost several times. Unfortunately, the number of available observations on bubble ornamentation is still very limited. It is hoped that bubble presence, their ecological function, and the variation in bubble number within genera and species can be further quantified with additional life-history observations.

Although rigorous experimental evidence on their fitness consequences is needed, for all of these potential defensive innovations there are plausible grounds for supposing that they would provide improved protection from parasitoid attack, as compared to the antecedent condition. Some or all might also increase leaf miner survival by making the mine more conspicuous, thereby deterring external herbivores from feeding on mined leaves, as recently suggested by Yamazaki (2010). Conversely, however, it is also possible that mine conspicuousness could promote discovery by enemies, similar to the way that feeding signs such as bite marks and frass presence are known to attract parasitoids that use visual or chemical cues (Heinrich, 1976; Heinrich and Collins, 1983; Roth et al., 1978; Turlings et al., 1991). For this reason, it seems plausible that the shift from blotch mining to leaf margin rolling, in the *Gracillaria* group and relatives, could represent yet another evolutionary escape from parasitism.

In addition to being interpretable as defensive innovations, the foregoing traits show striking phylogenetic conservatism, corroborated by significant PTP tests. Each

innovation appears to have originated once or at most twice, and to characterize most or all the species of a substantial lineage, suggesting that it has persisted long enough to have a marked effect on diversification rate. It is therefore of interest to ask whether the clades bearing these innovations show elevated net diversification over near relatives lacking the innovation. The clearest suggestion of such increased diversification is the case of tentiform mines. Our phylogeny suggests, albeit with only moderate support, that the sister-group of the tentiform-mining lineage *Phyllonorycter* + *Cremastobombycia,* which numbers over 400 species, is *Cameraria* + *Porphyrosela*. The latter two genera have a combined known diversity of approximately 80 species (De Prins and De Prins, 2010). This contrast in diversity is at least consistent with diversification spurred by reduced natural enemy attack.

Host preference, growth form, and shifts

Our results provide support that characters of larval feeding habit appear more conserved than host taxon association, a result that is concordant with the findings from other insect groups (e.g., Bucheli et al., 2002; Marvalidi et al., 2002; Nyman et al., 2006; Ronquist and Liljeblad, 2001; Winkler et al., 2009). Because the number of host switches are likely to be major underestimates with the taxon sampling of this study, it is plausible that major host shifts are more than ten or twenty times as frequent than changes in larval feeding habit.

Gracillariid host shifts were most frequent among host plants of the same family or order, but there were occasionally shifts to distantly related families such as the

Magnoliaceae and Ranunculaceae. Many host shifts have occurred back and forth between the Fagales, Fabales, Rosales, and the more distantly related Asterales, Ericales, and Sapindales, suggesting that other factors, such as geographic distribution and ecological properties of the plant taxa (e.g., host chemistry, morphology) are constraining host shifts. It would be valuable test to examine plant chemistry, as it has been promoted as the leading factor underlying host shifts (Feeny, 1975; Zangerl and Berenbaum, 1993). Specific information on secondary host chemistry is limited, but we have begun to examine how host chemistry (specifically tannin content) is correlated to gracillariid phylogeny in a separate study.

Evolutionary conservatism of phytophagous insects can sometimes lead to co-cladogenesis with host plants (Farrell, 1998b; Farrell and Mitter, 1990; Weiblen, 2001). However, a comparison of gracillariid and angiosperm phylogenies does not indicate co-cladogenesis, as repeated and convergent shifts occur among fabids and other plant taxa (Fig. 3.3). Our results are congruent with the general consensus that strong co-cladogenesis in phytophagous insects is rare (Nyman, 2010; Winkler and Mitter, 2008). Discordant insect and host phylogenies have also been reported in studies on seed- and leaf-mining moths (Bucheli et al., 2002; Kawakita et al., 2004; Lopez-Vaamonde et al., 2003), gall-inducing hymenopterans (Nyman, 2010; Nyman et al., 2006; Ronquist and Liljeblad, 2001), mining flies (Berlocher, 2000; Scheffer and Wiegmann, 2000; Smith and Bush, 1997; Winkler et al., 2009), and internally feeding beetles (Farrell and Sequeira, 2004; Jones, 2001; Morse and Farrell, 2005).

Of the life history traits examined, host growth form was the least conserved on phylogeny (Table 3.2, Fig. 3.4). Studies on other internal plant feeders, such as cynipid gall wasps (Schick et al., 2003) also show a weak correlation between host growth form and phylogeny, but some studies have demonstrated a correlation between the two, especially those in butterflies (e.g., Janz and Nylin, 1998). Physiological features of the plant, such as leaf width, tissue density, and sap viscosity vary across host growth forms, and may be of more importance in leaf-mining moths. It is clear that many additional tests, both ecological and evolutionary, will be necessary to characterize broad patterns in Gracillariidae.

Conclusions

This exploratory study serves to examine several general patterns of life history evolution in Gracillariidae. We conclude that characters associated with larval feeding habit are more conserved than host taxon associations. We observed numerous host shifts that frequently occurred within rosids, but there were also shifts to distantly related plants such as the Magnoliaceae and Ranunculaceae. A comparison of insect and host phylogeny reveals little indication of co-cladogenesis, supporting the trend that strong co-cladogenesis among phytophagous insects and their hosts is rare.

While our study revealed some broad patterns, we expect many more to be revealed with additional life history data and analysis. For instance, to further test the hypothesis that particular larval habits led to diversification in particular lineages (e.g., tentiform mining in *Phyllonorycter* + *Cremastobombycia*), we plan to use the Slowinski-

Guyer clade asymmetry statistic and conduct sister group tests for serpentine miners and blotch miners. Alternatively, we could create a gracillariid chronogram, and measure rates separately to examine if rates are higher for leaf rollers than for blotch miners. We propose to do these tests as part of our ongoing attempt to capture patterns of life history traits in Gracillariidae.

Table 3.1. Gracillariid life history traits examined for the present study.

Genus	species	LepTree code	Larval habit[a]	Host Family[b]	Host order[c]	Plant host	Plant form[d]	Bubbles	Dense frass
Acrocercops	*albinatella*	Aalne	B[18]	Fg	F	*Quercus sp.*[2]	T[2]		Y[18]
Acrocercops	*brongniardella*	Abrg	B[1]	Fg	F	*Quercus sp.*[2]	T[2]	Y	Y[18]
Acrocercops	*transecta*	Atran	B[18]	Er, J	A, F	*Carya, Juglans, Lyonia, Platycarya*[2]	T, S[2]		Y[18]
Amblyptila	*sp n.*	Atila	B[18]	Ap	L		H[2]		
Aristaea	*sp n.*	Aris	T[7]	As (?)	C		H[2]	?[7]	
Artifodina	*japonica*	Ajaa	B[10]	Mys	A	*Myrsine segunii*[2]	T[2]	Y[10]	N[10]
Callisto	*denticulella*	Cdel	R[3]	R	F	*Malus*[2]	T, S[2]	N[17]	Y[3]
Caloptilia	*bimaculatella*	Cbim	R[4]	Sp	M	*Acer*[4]	T[4]		
Caloptilia	*murtfeldtella*	Cmur	G[4]	Sc	L	*Penstemon*[4]	H[4]		
Caloptilia	*sapporella*	Csap		Fg	F	*Castanea, Quercus*[2]	T[2]		
Caloptilia	*stigmatella*	Cstg	R[3]	Sa	F	*Populus, Salix*[4]	T[4]		R[3]
Caloptilia	*obliquatella*	Pvob	R[9]	Fg	F	*Quercus sp.*[9]	T[9]		
Cameraria	*gaultheriella*	Caga	B[6]	Er	A	*Gaultheria*[2]	S[2]	N[17]	Y?
Cameraria	*sp.nov.*	Came087	B[6]					N[17]	Y?
Cameraria	*guttifinitella*	Cgut	B[4]	An	M	*Rhus, Toxicodendron*[4]	T, S[4]	N[4]	Y[18]
Cameraria	*ohridella*	Cohd	B[18]	Sp	M	*Aesculus*[2]	T, S[2]	N[17]	Y[3]
Chilocampyla	*dyariella*	Cdya	B[18]	Myr	R	*Eugenia*[2]	T, S[2]	N[17, 18]	Y[18]

Leucanthiza	*amphicarpeaefoliella*	Leuz	B[4]	Fb	F	*Amphicarpaea*[4]	H[4]	N[4]	Y[4, 18]
Leucospilapteryx	*venustella*	Lven	B[4]	As	C		H[4]	N[4]	
Leurocephala	*sp n.*	Gran	B[17]				?	Y[17]	
Liocrobyla	*lobata*	Lioc	B[18]	Fb	F	*Pueraria*[2]	V[2]		
Lithocolletinae	*genus novus*	Akgn126							
Macarostola	*japonica*	Mjap	R[7]	Sta	CE	*Euscaphis japonica*[7]	T[7]		
Marmara	*serotinella*	Msll	S[17]	R	F	*Prunus serotina*[2]	T[2]		Y[18]
Micrurapteryx	*salicifoliella*	Msai	B[4]	Sa	F	*Populus, Salix*[4]	T[4]	N[4]	N[18]
Neolithocolletis	*hikomonticola*	Nlik	B[18]	Fb	F	*Pueraria*[12]	V[12]	N[17]	
Neurobathra	*strigifinitella*	Neur	B[18]	Fg	F	*Castanea, Fagus, Quercus*[2]	T[2]	Y[15]	Y[18]
Parectopa	*robiniella*	Prbn	B[4]	Fb	F	*Robinia*[4]	T, S[4]	N[4]	Y[18]
Parornix	*anglicella*	Pxag	R[3]	R	F	*Crataegus, Sorbus*[2]	T, S[2]	N[17]	Y[18]
Phodoryctis	*stephaniae*	Pste	B[18]	Me	E	*Stephania*[2]	V[2]		
Phyllocnistis	*citrella*	Phcn	S[14]	Rt	M	*Citrus*[2]	T, S[2]		Y[18]
Phyllocnistis	*magnoliaeella*	Pmgl	S[18]	M	Mg	*Magnolia*[2]	T[2]	N[17, 18]	Y[18]
Phyllonorycter	*aberrans*	Anab	B[17]	Fb	F	*Desmodium sp.*[17]	S[17]	N[17]	Y[18]
Phyllonorycter	*basistrigella*	Pbas	T[4]	Fg	F	*Quercus sp.*[4]	T[4]	N[4]	Y[4]
Phyllonorycter	*n.sp. (malus)*	Phyl	B[18]	R	F	*Malus*[2]	T, S[2]	N[17]	Y?
Phyllonorycter	*insignitella*	Phzz	B[18]	Fb	F	*Trifolium, Ononis, Medicago*[2]	H, S[2]	N[17]	Y?
Phyllonorycter	*symphoricarpaeella*	Pmicp	T[4]	C	C	*Symphoricarpos*[4]	S[4]	N[4]	Y?
Phyllonorycter	*ostryaefoliella*	Posy	T[4]	B	F	*Ostrya*[4]	T[4]	N[4]	Y?
Phyllonorycter	*robiniella*	Proi	B[4]	Fb	F	*Robinia*[4]	T, S[4]	N[4]	Y?

Phyllonorycter	*lucetiella*	Ptea	B[4]	T	M	*Tilia*[4]	T[4]	N[4]	Y?
Porphyrosela	*sp.*	Pyla063		Fb	F		?[2]		
Porphyrosela n. gen.	*n. sp.*	Porp121		Fb	F		?[2]		
Psydrocercops	*wisteriae*	Pwis	B[18]	Fb	F	*Wisterea floribunda*[11]	S, V[11]		
Spulerina	*dissotoma*	Sput	B[18]	Fb	F	*Lespediza, Pueraria*[2]	S, V[2]		
Stomphastis	*sp.*	Stss							
Telamoptilia	*sp.nov.*	Tela	B[18]	Ma	M	*Hibiscus*[18]	S[18]		Y[18]

[a] Larval habit: B: bloch miner, G: galler, S: serpentine miner; T: tentiform blotch miner; R: roller,

[b] Host families: An: Anacardiaceae, Ap: Apocynaceae, Ar: Araliaceae, As: Asteraceae, B: Betulaceae, C: Caprifoliaceae, Ce: Celastraceae, Cl: Clusiaceae, Eb: Ebenaceae, Er: Ericaceae, Eu: Euphorbiaceae, Fb: Fabaceae, Fg: Fagaceae, J: Juglandacae, L: Lauraceae, M: Magnoliaceae, Ma: Malvaceae, Me: Menispermaceae, Mys: Myrsinaceae, Myr: Myrtaceae, O: Oleaceae, R: Rosaceae, Rb: Rubiaceae, Rt: Rutaceae, Sa: Salicaceae, Sp: Sapindaceae, Sc: Scrophulariaceae, Sta: Staphyleaceae, Sti: Stirculiaceae, T: Tiliaceae

[c] Host order: A: Asterid, C: Campanulid, CE: Core Eudicot; E: Eudicot, F: Fabid, L: Lamiid, M: Malvid, Mg: Magnoliid, R: Rosid

[d] Plant form: H: Herb, S: Shrub, T: Tree, V: Vine

References: De Prins and Steeman (2010)[1], De Prins and De Prins (2010)[2], Edmunds (2009)[3], Harrison (2010)[4], Kumata (1961)[5], (1963)[6], (1977)[7], (1978)[8], (1982)[9], (1985)[10], (1993)[12], (1998)[13], Kumata et al. (1988)[11], Suzuki (2010)[14], Wagner et al. (2000)[15], Atsushi Kawakita (pers. comm.)[16], Donald Davis (pers. comm.)[17], personal observations[18]

Table 3.2. PTP tests for significance for five groups in Gracillariidae. Numbers in parentheses are the number of taxa in each group. Abbreviated group names: *Acro* group = *Acrocercops* group; *Graci* group = *Gracillaria* group; *Parec* group = *Parectopa* group.

			PTP results				
Character	states	RI	Gracillariidae (67)	Lithocolletinae (22)	*Acro* group (16)	*Graci* group (8)	*Parec* group (11)
1: Larval feeding habit	6	0.8	**< 0.001***	**< 0.001***	**< 0.001***	**< 0.001***	**< 0.001***
2: Bubbles	2	0.5	**0.006***	**< 0.001***	0.27	**< 0.001***	n/a
3: Frass density	2	0.86	**< 0.001***	**< 0.001***	**< 0.001***	**< 0.001***	**< 0.001***
4: Growth form	4	0.06	0.614	0.428	0.424	**< 0.001***	**< 0.001***
5: Host order	10	0.31	**0.001***	**0.002***	0.089	**< 0.001***	**< 0.001***
6: Host subclass	3	0.27	**0.034***	0.1959	0.257	0.1025	**< 0.001***

Table 3.3. The 68 ingroup and 19 non-gracillariid taxa sampled in this study. Specimen localities, AToLep voucher ID numbers, and GenBank accession numbers for each gene are listed along with the life stage from which the extracts were made. "Div" refers to the number of described species in the gracillariid genus. "L Stg" refers to life stage. GenBank sequence numbers will be included as soon as they are assigned.

Family	Subfamily	Species	Div	Accession	Code	L Stg	CAD	DDC	ENO	ACC	109fin	265fin	268fin	3007fin
Gracillariidae	Gracillariinae	*Acrocercops albinatella*	337	TH-08-6107	Aalne	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Acrocercops brongniardella*	-	AYK-08-8215	Abrg	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Acrocercops transecta*	-	AYK-08-8201	Atran	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Amblyptila n. sp.*	2	AK-07-070	Atila	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Aristaea n. sp.*	12	GRACI054-07	Aris	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Artifodina japonica*	1	AYK-08-8206	Ajaa	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Callisto denticulella*	6	AYK-08-8214	Cdel	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Caloptilia bimaculatella*	325	DRD-05-0248	Cbim	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Caloptilia murtfeldtella*	-	TH-08-6096	Cmur	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Caloptilia obliquatella*	-	SWC-06-0265	Pvob	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Caloptilia sapporella*	-	JCS-08-1033	Csap	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Caloptilia stigmatella*	-	JDP-08-8081	Cstg	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Calybites auroguttella*	6	AYK-08-8218	Caug	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Chilocampyla dyariella*	2	EJN-06-2540	Cdya	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Conopomorpha cramerella*	4	AYK-08-8231	Ccrm	P	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Conopomorpha sp.*	-	AK-07-071	Cono	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Cuphodes diospyrosella*	19	AYK-08-8223	Cdio	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Dendrorycter marmaroides*	1	GRACI103-07	Dend	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Deoptilia heptadeta*	2	GRACI108-07	Deoa	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Dialectica sp.*	21	AK-07-011	Dtia	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Diphtheroptila scriptulata*	3	AYK-08-8224	Dscr	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Epicephala relictella*	40	JCS-06-0172	Epic	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Eteoryctis deversa*	4	AYK-08-8203	Edev	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Eucalybites aureola*	1	GRACI102-07	Euau	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Eucosmophora sp.*	16	AYK-08-9065	Euco	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Gibbovalva quadrifasciata*	7	GRACI105-07	Gibb	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Gracillaria syringella*	9	JDP-08-8042	Gsyg	P	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Leucanthiza amphicarpeaefoliella*	3	DRD-01-0064	Leuz	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Leucospilapteryx venustella*	3	TH-08-6105	Lven	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*"Leurocephala" n. sp.*	1	DRD-07-4001	Gran	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Liocrobyla lobata*	8	GRACI104-07	Lioc	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Macarostola japonica*	26	AYK-08-8238	Mjap	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Macarostola japonica*	-	AYK-09-5556	Mjap2	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx

Gracillariidae	Gracillariinae	*Marmara serotinella*	23	DRD-05-0265	Msll	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Micrurapteryx salicifoliella*	10	TH-08-6098	Msai	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Neurobathra strigifinitella*	3	DRD-01-0129	Neur	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Parectopa robiniella*	39	DRD-01-0009	Prbn	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Parornix anglicella*	72	AYK-08-8210	Pxag	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Phodoryctis stephaniae*	4	AYK-08-8204	Pste	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Psydrocercops wisteriae*	1	AYK-08-8221	Pwis	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Spulerina dissotoma*	16	AYK-04-5630	Sput	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Stomphastis sp.*	17	AK-07-025	Stss	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Gracillariinae	*Telamoptilia n. sp.*	5	AYK-08-8113	Tela	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Cameraria gautheriella*	72	DRD-01-0113v	Caga	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Cameraria guttifinitella*	-	TH-08-6116	Cgut	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Cameraria ohridella*	-	JDP-08-8021	Cohd	P	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Chrysaster hagicola*	2	AYK-08-8202	Chag	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Chrysaster ostensackenella*	-	DRD-05-0247	Cyosk	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Cremastobombycia ambrosiella*	6	TH-08-6114	Camb	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Cremastobombycia solidaginis*	-	TH-08-6130	Cmys	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Cremastobombycia n. sp.*	-	KN-06-0004	Crby	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Hyloconis wisteriae*	5	GRACI111-07	Hwis	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Lithocolletinae n. gen.*	1	AK-07-126	Akgn126	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*"Macrosaccus" robiniella*	1	JDP-08-8001	Proi	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Neolithocolletis hikomonticola*	3	GRACI110-07	Nlik	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Phyllonorycter basistrigella*	404	TH-08-6111	Pbas	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Phyllonorycter n. sp. (malus)*	-	TPF-89-1114	Phyl	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Phyllonorycter insignitella*	-	GRACI147-07	Phzz	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Phyllonorycter symphoricarpaeella*	-	TH-08-6128	Pmicp	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Phyllonorycter ostryaefoliella*	-	TH-08-6103	Posy	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Phyllonorycter lucetiella*	-	TH-08-6117	Ptea	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*Porphyrosela sp.*	10	AK-07-063	Pyla063	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Lithocolletinae	*"Porphyrosela" (n. gen.) n. sp.*	1	AK-07-121	Porp121	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Oecophyllembiinae	*Coryxethis sp.*	6	AYK-08-8220	Crym	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Oecophyllembiinae	*Eumetriochroa hederae*	4	AK-08-8111	Ehdr	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Phyllocnistinae	*Phyllocnistis citrella*	87	JBA-06-0025	Phcn	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Gracillariidae	Phyllocnistinae	*Phyllocnistis magnoliella*	-	DRD-05-0244	Pmgl	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx

Gracillariidae	undetermined	*"Anartioses" aberrans*	1	DRD-01-0010	Anab	L	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Outgroups														
Alucitidae	–	*Alucita sp.*		JBA-05-0004	Alsp	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Bucculatricidae	–	*Bucculatrix staintonella*		TH-08-6083	Bsta	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Bucculatricidae	–	*Bucculatrix sp.*		DRD-05-0270	Bucc	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Choreutidae	–	*Hemerophila felis*		DRD-01-0218	Hfel	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Cosmopterigidae	Antequerinae	*Euclemensia bassettella*		JWB-05-0044	Cole	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Douglasiidae	–	*Klimeschia transversella*		DRD-01-0017	Ktr	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Douglasiidae	–	*Tinagma gaedikei*		TH-08-6082	Tgak	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Douglasiidae	–	*Tinagma ocnerostomella*		DRD-01-0013	Tocn	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Elachistidae	Ethminae	*Ethmia eupostica*		MJM-96-0277	Eeu	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Pterophoridae	Pterophorinae	*Emmelina monodactyla*		CWM-05-1000	Emon	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Roeslerstamiidae	–	*Agriothera elaeocarpophaga*		DRD-01-0143	Agel	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Roeslerstamiidae	–	*Roeslerstammia pronubella*		DRD-01-0142	Rstm	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Tineidae	Tineinae	*Tinea columbariella*		EN-91-0006	Tco2	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Tineidae	Tineinae	*Tineola bisselliella*		JG-06-0009	Tbi3	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Tortricidae	–	*Argyrotaenia alisellana*		CWM-94-0262	Arga	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Tortricidae	Tortricinae	*Platynota ideausalis*		JWB-05-0029	Pida2	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Urodidae	–	*Urodus decens*		DA-03-3349	Ursp	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Yponomeutidae	–	*Eucalantica sp.*		DA-03-3364	Ysp	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
Yponomeutidae	Attevinae	*Atteva punctella*		JWB-05-0041	Atpu2	A	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx

Table 3.4. Gracillariid moth sequences amplified as larvae in the present study that had high matching similarity values with chalcidoid wasp sequences in GenBank. All sequences were generated from larval extract.

Moth	Gene	Genbank match	Genbank accession	BLASTn E-value
Parornix angicella	109fin	*Nasonia vitripennis* (Hymenoptera, Chalcidoidea)	XM_001600207	3e-120
Telamoptilia sp. nov.	109fin	*Nasonia vitripennis* (Hymenoptera, Chalcidoidea)	XM_001600207	3e-89
Telamoptilia sp. nov.	265fin	*Nasonia vitripennis* (Hymenoptera, Chalcidoidea)	XM_001605062.1	9e-23
Telamoptilia sp. nov.	DDC	Semiotellus sp. (Hymenoptera, Chalcidoidea)	DQ990771	2e-89

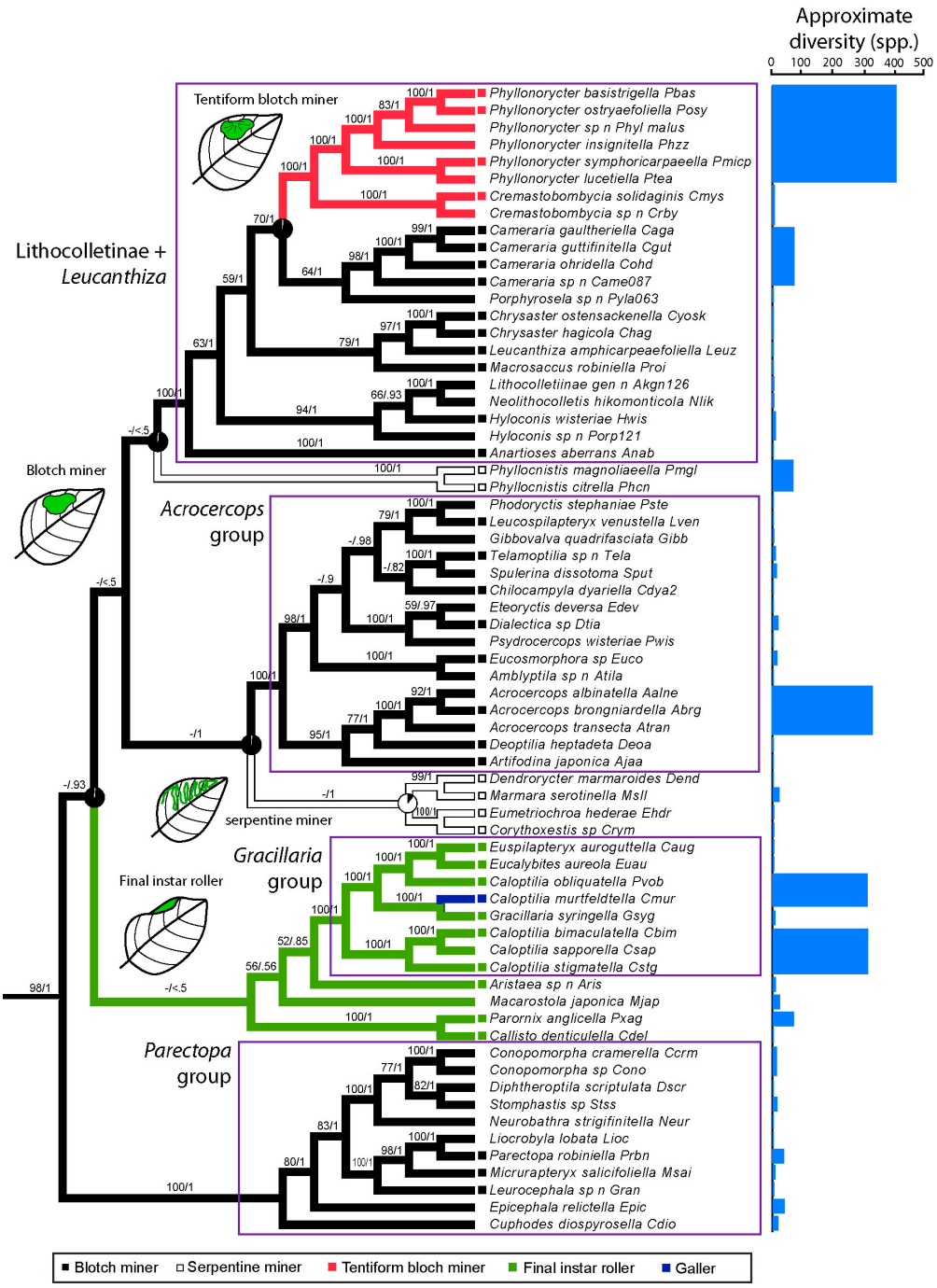


Fig. 3.1. Larval habit mapped onto phylogeny. Bootstrap support values and posterior probabilities are shown above branches. Pie charts of ML ancestral character state probabilities are included for relevant nodes. Approximate species diversity for each genus is included as a bar graph the right of the tree.

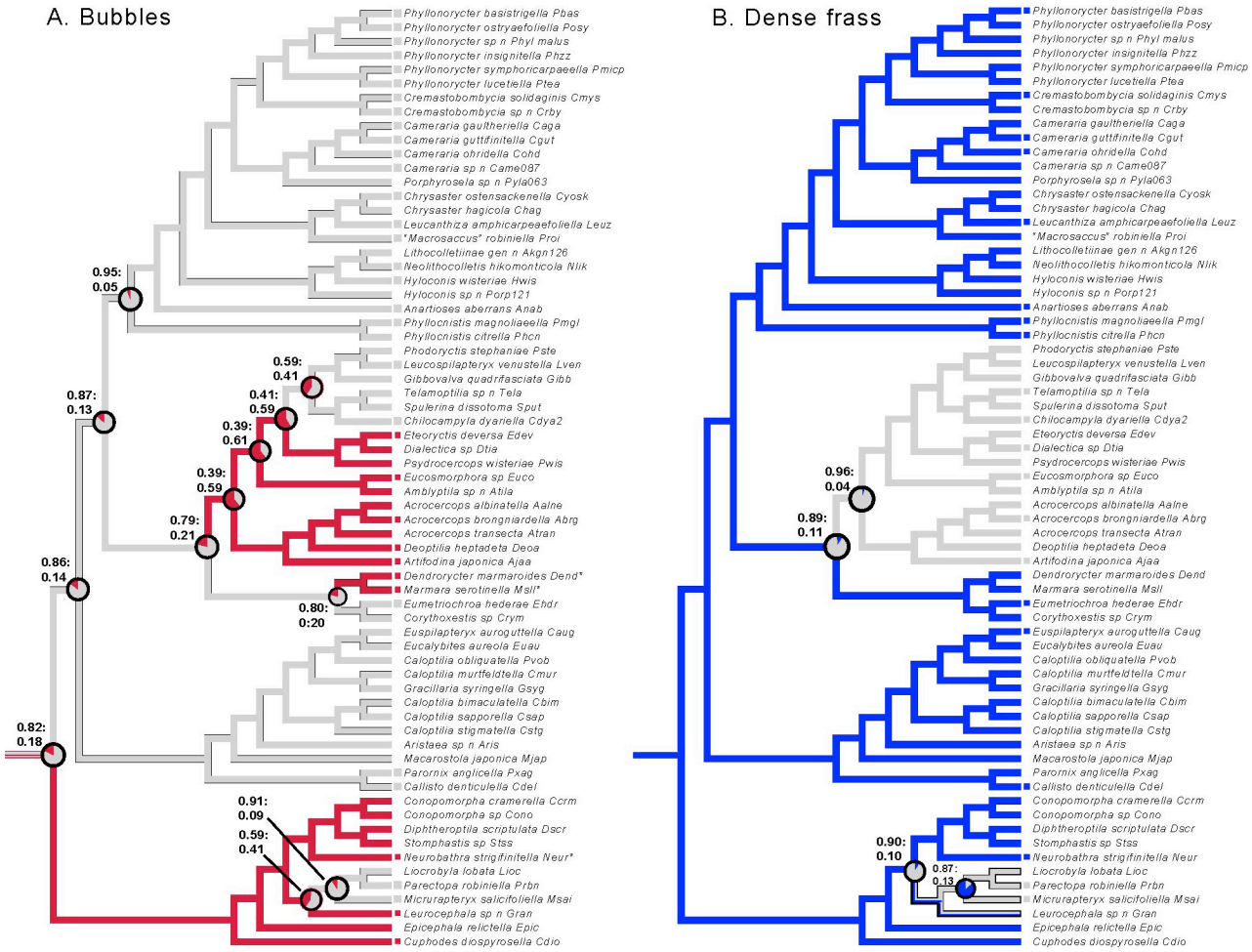


Fig. 3.2. Bubble ornamentation and dense frass deposition mapped onto gracillariid phylogeny. Colored lines indicate the presence of the trait. **A.** Bubble ornamentation. Asterisks indicate taxa that are known to ornament their cocoons with ≥ 10 bubbles. **B.** Dense frass presence in mine.

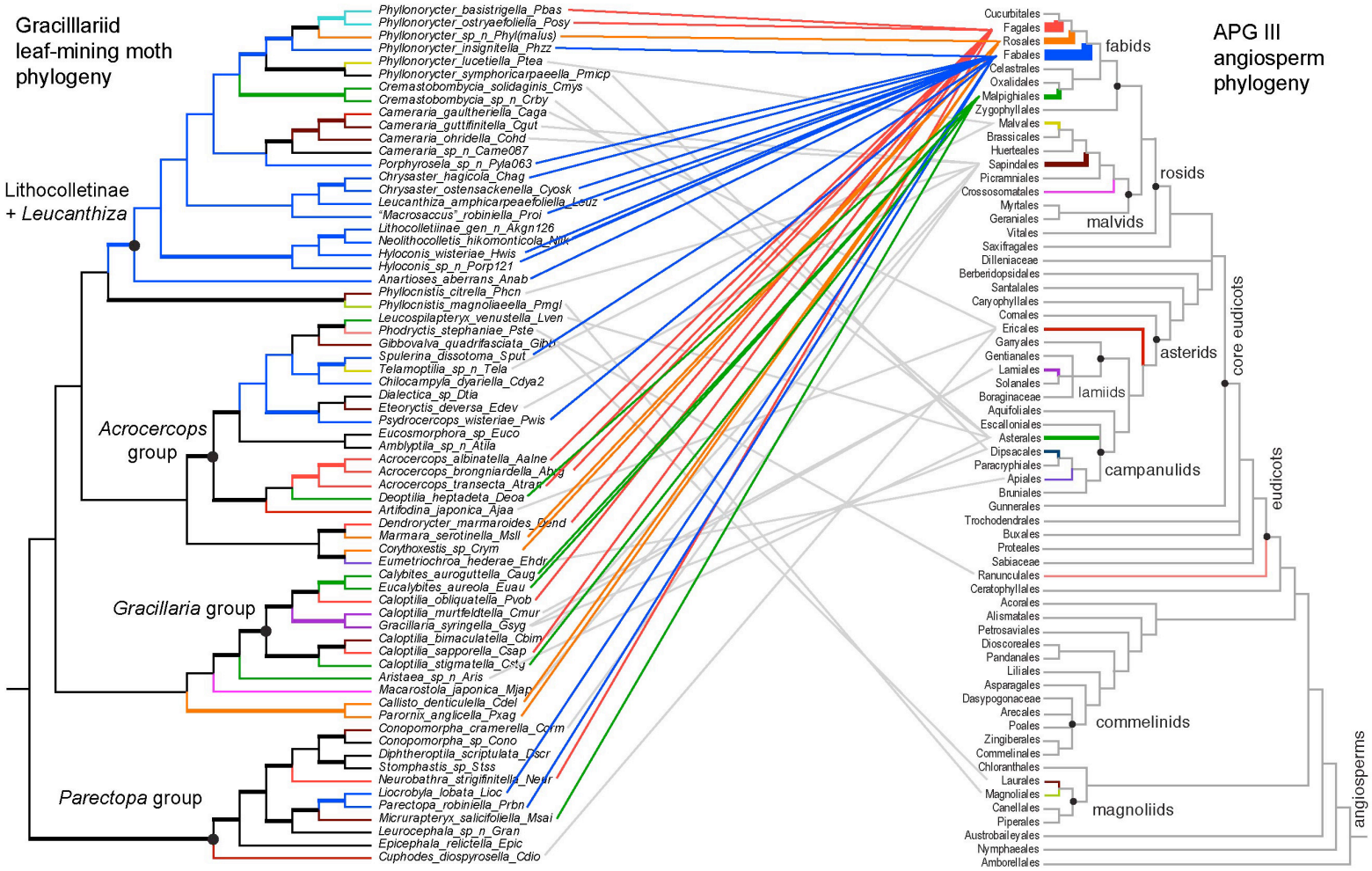


Fig. 3.3. Gracillariid phylogeny and the plant phylogeny of APG III (2009), showing known host associations. Thick branches on moth phylogeny indicate groups with strong (BP > 80%, PP = 1.0) branch support; thick branches on host phylogeny correspond to the number of gracillariid species from the present study that utilizes that plant order. Fabid associations are highlighted in color.

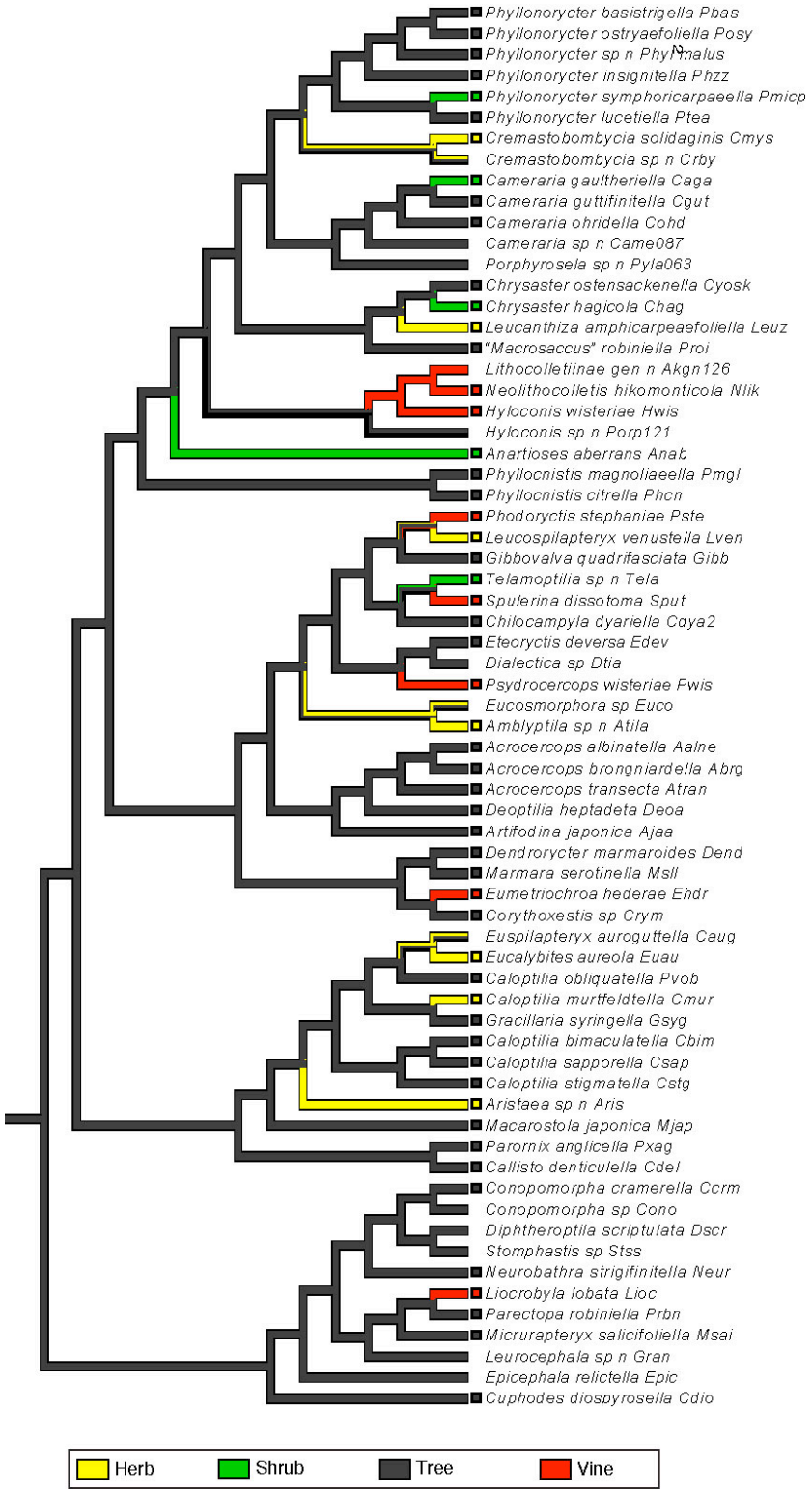


Fig. 3.4. Host plant growth form mapped onto gracillariid phylogeny.

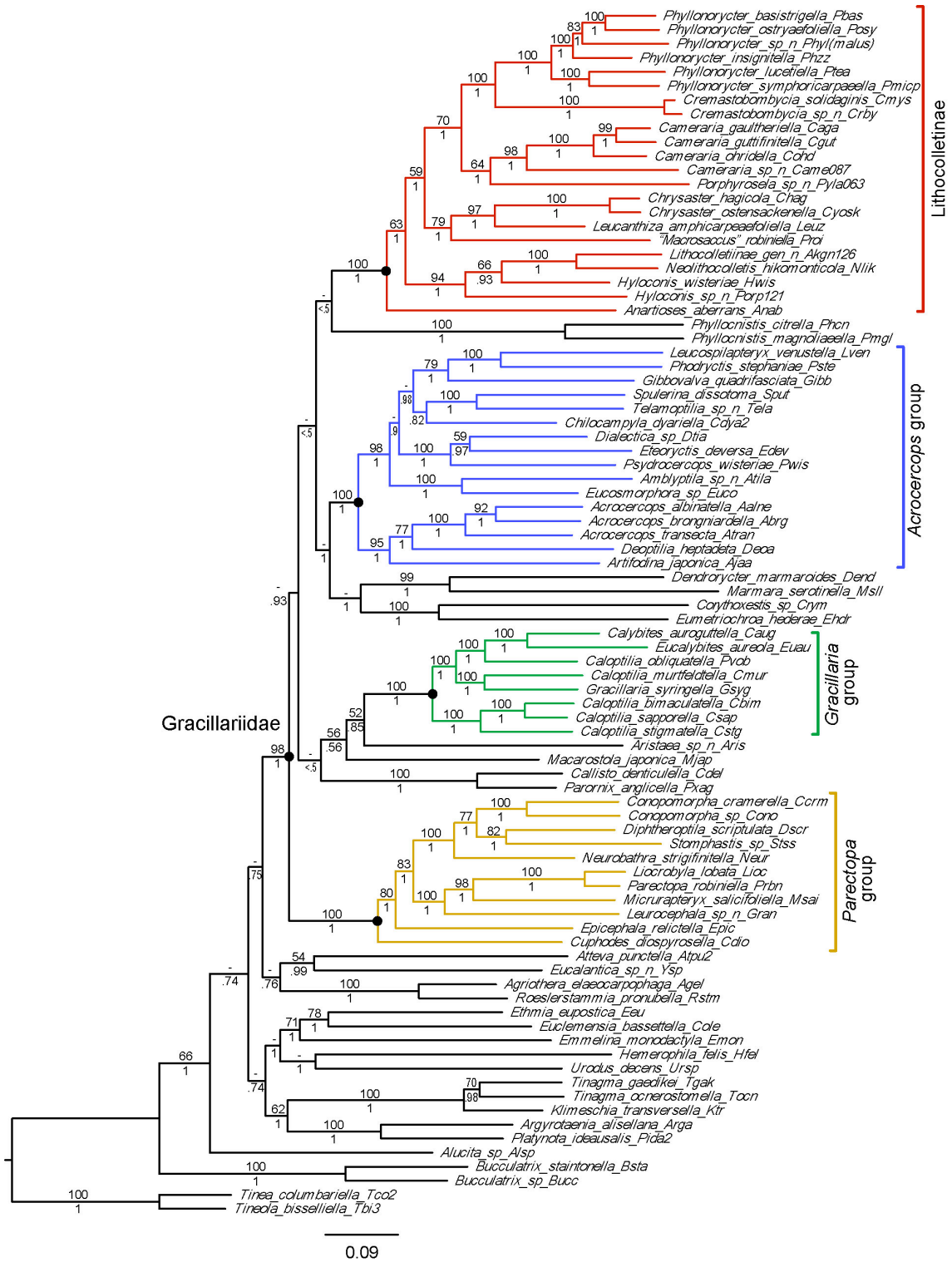


Fig. 3.5. All-nucleotide ML tree showing branch lengths and branch support. Support above branches are bootstrap values, values below are Bayesian posterior probabilities. Five key nodes with strong support are highlighted.

CHAPTER 4

On the taxonomic history of *Phyllocnistis* Zeller 1848 (Gracillariidae)

Abstract

For over 150 years, the proper taxonomic placement of *Phyllocnistis* Zeller has remained largely uncertain. The genus shares morphological and life history traits with several different families of microlepidoptera, and these characteristics have made it challenging for microlepidopterists to correctly place the genus. *Phyllocnistis* includes *P. citrella* Stainton, a globally important economic pest of citrus. We review the taxonomic history of *Phyllocnistis* and provide a comprehensive list of references.

Introduction

The leaf-mining moth genus *Phyllocnistis* Zeller, 1848 has been one of the 'poster-child' examples of a poorly studied genus whose taxonomic placement has vacillated among many different families. Eighty-seven species of *Phyllocnistis* are described worldwide (De Prins and De Prins, 2009; De Prins and De Prins, 2005), 36 species from the Oriental region, 17 from Australasia, 15 from the Palaearctic, and 12 each from the Nearctic and Neotropical regions. Only five are known to occur in the Afrotropical region (De Prins and De Prins, 2009; De Prins and De Prins, 2005). The distribution of most species is restricted to one biogeographical region. However, five species cross biogeographical boundaries: *P. saligna* (Zeller, 1839) occurs in the

Palaearctic, Afrotropical and Oriental regions, *P. selenopa* Meyrick, 1915 in the Oriental and Australian regions, *P. toparcha* Meyrick, 1918 in the Palaearctic and Oriental regions, and *P. vitegenella* Clemens, 1859 has a Holarctic distribution. *Phyllocnistis citrella* Stainton, 1856 has a cosmopolitan distribution. There are currently more than 800 publications on *Phyllocnistis,* most of which focus on the pest species *Phyllocnistis citrella* (Fig. 4.1).

Phyllocnistis is very similar to the lyonetiid genus *Leucoptera* Hübner, [1825] in forewing pattern, but differs in having a smoothly scaled head. Unlike most genera of Gracillariidae, all larval feeding instars of *Phyllocnistis* are sap feeding, creating a long, slender, serpentine, subepidermal mine, that contains a dark median frass line deposited under the leaf epidermis. There are no tissue-feeding instars, hence no granular frass, but only three sap-feeding instars and one non-feeding, highly specialized, spinning instar. The mine terminates in a slightly enlarged cavity, usually near the edge of the leaf in which the last instar constructs a flimsy cocoon and pupates (Emmet, 1985; Parenti, 2000). *Phyllocnistis* is very successful in its ability to exploit a wide range of host plants as it feeds on 26 plant families (Davis, 1987; De Prins and De Prins, 2010). Some species of *Phyllocnistis* (e.g., *P. citrella*) are cosmopolitan, fast spreading pests, causing substantial economic damage (Causton et al., 2006; Davis, 1994; Heppner and Dixon, 1995; Hoy, 1996; Jahnke et al., 2006; Jahnke et al., 2007). The present paper aims to summarize the taxonomic history of *Phyllocnistis.*

Taxonomic history

Zeller (1848) described *Phyllocnistis* (Fig. 4.2) as a genus of "leaf-mining moths with eye caps" placing it just after *Lyonetia* Hübner, [1825]. Soon thereafter, Herrich-Schäffer (1853-1855) placed *Phyllocnistis* in Tineidae, together with many other genera of small Lepidoptera. Stainton, in his lists (1854a; 1854b; 1854c; 1859), placed *Phyllocnistis* in the family Lyonetidae [sic], and this was followed by Frey (1856) and Wocke (1861; 1871). According to Stainton (1854a) the family Lyonetiidae contained five genera*: Bucculatrix* Zeller, 1839, *Cemiostoma* Zeller, 1848, *Lyonetia* Hübner, [1825], *Opostega* Zeller, 1839, and *Phyllocnistis* Zeller, 1848. However, in his lecture of 7 January 1856 to the Entomological Society of London, Stainton (1856) presented '*Phyllocnistis citrella* Atkinson in litt.' as a new species of Indian Microlepidoptera feeding on *Citrus*. Stainton did not place this global economic pest into any of the then recognized lepidopteran families. He only indicated that the new species is similar to the European *Phyllocnistis saligna* (Zeller, 1839) and *suffusella* (Zeller, 1847). Wocke (1861) added *Phyllobrostis* Staudinger, 1859 to the list of Lyonetidae [sic] and later (1871) added *Opogona* Zeller, 1853. At about the same time, Herrich-Schäffer (1857) recognized Phyllocnistina as a separate group, and included three genera into it: *Bucculatrix, Cemiostoma,* and *Phyllocnistis.* On the basis of wing venation, Clemens (1859) transferred *Phyllocnistis* into Lithocolletidae, together with *Leucanthiza* Clemens, 1859, *Lithocolletis* Hübner, [1825], and *Tischeria* Zeller, 1839. Clemens (1859) placed these four genera in Lithocolletidae, but noted that his classification was in contrast to European authors who treat *Leucanthiza* and *Tischeria* as Lyonetidae [sic]. Unfortunately, Clemens did

not indicate who the European authors were. Clemens also stated that he did not support the separation of these four genera into distinct families. At that time *Phyllocnistis* was placed in Tineina, which included many different genera of small moths (Chambers, 1875; Clemens, 1863; Frey and Boll, 1876; van Deventer, 1904; Zeller, 1873; Zeller, 1877). Stainton (1863) summarized the generic characters of twenty genera of leaf-mining Lepidoptera. He placed *Phyllocnistis* in a group with *Bucculatrix* Zeller, 1839, *Cemiostoma* Zeller, 1848, *Lithocolletis* Hübner, [1825], *Lyonetia* Hübner, [1825], and *Nepticula* Heyden, 1843. All genera except *Bucculatrix* share a mining larva and *Lithocolletis* and *Phyllocnistis* pupate within the mine (Stainton, 1863). Chambers (1871) noted that the larva of *Phyllocnistis* resemble the young cylindrical larva of *Lithocolletis* in general appearance and compared adult *Phyllocnistis* with the white species of *Lithocolletis*. In his work on Australian Microlepidoptera, Meyrick (Meyrick, 1880`: 136) made an attempt to classify the species he was describing and placed *Phyllocnistis* into Lyonetidae [sic], and stated "[*Phyllocnistis*] appears by its quite smooth head and apodal larva to be an extreme development of [*Opostega* and *Cemiostoma*]". Heinemann and Wocke (1877) discriminated Phyllocnistidae as a separate family and included three genera within: *Phyllocnistis*, *Cemiostoma*, and *Bucculatrix*.

Even at the turn of the century, the definition and placement of *Phyllocnistis* differed among microlepidopterists. Noting similarities in early stages and habits of the American species, Busck (1900) proposed to broaden the definition of *Phyllocnistis.* He described *P. intermediella* from Florida*,* which has morphological

features that are somewhat different from the species that had previously been described in the genus. Rebel (1901) allocated *Phyllocnistis* to the subfamily Phyllocnistinae along with *Bucculatrix* Zeller, 1839, *Cemiostoma* Zeller, 1848, *Opogona* Zeller, 1853 and *Opostega* Zeller, 1839, but placed Phyllocnistinae into family Lyonetiidae. Kirby (1903) divided Lyonetiidae into two subfamilies: Lyonetiinae and Phyllocnistiinae [sic]. Meyrick (1895) transferred *Phyllocnistis* to Tineidae and in 1906 he placed it along with *Epicnistis* Meyrick, 1906, *Exorectis* Meyrick, 1906, *Leucoptera* Hübner, [1825], *Nepticula* Heyden, 1843, and *Setomorpha* Zeller, 1852. Spuler (1910) recognized three species of *Phyllocnistis*, *P. suffusella* Zeller, 1847, *P. sorhageniella* Lüders, 1900 and *P. saligna* (Zeller, 1839) and placed the genus in its own family Phyllocnistidae. Meyrick (1915a; 1915b) continued to include *Phyllocnistis* in Lyonetiidae, which he spelled in different ways (Meyrick, 1915a; Meyrick, 1915b; Meyrick, 1916; Meyrick, 1920; Meyrick, 1921). Other authors followed to include *Phyllocnistis* in Lyonetiidae (e.g. Braun, 1925; Turner, 1923). Braun and Meyrick independently[1] transferred *Phyllocnistis* from Lyonetiidae to Gracillariidae (Braun, 1927; Meyrick, 1928a; Meyrick, 1928b; Meyrick, 1935; Meyrick, 1936), and such a placement has since been widely accepted (Davis and Robinson, 1998; Nye and Fletcher, 1991; Turner, 1947). However, some authors have treated *Phyllocnistis* as a separate family until recently (Emmet, 1985; Kuznetzov and Stekol'nikov, 1987; Seksjaeva, 1981).

Placement of *Phyllocnistis* in Phyllocnistinae

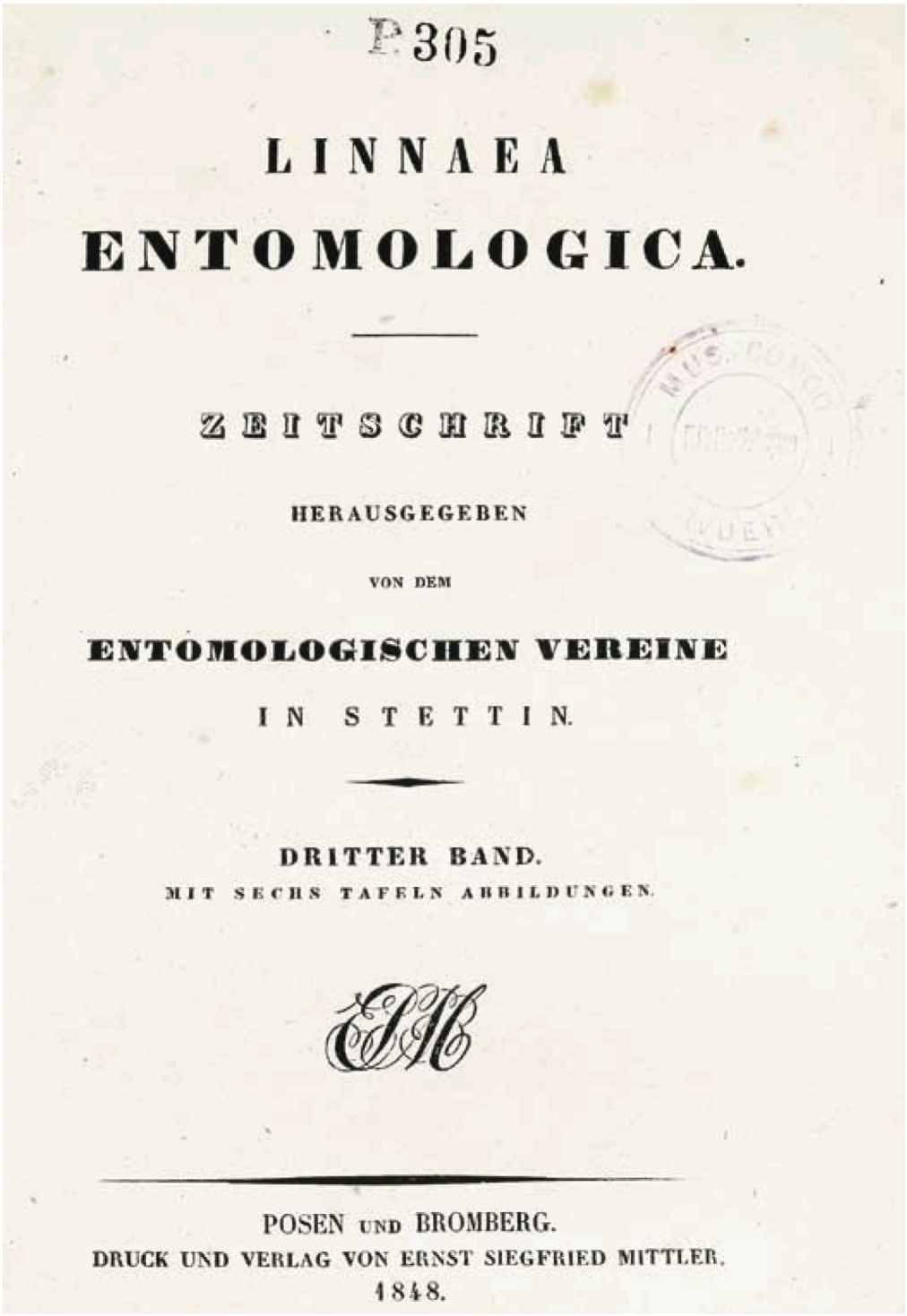
Most modern authors divide Gracillariidae into three subfamilies: Gracillariinae, Lithocolletinae and Phyllocnistinae (Common, 1990; Dall'Asta et al., 2001; Davis, 1983; Davis and Miller, 1984; Davis and Robinson, 1998; De Prins and De Prins, 2005; Heppner, 2004; Kuznetsov and Baryshnikova, 1998; Parenti, 2000). However, some other authors have proposed to erect additional subfamilies: Oecophyllembiinae (Kumata, 1998; Réal and Balachowsky, 1966), Ornichinae (Kuznetzov and Stekol'nikov, 1987); misspelled as 'Orniginae' (Kuznetsov and Stekol'nikov, 2001; Kuznetzov and Baryshnikova, 2001)), and Ornixolinae (Kuznetzov and Baryshnikova, 2001). In the checklist of the Moths of America North of Mexico, Davis (Davis, 1983) included *Phyllocnistis* Zeller, 1848 and *Metriochroa* Busck, 1900 in Phyllocnistinae, while Kuznetsov (1981) considered *Metriochroa* Busck, 1900 belonging to Gracillariinae. Later Davis and Robinson (Davis and Robinson, 1998) included *Cryphiomystis* Meyrick, 1922, *Metriochroa* Busck, 1900, *Phyllocnistis* Zeller, 1848 and *Prophyllocnistis* Davis, 1994 in Phyllocnistinae. Kumata (1998) then transferred all but *Phyllocnistis* to Oecophyllembiinae based on hindwing venation and position of the larval thoracic spiracles. In the classification and checklist of the Lepidoptera species recorded in southern Africa, Vári *et al*. (2002) treated Oecophyllembiinae as a synonym of Phyllocnistinae and included *Cryphiomystis* Meyrick, 1922, *Metriochroa* Busck, 1900 and *Phyllocnistis* Zeller, 1848 into Phyllocnistinae. De Prins & De Prins (2010; 2005) recognized seven genera in Phyllocnistinae: *Angelabella* Vargas & Parra, 2005, *Corythoxestis* Meyrick, 1921b, *Eumetriochroa* Kumata, 1998, *Guttigera* Diakonoff, 1955, *Metriochroa* Busck, 1900,

Phyllocnistis Zeller, 1848, and *Prophyllocnistis* Davis, 1994. It still remains largely uncertain whether these groups are monophyletic, and we hope that future phylogenetic studies based on morphological and molecular characters of Gracillariidae will shed light on the phylogenetic position of *Phyllocnistis*, and its placement in the classification of Gracillariidae.

Footnote:

[1] Although the publication of Braun (1927) preceded the publication of Meyrick (1928b), we consider that both authors came to the conclusion to include *Phyllocnistis* into Gracillariidae independently and at the same time. Braun (1927) published the description of *Phyllocnistis finitima* Braun, 1927, which she placed into Gracillariidae. Meyrick (1928b) significantly revised his monumental monograph of 914 pages, which includes the identification keys of genera, species, illustrations of wing venation and short species descriptions. He discriminated six genera within Gracillariidae: *Acrocercops* Wallengren, 1881, *Gracilaria* [sic] Haworth, 1828, *Lithocolletis* Hübner, 1825, *Ornix* Treitschke, 1833, *Parectopa* Clemens, 1860, and *Phyllocnistis* Zeller, 1848. The preface of his revised handbook was written on 28th September 1927, the same year as the paper of Braun (1927) was published. We believe both lepidopterists communicated with each other on the placement of *Phyllocnistis.*

Fig. 4.1. *Phyllocnistis citrella* Stainton. Italy, Piemonte, Asti, fraz. Valgera, 120 m, 2–15.11.2002, e.l. *Citrus* sp., leg. G. Baldizzone, coll. MHNG.



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LINNAEA

ENTOMOLOGICA.

ZEITSCHRIFT

HERAUSGEGEBEN

VON DEM

ENTOMOLOGISCHEN VEREINE

IN STETTIN.

DRITTER BAND.

MIT SECHS TAFELN ABBILDUNGEN.

POSEN UND BROMBERG.

DRUCK UND VERLAG VON ERNST SIEGFRIED MITTLER.

1848.

Fig. 4.2. The text of the original description of *Phyllocnistis* Zeller in Linnaea Entomologica. Zeitschrift herausgegeben von dem Entomologischen Vereine in Stettin 3 (1848).

CHAPTER 5

Systematics, host plants, and life histories of three new *Phyllocnistis* species from the central highlands of Costa Rica (Lepidoptera, Gracillariidae, Phyllocnistinae)

Abstract

Three new species of *Phyllocnistis* Zeller are described from the central highlands of Costa Rica: *Phyllocnistis drimiphaga* sp. n., *P. maxberryi* sp. n., and *P. tropaeolicola* sp. n. Larvae of all three are serpentine leaf miners. *Phyllocnistis drimiphaga* feeds on *Drimys granadensis* (Winteraceae), *P. maxberryi* on *Gaiadendron punctatum* (Loranthaceae), and *P. tropaeolicola* on *Tropaeolum emarginatum* (Tropaeolaceae). All specimens were collected as larvae or pupae in their mines and reared in captivity. Parasitoid wasps were reared from *P. drimiphaga* and *P. maxberryi*. Description of the adults, pupae, and life histories are supplemented with photographs, illustrations, and scanning electron micrographs.

Introduction

Phyllocnistis Zeller includes 87 described species, many of which are very small, with silvery vestiture, and similar in appearance (De Prins and De Prins, 2005[De Prins, 2009 #497). The genus has been generally poorly studied because of its small size and difficulty to identify species. The precise taxonomic placement of the genus has also remained questionable because of a lack of shared adult

morphological characters with other microlepidoptera (De Prins and Kawahara, 2009).

Only two species of *Phyllocnistis* were known to occur in Costa Rica (De Prins and De Prins, 2009; De Prins and De Prins, 2005), one of which is citrus leaf miner, *P. citrella* Stainton, 1856, and the other, the mahogany leaf miner, *P. meliacella* Becker, 1974. *Phyllocnistis citrella*, originally from the Old World, was first reported in the Americas in 1993 (Heppner, 1993) and has since become established in nearly every major citrus growing region in the New World. The larva of *citrella* is restricted to the plant family Rutaceae, and the larva of *meliacella* is known to feed only on members of the Meliaceae.

The larva of *Phyllocnistis* is unusual in having three or more sap-feeding instars and one non-feeding, highly specialized cocoon-spinning instar (Davis, 1987). The larva creates a long, slender, subepidermal serpentine mine with a characteristic median frass line at the terminus of which a pupal chamber (pupal cocoon fold) is constructed, usually from the curled edge of the leaf (Davis, 1994). On the basis of its unique mine, a phyllocnistine fossil has been identified as the oldest fossil in the Ditrysia, dated from leaf impressions from the Cretaceous (Grimaldi and Engel, 2005; Labandeira et al., 1994), the bedrock which was recently reevaluated to be ~ 102 million years Ma (Brenner et al., 2000).

In general, larval morphological characters poorly define species of

Phyllocnistis. From our experience rearing North American *Phyllocnistis* with David Wagner and others, pupal morphology provides the most informative characters for distinguishing species in the genus. In particular, we have found the shape of the frontal ridge (cocoon-cutter) and hooks on the dorsal surface of the abdominal segments to be very useful. These structures are respectively used to cut the cocoon and anchor it during adult emergence. We describe the adults, pupae, and life histories of the three new species of *Phyllocnistis* found in the central highlands of Costa Rica.

Methods

Study sites and habitats. Field studies were conducted at four high elevation sites between 1950–3100 m in the central region of Costa Rica during July 2001, April–May and November 2002, February–April 2003, December 2003–January 2004, March–April 2004, May 2005, September 2008, and July 2009. Three sites were located on Cerro de la Muerte, in the northern to central region of Cordillera de Talamanca (Fig. 5.1A). This region is cold and humid with 1–2 months of dry season (Herrera and Gómez, 1993). According to Kappelle (1996), annual rainfall ranges from 2000 to 3500 mm and average daily temperature is 11°C, with temperatures at night occasionally falling below 0 °C during the dry season. Sleet and heavy frost has been observed at Mills region (Oscar Abarca, pers. comm.). One of the sites on Cerro de la Muerte was near Villa Mills, at the 95 km mark of the Pan-American Highway (09°33'30.0"N, 083°43'25.8"W, 3100 m; Fig. 5.1, H). Another site was near the road leading to El Paraíso del Quetzal at the 70 km mark of the Pan-American Highway

(2774 m, 09°33'45.6"N, 083°50'50.1"W; Fig. 5.1C). This road divides Parque Nacional Tapantí-Macizo de la Muerte and Parque Nacional Los Quetzales/Reserva Forestal Los Santos of San José Province. The third site on Cerro de la Muerte was on the road to the Genesis II Cloud Forest Preserve, 4 km NE of La Cañón in Cartago Province (09°42'23.4"N, 083°54'35.9"W, 2385 m).

The fourth site was in Cordillera Volcánica Central, 6 km ENE of Vara Blanca, part of Volcán Barva in Parque Nacional Braulio Carrillo (10°10'51"N, 084°06'20"W, 1950–2050 m; Fig. 5.1B). This collecting site was near the edge of a swampy open field and oak forest. The weather of this locality is consistently cool and humid throughout the year (Herrera and Gómez, 1993). Typical weather at this site is rainy and windy, with a few hours of daily sunshine and temperatures ranging from 5–11 °C (Nishida, 2006).

Leaf mine sampling and rearing. Leaf mines were collected and placed in transparent plastic bags or vials and larvae were reared at Universidad de Costa Rica, San José (1200 m elevation). Each day, mines were placed in a refrigerator (7.0–8.0 °C) and transferred to ambient temperature (~ 20 °C) to simulate natural conditions at high elevations. Reared parasitoids and samples of the mature larva and pupa of each species were preserved in 75–80 % EtOH. Adult moths were pinned, spread, and doublemounted. All adult specimens in this study were obtained from reared immatures.

Photography and dissection. Photographs of leaf mines were taken primarily in the field using Nikon Coolpix 4500, 8700, and Canon G7 digital cameras. Some pupae were dried and sputter-coated with a 60:40 mixture of gold-palladium for examination with a scanning electron microscope (SEM). SEM photographs were taken using an Amray 1810 SEM with a lanthanum hexaboride (LaB6) source at an accelerating voltage of 10 kV. Illustrations of the genitalia were sketched with a camera lucida attached to a stereomicroscope.

Type deposition, nomenclature, and diagnosis. Type specimens are deposited in the United States National Museum of Natural History, Smithsonian Institution (USNM), Museo de Zoología, Escuela de Biología, Universidad de Costa Rica (UCR), and Instituto Nacional de Biodiversidad, Santo Domingo de Heredia, Costa Rica (INBio). Scientific names of plants follow Missouri Botanical Garden (2009). Adult wing pattern nomenclature is explained in Fig. 5.3; diagnostic features of the three species are summarized in Table 5.1.

Adult, pupa, and life history descriptions

Phyllocnistis drimiphaga Kawahara, Nishida & Davis, sp. n.

Diagnosis (Table 5.1). *Phyllocnistis drimiphaga* is similar to *P. maxberryi*, but is larger and has slender, sharply angled costal fascia, V-shaped transverse fascia, three costal strigulae, and dissimilar signa. *Phyllocnistis drimiphaga* differs from *P. tropaeolicola* in having broad longitudinal fascia, genital valva that are only ~ 1.8×

the length of the vinculum, and paired signa. The pupa has curved, flattened frontal processes, which are reduced in *P. maxberryi* and conical in *P. tropaeolicola*.

Adult (Fig. 5.2A). Forewing length 2.9–3.5 mm. Head. Vestiture consisting of smooth, broad, silvery-white scales that overlap anterior margin of eye. Antenna ~ equal to length of forewing, scape and pedicel enlarged laterally and covered with lanceolate scales, a single row of fine short scales completely encircling each flagellomere. Labial palpus long, slender, ~ 1.0 mm in length, covered with lustrous white scales. Thorax. Forewing silvery white; with a long pale yellowish-orange longitudinal fascia with dark-gray margins extending 2/3 length of forewing slightly diagonal from base of costa to strongly oblique, costal fascia of similar color across distal third of wing; apex of forewing with three slender, fuscous, costal strigulae; apical to subapical pale yellowish orange bordered by gray; three apical, fuscous strigulae arising from small black apical spot, and one tornal, fuscous strigula also from apical spot; ventral surface mostly dark brown. Hindwing creamy white. Legs mostly silvery white; foretibia fuscous dorsally; foreand mid-tarsomeres lightly suffused with cream scales dorsally. Abdomen. Length ~ 2.0 mm, covered in long silver scales. Coremata present on segment VIII of male, consisting of a pair of elongate, inflatable tubular extensions bearing a terminal cluster of long slender scales (Fig. 5.4A). Male genitalia (Figs 5.4A–C). Uncus absent; tegumen complex, consisting of a narrow, sclerotized dorsal arch, continuing caudally, often slightly beyond apex of valva, as an elongate, mostly membranous, basally spinose cylinder that encloses the anal tube; vinculum well developed, ~ 0.6× length of valva, to V-

shaped with relative narrow anterior end; valva (Fig. 5.4B) relatively long, ~1.8× length of vinculum, generally slender with a moderately broad base, very slender for most of its length, then broadening apically to form a prominent dorsal lobe and a smaller ventral lobe (Fig. 5.4A); transtilla arising from mesal base of valva as an elongate, acute process, and continuing mesally to articulate at midline with process from opposite valva. Aedeagus (Fig. 5.4C) slender, weakly sclerotized, externally finely wrinkled cylinder, ~ equal to length of valva; cornuti absent; phallobase greatly extended as a membranous tube ~ 1.7–2.0× length of aedeagus; terminal hood of phallobase abruptly inflated and curved at right angle to phallobase. Genitalia slide USNM 33208. Female genitalia (Figs 5.4D, E). Oviscapt greatly reduced; posterior apophyses very short, ~ 0.8× length of papillae anales; anterior apophyses slightly longer, ~ 1.3× length of posterior apophyses; ostium bursae opening in membrane between sterna 7 and 8; ductus bursae completely membranous, slender, elongate, over 7.5× length of papillae anales and terminating near caudal fifth of corpus bursae; corpus bursae greatly enlarged, ~ 0.7× length of ductus bursae; walls of corpus bursae membranous except for a pair of ligulate and very dissimilar signa; longest signum ~ 3× length of shorter member and with 5 short, acute to rounded, flattened spines projecting from one side of signum; shorter signum with a single, blunt, flattened, rounded spine projecting from middle; length of spines ~ equal to width of signa; ductus seminalis extremely slender, elongate, ~ 2.3× length of corpus bursae and arising from anterior end of corpus bursae. Genitalia slides USNM 33207, 33273.

Larva (Figs 5.10F, G). Mature sap-feeding larva ~ 6.5 mm long, yellowish

white, head capsule translucent pale brown (Fig. 5.10F). Last instar (cocoon-spinning) larva yellowish white, head capsule yellowish white; ~ 6.2 mm long (Fig. 5.10G).

Pupa (Figs 5.7, 10I). Dark brown, up to ~ 3.8 mm long, diameter ~ 0.75 mm. Vertex with a stout, triangular frontal process (cocoon-cutter) transversed by a pair of shorter, curved spines (Figs 5.7A–E), and single pair of long setae at base of frons (Fig. 5.7C). Dorsum of A2–A7 with a pair of curved, large spines, arranged roughly in the shape of a V, in between which is a concentration of smaller spines projecting posteriorly (Figs 5.7F–H); each segment with a pair of long, lateral, sensory setae (Fig. 5.7K). A10 prominently furcated (Figs 5.7I, J, L), with a pair of slightly divergent acute processes from caudal apex. Pupal slide USNM 34034.

Types. Holotype (Fig. 5.2A): ♀, COSTA RICA: Prov. Heredia, 6 km ENE Vara Blanca, 2050 m, 10°10'34"N, 084°06'41"W, 27 Jan 2004, adult emergence, INBio-OET-ALAS transect, col./rear Kenji Nishida, pupa collected 30 Dec 2003, host plant *Drimys granadensis*. Leaf miner on underside (USNM). Paratypes: Immatures: Prov. Cartago: Cerro de la Muerte, La Cañón, Genesis II Cloud Forest Preserve, 2422 m, 09°42'23.4"N, 83°54'36.1"W: 2 sap-feeding larvae, 1 pupa, 12 Sep 2008, Kenji Nishida, host *Drimys granadensis*; Prov. San José: Cerro de la Muerte, Paraíso del Quetzal: 2 pupae, USNM 34034. Adults: same locality as holotype: 1♂, 26 Jan 2004, USNM 33208; 1♀, 26 Jan 2004, USNM 33207; 1♂, 1♀ (USNM 33273), 28 Jan 2004. 1♀ adult paratype at INBio and UCR, the remaining paratypes at USNM.

Life history (Fig. 5.10). Mines are narrow, long, and serpentine, with a brown median frass line (Figs 5.10A, C, D) covering most areas of the leaf on small leaves (< 6 cm) or half the area in larger leaves. Mines were found on relatively young leaves near the apex of branches, from branches close to the ground up to ~ 3 m on young trees, along shaded areas of forest trails (Fig. 5.1C) or in the understory. We observed 43 of 48 mines on the abaxial side of the leaf (Fig. 5.10A), and the remaining mines on the adaxial side (Fig. 5.10D). Most mines were singly found on a leaf; however seven of 38 mined leaves carried two mines, either two on the abaxial side or one on both sides. All but one adaxial mine began near the mid-vein and extended along it (Fig. 5.10D). Mature mines are yellowish green in color (Fig. 5.10C). Mining on small, soft, young leaves frequently caused the leaf margin to curl. We were unable to study the upper canopy for leaf mines.

Early stage mines were typically in the shape of a whorl (Figs 5.10A–C). Flat, oval egg shells were found attached to the leaf surface in the middle of an early mine whorl (Fig. 5.10B). A pupal cocoon fold (~ 6.5 mm long), typical of *Phyllocnistis*, was found along leaf margins (Figs 5.10A, H, J) both on the adaxial (Fig. 5.10H) and abaxial sides (Figs 5.10A, J).

In 70 examined mines, only 20 had a live larva or pupa. Remaining mines either were empty or contained dead, early to middle stage sap-feeding larvae. Mortality of sap-feeding stages was most likely caused by desiccation after rupturing of the

epidermal layer and by a cf. *Ceraphron* (Ceraphronidae) parasitoid wasp. In some pupal folds, a pupal shell of an entedonine wasp (Eulophidae) was found with a shrunken *P. drimiphaga* pupal shell. In others, cocoons of *Ageniaspis* sp. (Encyrtidae) were found in a last instar (cocoon-spinning) larval pelt (Fig. 5.10K).

We also discovered active mines of *Marmara* sp. (Gracillariidae) on the abaxial side of same host along the road to El Paraíso del Quetzal. Compared to those of *P. drimiphaga*, mines were much narrower, whiter, less serpentine, and were typically found near leaf margins.

Host. *Drimys granadensis* L. f. (Winteraceae) (Fig. 5.1D). *Drimys* Foster & Forster is the only genus in the family Winteraceae found in the New World tropics (Doust and Drinnan 2004). All other genera of Winteraceae are found in the Old World southern hemisphere with a center of diversity in Southeast Asia (Gentry, 1996; Hartshorn, 1983). *Drimys granadensis*, commonly known as 'chilemuelo' or 'quiebra muelas', has been recorded from central Mexico (~20°N) south through Central America to northern Peru (~ 5°S) (Missouri_Botanical_Garden, 2009). Trees grow to nearly 15 m in height and are characterized by pepper-flavored leaves with white underside surfaces and aromatic bright, white flowers (Fig. 5.1E), found mostly in primary forest (Alfaro-Vindas, 2003). In Costa Rica, the species has been recorded between 1100 and 3700 m elevations on both Pacific and Atlantic slopes. Large young leaves are pale green color, sized ~ 10–15 cm long and 2–4 cm wide (Kenji Nishida, pers. obs.).

Distribution. Known only from cloud forests above 2000 m in Cordillera de Talamanca and Cordillera Volcánica Central. More specifically, specimens have been collected from Heredia Province, 6 km ENE of Vara Blanca; San José Province, Cerro de la Muerte, Paraíso del Quetzal; and Cartago Province, Cerro de la Muerte, Genesis II Cloud Forest Preserve. In February 2009, several additional old leaf mines were observed in Chirripó National Park along the main trail between 2200 and 2700 m elevation.

Etymology. The species name, *drimiphaga*, comes the host plant genus, *Drimys*, and the Greek word phaga, meaning "to eat".

Phyllocnistis maxberryi Kawahara, Nishida & Davis, sp. n.

Diagnosis (Table 5.1). *Phyllocnistis maxberryi* differs from *P. drimiphaga* and *P. tropaeolicola* in having an oviform costal fascia with a broad margin, a C-shaped transverse fascia, two costal strigulae, and paired signa that are similar in shape. Unlike *drimiphaga* and *tropaeolicola*, the pupa of *maxberryi* has less developed frontal processes and two parallel rows of spines on the dorsal surface of abdominal segments. Of the three new *Phyllocnistis* species proposed in this paper, *P. maxberryi* is morphologically most similar to *P. meliacella* Becker. *Phyllocnistis maxberryi* may be distinguished from the latter by its broader apex of the valva and proportionately larger signa.

Adult (Fig. 5.2B). Forewing length 2.2–3.7 mm. Head. Vestiture silvery white, completely covered with smooth, broad, scales that overlap anterior margin of eye; occipital scales cream. Antenna ~ equal or slightly longer than length of forewing, scape and pedicel enlarged laterally and covered in long silvery scales, a single row of slender mostly silvery-white scales completely encircling each flagellomere; dorsal surface of antenna with a pale-golden luster. Labial palpus slender, ~ 0.5 mm in length, with silvery-white scales. Thorax. Forewing silvery white, with a single, broad, light-brown longitudinal fascia with a dark brown posterior margin extending slightly diagonal from base of costa joining costal fascia at ~ midway to apex; costal fascia oblique, pale gold, oviform, with a broad, inner dark-brown margin; transverse fascia C-shaped, pale gold with dark margin; apical to subapical area pale yellow; two faint, dark-brown costal strigulae present; a single, small black spot at wing apex from which two dark-brown apical strigulae arise. Hindwing silvery white. Legs mostly silvery white, with a faint suffusion of pale gold dorsally over most segments. Abdomen. Length ~1.5–2.0 mm, silvery white; coremata similar to *P. drimiphaga*. Male genitalia (Figs 5.5A–C). Similar to *P. drimiphaga* except vinculum relatively broader and more U-shaped. Valva ~ 2× length of vinculum, nearly straight with apex only slightly enlarged (Fig. 5.5A). Genitalia slide USNM 33279. Female genitalia (Figs 5.5D–F). Oviscapt greatly reduced as in *P. drimiphaga*; ductus bursae completely membranous, slender, elongate, over 12× length of papillae anales and terminating near middle of corpus bursae; corpus bursae greatly enlarged, ~ 0.7× length of ductus bursae; signa paired, closely similar in shape and size (fusiform), with more posterior signum ~ 1.2–1.5× longer than anterior signum; each signum

with a single, acute, flattened spine projecting from middle (Fig. 5.5F); length of spines slightly more than width of signa; ductus seminalis extremely slender, elongate, ~ 1.9× length of corpus bursae and arising from anterior end of corpus bursae. Genitalia slides USNM 33280, 33286.

Larva (Figs 5.11C–F). Mature sap-feeding larva ~ 6.0 mm long, translucent orange, head capsule brown, prothoracic shield brown (Figs 5.10C–E). Last instar (cocoon-spinning) larva orange, head capsule orange, ~ 6.3 mm long (Fig. 5.10F).

Pupa (Figs 5.8; 11H, I). Brown, length up to ~ 4.0 mm; diameter ~ 0.85 mm. Vertex with a long, dorsally curved, spine-like process (cocoon-cutter) (Figs 5.8A, B, D, E), and two pairs of short setae (Fig. 5.8C). Dorsum of A2–A7 with a pair of laterally curved, large spines in between which is a concentration of smaller spines, projecting posteriorly that are roughly arranged in two parallel rows (Figs 5.8F–H); each segment with a pair of long, lateral, sensory setae (Fig. 5.8K). A10 with a pair of slightly divergent processes from caudal apex (Figs 5.8I, J, L).

Types. Holotype (Fig. 5.2B): ♀, Costa Rica: Prov. San José, Cerro de la Muerte, Villa Mills, 3100 m, 13 Mar 2003 (adult emergence), host *Gaiadendron* punctatum, upper epidermis leaf miner, col./rear Kenji Nishida, DRD 4474 (USNM). Paratypes: Immatures: same locality as holotype: 3 pupae (USNM 33732), 5 Mar 2003, K. Nishida; 3 larvae, 2 pupae, 2 Apr 2003, K. Nishida; 1 larva, 21 May 2002, K. Nishida; 3 larvae, 1 pupa (USNM 34024), 10 Mar 2004, K. Nishida. One pupa,

Villa Mills, trail front of La Georgina, 3103 m, 12 Sep 2008, K. Nishida, host *Gaiadendron* punctatum. Two larvae, 1 pupa, Prov. Heredia, 6 km ENE Vara Blanca, 10°11'N, 84°07'W, 2050 m, 10 May 2005, K. Nishida; 1 pupa, 23 Nov 2002, K. Nishida. Adults: same locality as holotype: 1♂, 22 Mar 2003, K. Nishida; 2♂, 26 Mar 2003, K. Nishida; 2♂, 2♀, Prov. Heredia, 6 km ENE Vara Blanca, 10°11'N, 84°07'W, 1950–2050 m, 2 Feb 2003, K. Nishida; 2♂, 9 Apr 2002, 1900 m, emerged 22–28 Apr 2002, host *Gaiadendron* punctatum, D. and M. Davis. ♂ slide USNM 33279; ♀ slides USNM 33280, 33286. One paratype, unknown sex (missing abdomen) at UCR, remaining paratypes at USNM.

Life history (Fig. 5.11). Active mines were found on fully open young leaves near the tip of a branch. The smallest leaf with an active mining larva measured 12 × 30 mm. Mines were generally found on young plants about 30 cm to 1.5 m tall, in open fields or along exposed dirt roads or trails. In an open swampy field at the ALAS transect near Vara Blanca, many active mines were found on new leaves on young plants less than 1.5 m tall (Fig. 5.11A) and very few active mines were found on larger plants bearing flowers or fruit.

Thirty-six of 42 leaves had mines on the adaxial side and the rest had mines on the abaxial side or on both. Up to three mines were observed on a single leaf. These mines were relatively short, serpentine mines with a brown median frass line that became dark brown as the mine widened (Fig. 5.11C).

We recognize a general mining pattern for *P. maxberryi*: the egg is laid on the mid-vein, near the center of the leaf (Fig. 5.11C). After hatching, the larva enters the leaf and mines proximally towards the leaf petiole along the mid-vein and turns toward the leaf apex near or at the leaf petiole and mines along the leaf margin. Before reaching the midpoint along the axis of the leaf, the larva travels inward between the mid-vein and leaf margin and travels towards the leaf apex. After nearing the apex, the larva crosses the mid-vein and begins mining the other half of the leaf in a relatively straight line turning back towards the petiole. Once near the petiole, the larva constructs an oval-shaped chamber and molts within. After molting, the cocoon-spinning instar folds the margin while spinning its cocoon. This pupal fold was typically ~ 7.0 mm long (Figs 5.11B, G). Under rearing conditions, the pupal stage lasts between 21–28 days (n = 7). Five female specimens of *Chrysocharis* sp. (Eulophidae: Entedoninae) were reared from pupal cocoon folds collected at Villa Mills, Cerro de la Muerte.

Host. *Gaiadendron punctatum* (Ruiz & Pav.) G. Don (Loranthaceae) (Fig. 5.1G). The free-standing root parasite/epiphyte tree genus *Gaiadendron* includes approximately 15 species occurring in the New World (Gentry, 1996; Missouri_Botanical_Garden, 2009). *Gaiadendron punctatum* is distributed from Nicaragua through southern Central America to Bolivia (~ 17°50'S) between 600 and 4100 m elevation (INBio, 2009; Missouri_Botanical_Garden, 2009). Trees are typically 2–5 m in height with bright yellow/orange flowers (Kappelle, 2008). Young leaves are pale green to reddish brown, about 3–6 cm long and 1–3 cm wide (Kenji

Nishida, pers. obs.). Among species in the genus, only *G. punctatum* is known from Costa Rica, and it has been recorded above 1500 m in open areas and along trails in cloud forests (INBio, 2009; Kappelle, 2008).

Distribution. This species appears to have a greater elevational range than the other two, being found between 1950 and 3100 m. Specimens have been collected from Heredia Province, 6 km ENE of Vara Blanca, in the Cordillera Volcánica Central; and Cartago Province, Cerro de la Muerte, Villa Mills, in Cordillera de Talamanca.

Etymology. Named for the Honorable Max N. Berry of Washington, D.C., an honorary member of the Smithsonian National Board.

Phyllocnistis tropaeolicola Kawahara, Nishida & Davis, sp. n.

Diagnosis (Table 5.1). *Phyllocnistis tropaeolicola* differs from *P. drimiphaga* and *P. maxberryi* in its larger size, having a slender longitudinal fascia, valva that are ~2.4× the length of the vinculum, and a single, band-shaped signa. The pupa of *P. tropaeolicola* has conical frontal processes and dorsal abdominal spines on each segment are arranged in a V.

Adult (Fig. 5.2C). Forewing length 2.6–5.0 mm. Head. Vestiture silvery white, completely covered with smooth, broad, scales slightly overlapping anterior margin of eyes. Antenna ~ equal to length of forewing, scape and pedicel enlarged laterally

and covered in long silvery scales, a single row of fine short scales completely encircling each flagellomere. Labial palpus long, slender, ~ 1.0 mm. Thorax. Forewing silvery white; with a slender, dark-brown, longitudinal fascia extending 2/3 length of wing to meet distally at junction of brown, costal and transverse fasciae; costal fascia slender and strongly oblique with dark-brown border; transverse fascia V-shaped, with a dark-brown border; apical to subapical area pale yellowish orange with a small black spot; three slender, dark-brown costal strigulae, three slender dark-brown apical strigulae, and one faint brown tornal strigula arising from black apical spot; fringe along tornal margin white with a dark-brown basal band of broad scales. Hindwing mostly white except for a band of pale brown scales extending length of costal margin. Legs similar to *P. drimiphaga*, silvery white except dark brown over dorsal surface of femur, tibia and tarsus of foreleg. Abdomen. Length ~ 2.0 mm, mostly brownish gray dorsally, silvery white ventrally. Coremata similar to *P. drimiphaga*. Male genitalia (Figs 5.6A–C). Similar to *P. drimiphaga* except valva relatively longer and more slender, ~ 2.4× the length of vinculum, nearly straight, with ventral lobe of apex slightly re-curved dorsad (Fig. 5.6A). Genitalia slide USNM 33281. Female genitalia (Figs 5.6D, E). Oviscapt greatly reduced as in *P. drimiphaga*; ductus bursae completely membranous, slender, elongate, ~ 8.5× length of papillae anales and terminating at posterior end of corpus bursae; corpus bursae ~ 0.6× length of ductus bursae; a single elongate signum present as a narrow band partially encircling middle of corpus bursae; signum with 2 acute, flattened spines projecting inwards from band; length of spines slightly more than width of signa; ductus seminalis extremely slender, elongate, ~ 2.4 × length of corpus bursae and

arising from near middle of corpus bursae. Genitalia slide USNM 33282, 33285, 33288.

Larva (Figs 5.12A, C–F). Young sap-feeding larva translucent yellow (Fig. 5.12A). Mature sap-feeding larva ~7.5 mm long, translucent yellow, head capsule translucent pale brown, prothoracic shield dark brown (Figs 5.12C–F). Cocoon-spinning larva whitish yellow, head capsule pale gray brown; ~ 6.5 mm long (Fig. 5.12F).

Pupa (Figs 5.10, 12H). Brown, length up to ~ 5 mm; diameter ~ 1.0 mm. Vertex with a short, stout, process (cocoon-cutter) flanked by two, flattened, slightly longer processes (Figs 5.9A, B, D, E) and two pairs of short setae (Fig. 5.9C). Dorsum of A2–A7 with a pair of laterally curved, large spines in between which is a concentration of smaller spines, arranged in a triangular, V-shaped pattern (Figs 5.9F, G); each segment with a pair of long, lateral, sensory setae (Fig. 5.9L) that are shortest on A9–10 (Figs 5.9J, K). A10 with a pair of slightly divergent processes from caudal apex (Figs 5.9I, J).

Types. Holotype (Fig. 5.2C): ♂, Costa Rica: Prov. Cartago, Cerro de la Muerte, Villa Mills, 3100 m, 13 Mar 2003 (adult emergence), host *Tropaeolum emarginatum*, col./ rear Kenji Nishida, mine with pupal fold collected 6 Mar 2003 (USNM). Paratypes: Immatures: 1 prepupa, 1 pupa (USNM 34036), Villa Mills, Georgina, 9°33'30"N, 83°43'25.8"W, 3103 m, 12 Sep 2008, K. Nishida, host *Tropaeolum*

emarginatum. Adults: same locality as holotype, 6♂, 4♀: ♂ slide USNM 33281, ♀ slide USNM 33285; 2♂, 2♀ (USNM 33280, 33282) with adult emergence 11 Mar 2003; 1♂, with adult emergence 15 Mar 2003. 1♀ adult paratype at INBio and UCR, the remaining paratypes at USNM.

Life history (Fig. 5.12). Mines of *P. tropaeolicola* were readily found on plants growing along the Pan-American Highway (Fig. 5.1H). Most mines occurred on full-grown new leaves (Figs 5.12B, C) but some were found on developing leaves (Fig. 5.12A). Thirteen had a single mine, two leaves had two, and one had three. All mines were found on the adaxial side, and the late sap-feeding instar fed on the mesophyll (Fig. 5.12E).

The mine characteristically begins as a narrow, irregular serpentine gallery (Fig. 5.12B) that widens as it extends along or near the leaf margin (Figs 5.12B, C). It is relatively narrow, pale green to white with a less conspicuous dark green median frass line. Pupal cocoon folds were ~ 5.5 mm long and were found near the leaf margin (Figs 5.12B, G). Adults emerged 5–9 days after pupal cocoon folds were collected.

We found mines of an unidentified fungus gnat (Diptera: Mycetophilidae) at same site on the same plant. The mines, which usually occur several on a single leaf, are irregularly shaped blotch mines with dark-green frass scattered randomly within. The fly larva causes curling, drying, necrosis, and yellowing of the leaves, and was more abundant than *P. tropaeolicola* mines. Several leaves were infested with both

mycetophilid and *P. tropaeolicola* larvae.

Host. *Tropaeolum emarginatum* Turcz (Tropaeolaceae) (Fig. 5.1I). *Tropaeolum*, the only genus recognized in Tropaeolaceae, is Neotropical and contains approximately 90 species, many of which are found in Andean cloud forests (Gentry 1996). Four species occur in Costa Rica, and *T. emarginatum* is present on both the Atlantic and Pacific slopes between 700 and 3200 m (Alfaro-Vindas, 2003; INBio, 2009). Outside Costa Rica, *T. emarginatum* has been recorded from Chiapas, Mexico to Cotopaxi, Ecuador (Missouri_Botanical_Garden, 2009). The tenuous, soft, and succulent vines of *T. emarginatum* are usually found in forest edges and disturbed areas, and the flowers are red to yellow orange (Alfaro-Vindas, 2003; Gentry, 1996). Most of the leaves are between 5 and 8 cm wide (Kenji Nishida, pers. obs.).

Distribution. Known only from the type locality, Cerro de la Muerte, Villa Mills, at 3100 m elevation in the Cordillera de Talamanca.

Etymology. The species name, *tropaeolicola*, is formed from its host plant genus name, *Tropaeolum*, and the Latin word cola, meaning “inhabitant”.

Table 5.1. Diagnostic features of the three new *Phyllocnistis* species described in this study.

	Host	Costal fascia	Longitudinal fascia	Transverse fascia	Costal strigula	Valva	Signa
P. drimyphaga	*Drimys granadensis* (Winteraceae)	Slender, margin narrow	Broad, yellow-orange	V-shaped	3	~ 1.8X length of vinculum, very slender but broadening apically forming a prominent dorsal lobe and a smaller ventral lobe	Paired, dissimi in shap
P. maxberryi	*Gaiadendron punctatum* (Loranthaceae)	Oviform, margin broad	Broad, light brown	C-shaped	2	~ 2X length of vinculum, nearly straight with apex only slightly enlarged	Paired, similar shape
P. tropaeolicola	*Tropaeolum emarginatum* (Tropaeolaceae)	Slender, margin broad	Slender, brown	V-shaped	3	~ 2.4X the length of vinculum, nearly straight, ventral lobe of apex slightly recurved dorsad	Single, band-shaped

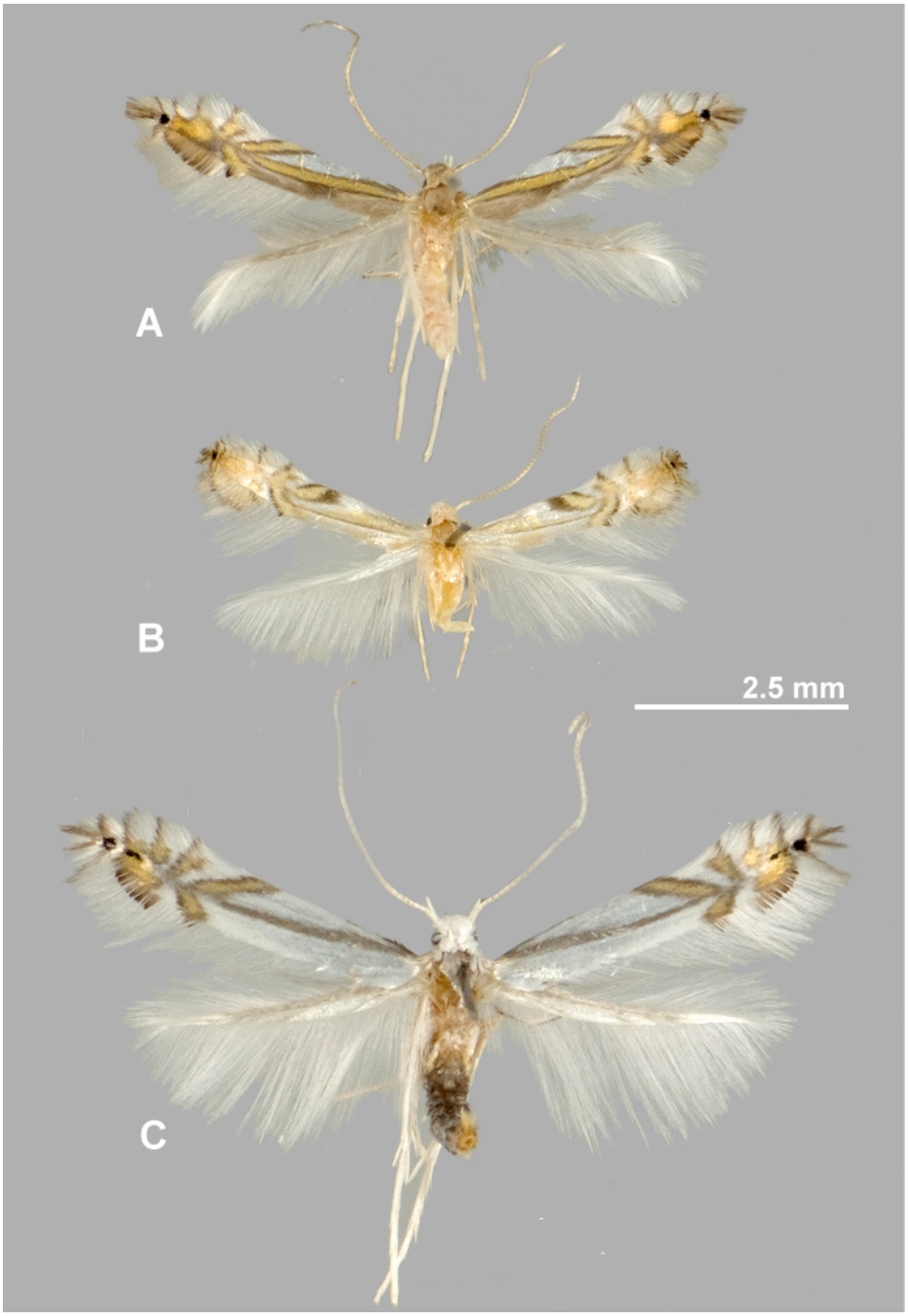


Fig. 5.1. Adults of three new *Phyllocnistis* from Costa Rica. **A)** *Phyllocnistis drimyphaga* sp. n., holotype female; **B)** *P. maxberryi* sp. n., holotype female (abdomen removed for dissection); **C)** *P. tropaeolicola* sp. n., holotype male.

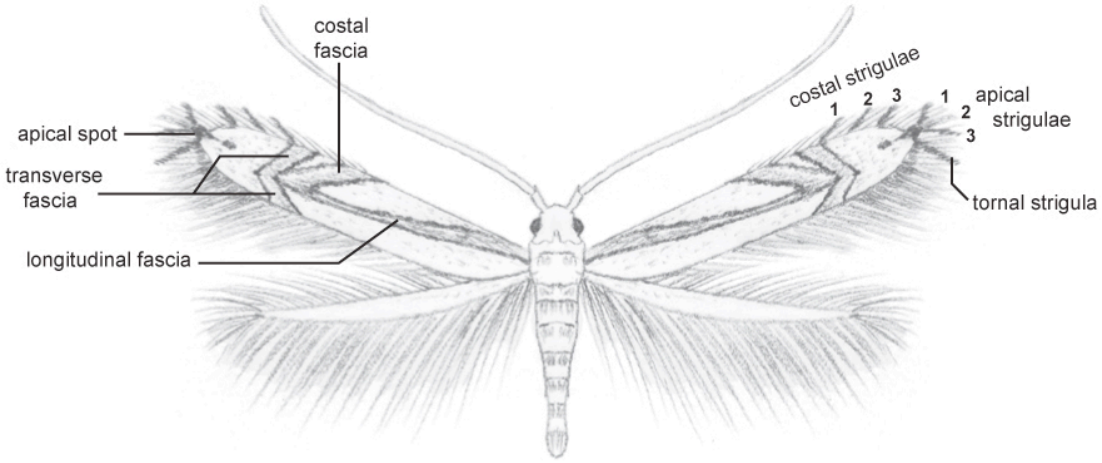


Fig. 5.2. Nomenclature of *Phyllocnistis* forewing fasciae and strigulae.

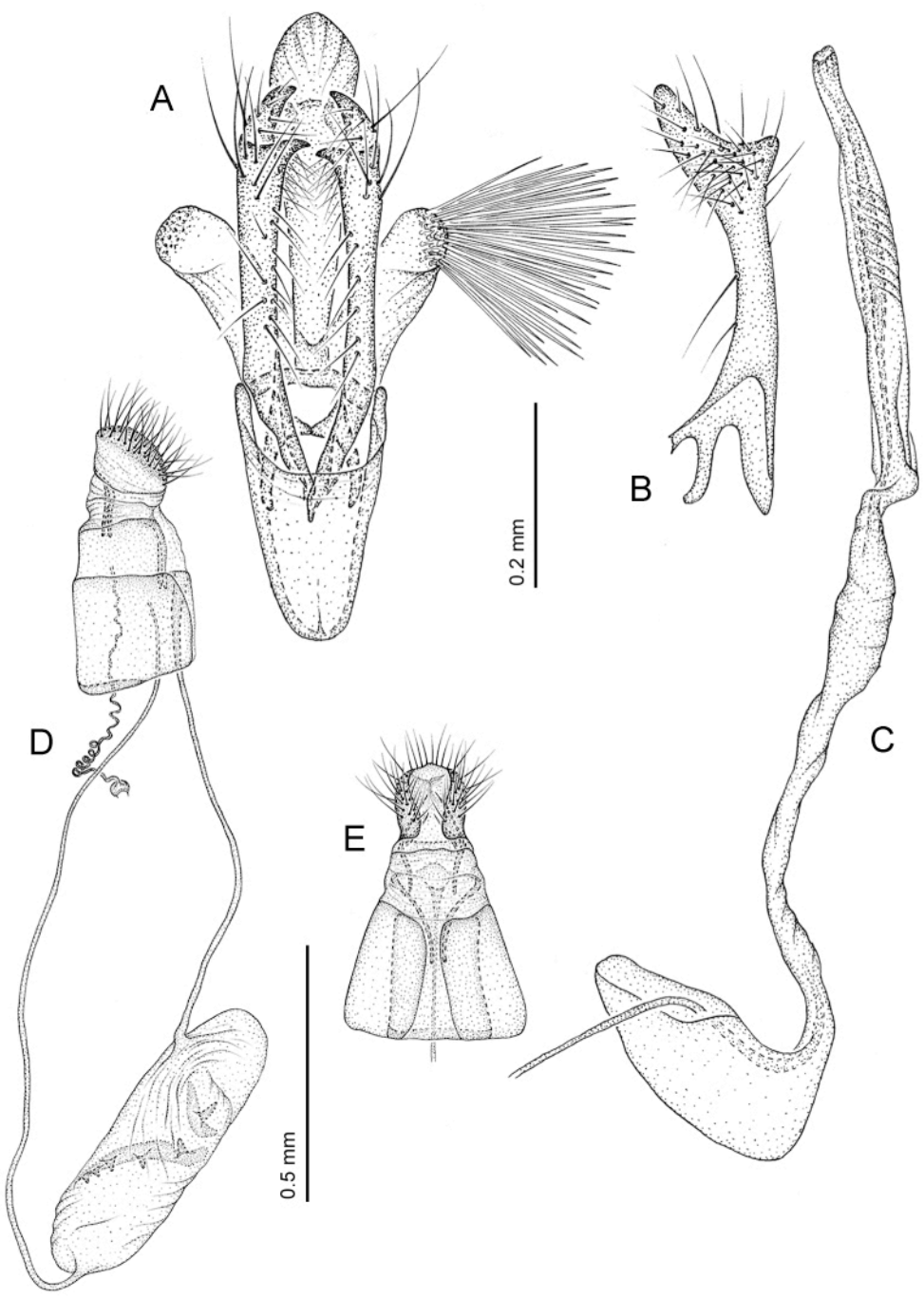


Fig. 5.3. *Phyllocnistis drimyphaga*, genitalia. **A)** Male, ventral view; **B)** right valva, mesal view; **C)** aedoeagus; **D)** female, lateral view; **E)** ventral view of figure D. (Scale bar 0.5 mm except for figure B, 0.2 mm.)

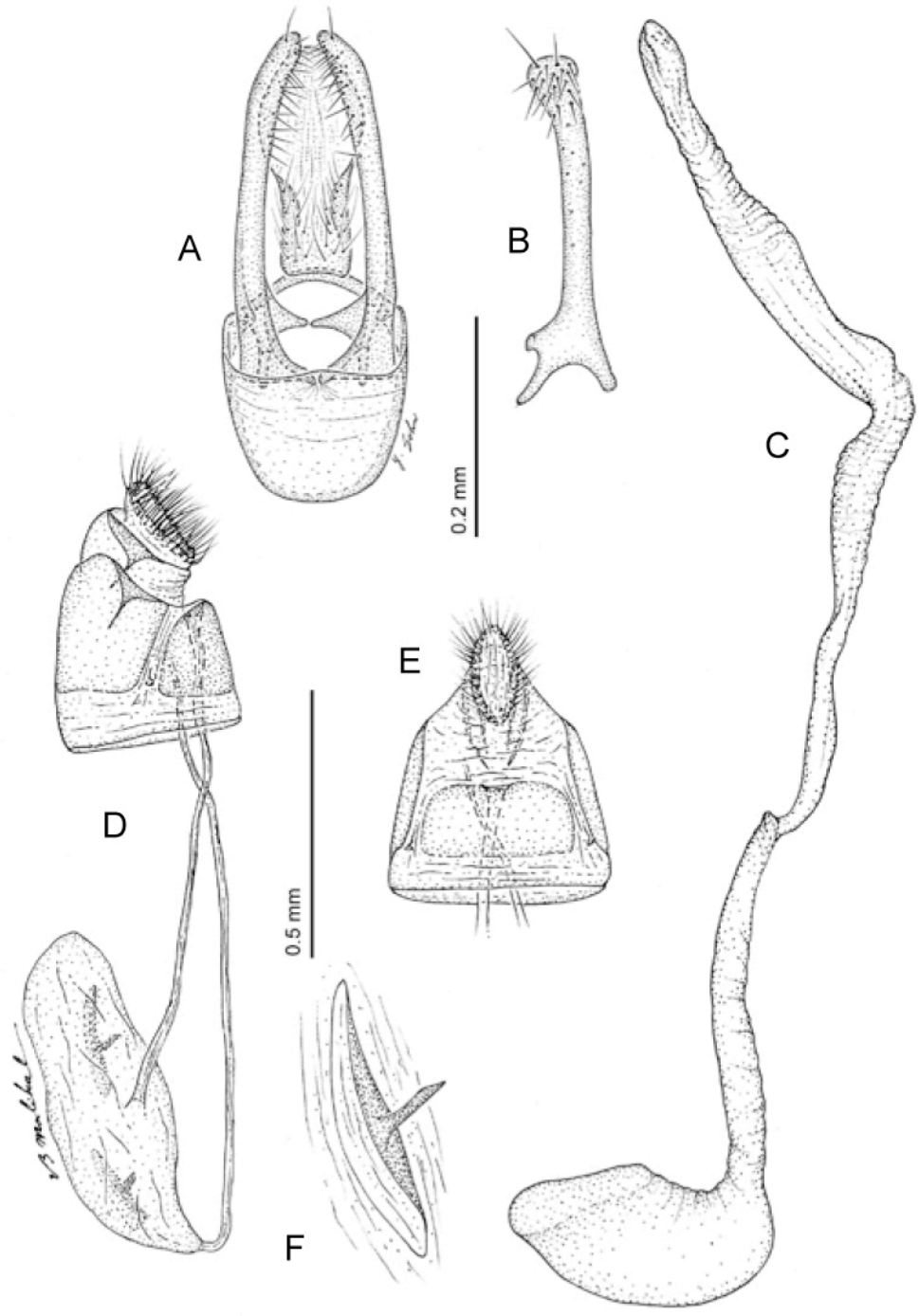


Fig. 5.4. *Phyllocnistis maxberryi*, genitalia. **A)** Male, ventral view; b right valva, mesal view; **C)** aedoeagus; **D)** female, lateral view; **E)** ventral view of figure D; **F)** signa. (Scale bar 0.5 mm except for figure B, 0.2 mm.)

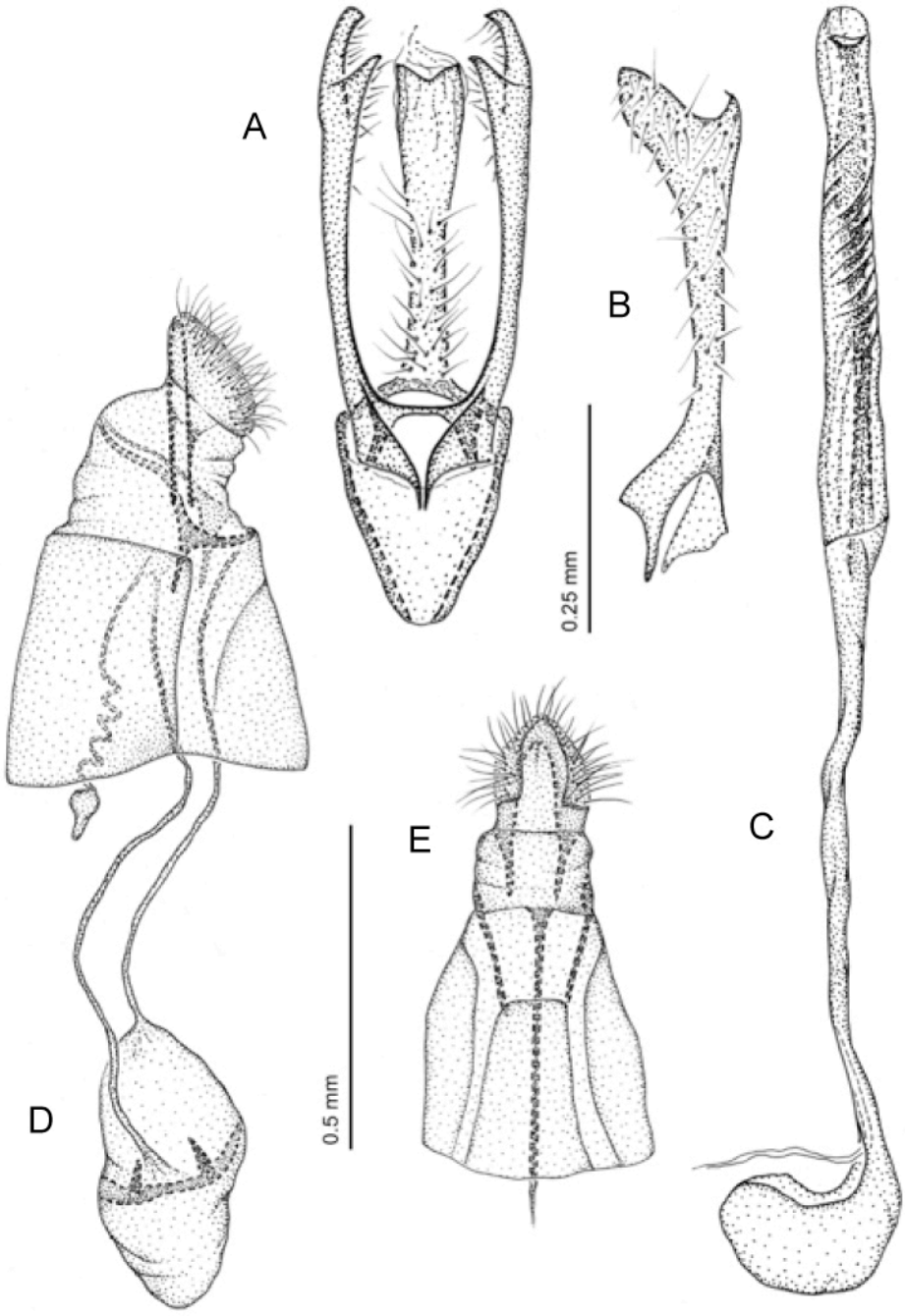


Fig. 5.5. *Phyllocnistis tropaeolicola* , genitalia. **A)** Male, ventral view; **B)** right valva, mesal view; **C)** aedoeagus; **D)** female, lateral view; **E)** ventral view of figure D. (Scale bar 0.5 mm except for figure B, 0.25 mm.)

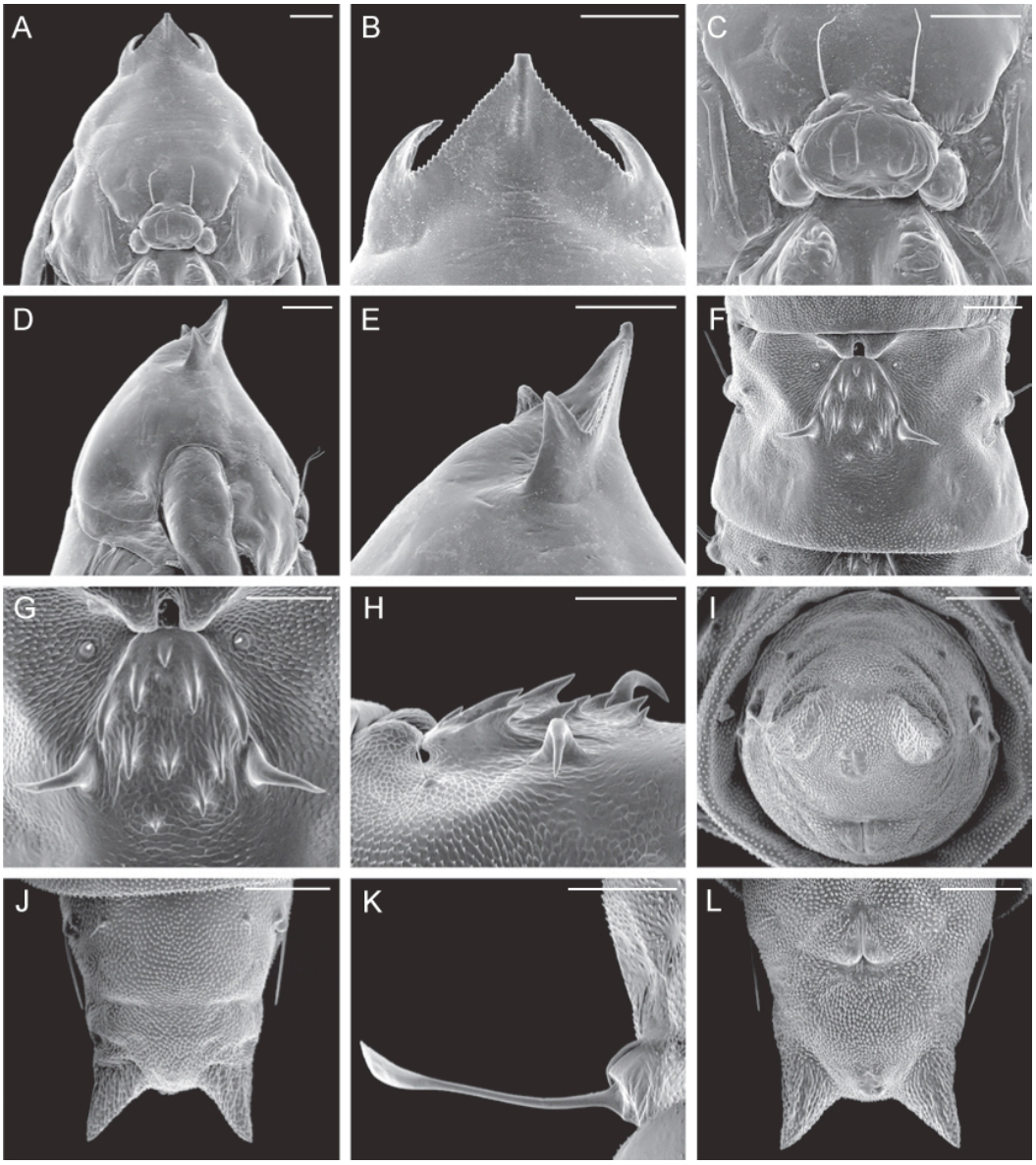


Fig. 5.6. *Phyllocnistis drimyphaga* sp. n., pupa. **A)** Ventral view of head; **B)** detailed ventral view of cocoon cutter; **C)** detailed view of frons; **D)** lateral view of head; **E)** detailed lateral view of cocoon cutter; **F)** fifth abdominal tergum, dorsal; **G)** detailed view of spines on fifth abdominal tergum; **H)** detailed lateral view of spines on fifth abdominal tergum; **I)** caudal view of abdominal tip; **J)** dorsal view of A9–10; **K)** detailed view of lateral seta on seventh abdominal tergum; **L)** ventral view of A9–10. Scale bar 100 μm.

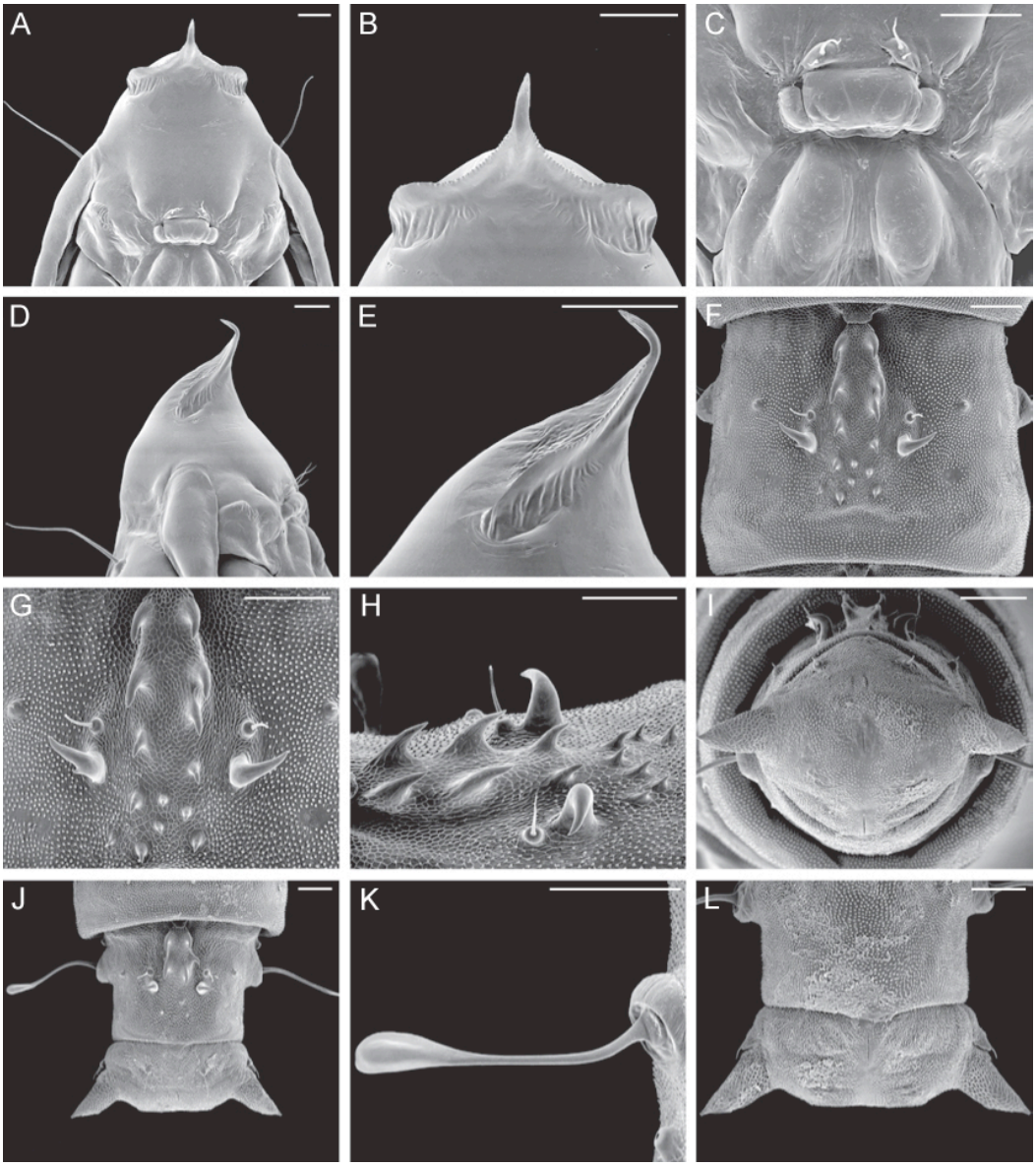


Fig. 5.7. *Phyllocnistis maxberryi* sp. n., pupa. **A)** Ventral view of head; **B)** detailed ventral view of cocoon cutter; **C)** detailed view of frons; **D)** lateral view of left side head; **E)** detailed lateral view of cocoon cutter; **F)** dorsal view of sixth abdominal tergum; **G)** detailed view of spines on sixth abdominal tergum; **H)** detailed lateral view of spines on seventh abdominal tergum; **I)** caudal view of abdominal tip; **J)** dorsal view of A9–10; **K)** detailed view of lateral seta on sixth abdominal tergum; **L)** ventral view of A9–10. Scale bar 100 μm.

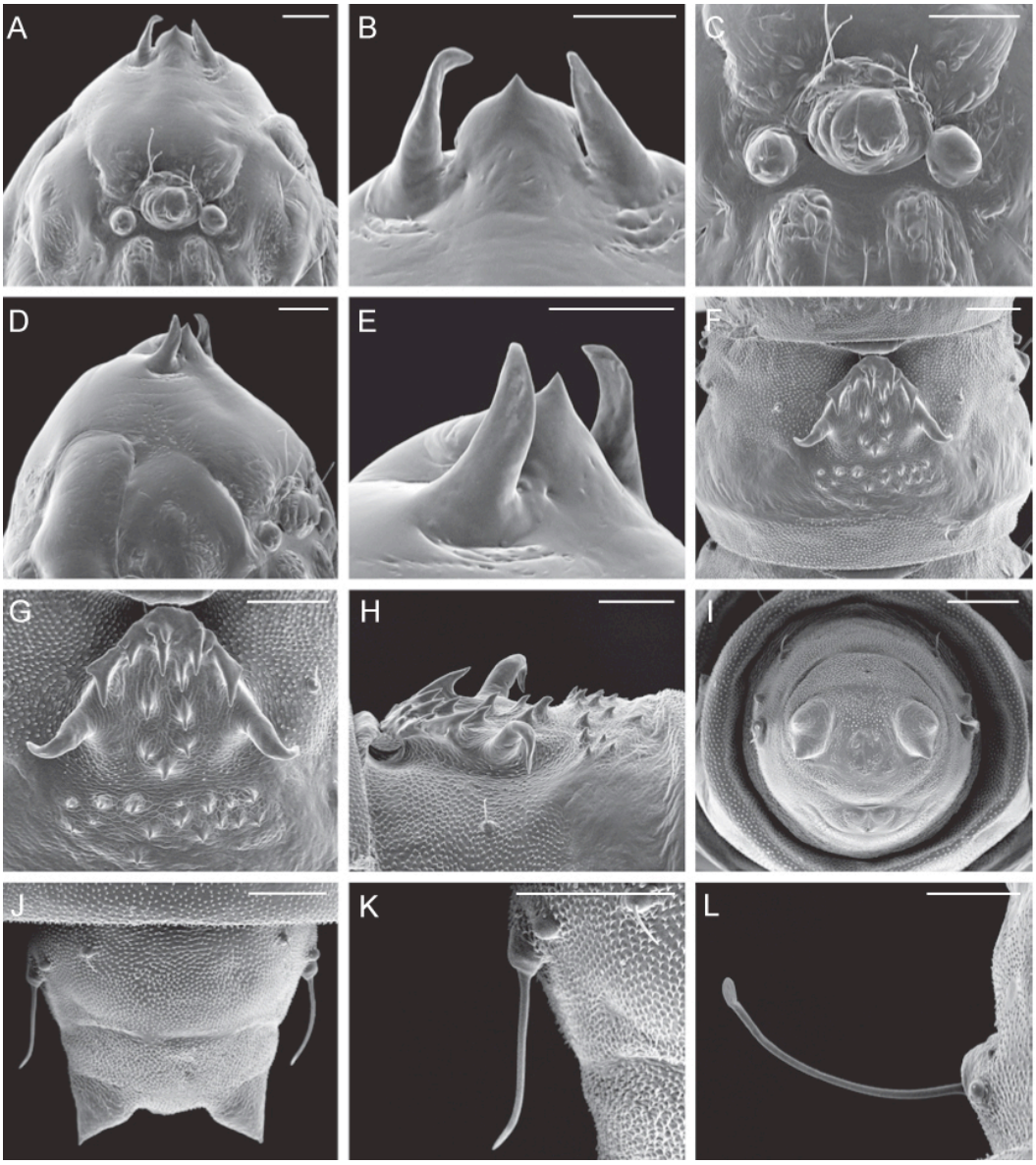


Fig. 5.8. *Phyllocnistis tropaeolicola* sp. n., pupa. **A)** Ventral view of head; **B)** detailed ventral view of cocoon cutter; **C)** detailed view of frons; **D)** lateral view of head; **E)** detailed lateral view of cocoon cutter; **F)** fourth abdominal tergum, dorsal; **G)** detailed view of spines on fourth abdominal tergum; **H)** detailed lateral view of spines on fourth abdominal tergum; **I)** caudal view of abdominal tip; **J)** dorsal view of A9–10; **K)** detailed view of lateral seta on A9–10; **L)** detailed view of lateral seta on seventh abdominal tergum. Scale bar 100 μm.

Fig. 5.9. (See legend on following page).

Fig. 5.9. Habitats and larval host plants of *Phyllocnistis* species. **A)** Cerro de la Muerte, Villa Mills region, 3000 m and below, in Cordillera de Talamanca; **B)** Barva Volcano, ALAS transect, 2000 m, in Braulio Carrillo National Park; **C)** habitat of *P. drimyphaga* in Cerro de la Muerte, km 70 Pan-American Hwy, road to El Paraíso del Quetzal, 2700 m, arrow pointing to host plant where mines were found; **D)** young stem shoots and leaves of *Drimys granadensis* of figure C, growing from base of the tree; **E)** flowers and leaves of *D. granadensis*; **F)** habitat of *P. maxberryi* in Cerro de la Muerte, km 95 Pan-American Hwy, trail front of La Georgina in Villa Mills, 3100 m, arrow pointing to host plant where mines were found; **G)** young vigorous growth of *Gaiadendron punctatum* in front, and mature trees with yellow fruits in behind, at ALAS transect in Vara Blanca, 2000 m; **H)** habitat of *P. tropaeolicola* in Cerro de la Muerte, on km 95 Pan-American Hwy, near La Georgina in Villa Mills, 3100 m, arrow pointing to host plant where mines were found; **I)** *Tropaeolum emarginatum*, details of host plants shown in figure H.

Fig. 5.10. (See legend on following page).

Fig. 5.10. Life history of *Phyllocnistis drimyphaga*. **A)** Leaf mines on abaxial leaf surface, arrow pointing to pupal cocoon fold, white square enclosing early mine; **B)** close-up view of early mine, arrow pointing to remaining of egg shell; **C)** same as figure B, but showing frass pattern via projecting sunlight through the leaf from behind; **D)** nearly mature old mine on adaxial surface; **E)** nearly mature old mine on abaxial surface seen from the underside; **F)** opened mine showing mature sap-feeding larva *in situ*; **G)** opened young pupal cocoon fold showing cocoon-spinning larva *in situ*; **H)** pupal cocoon fold on adaxial mine (arrow); **I)** opened pupal cocoon fold showing pupa *in situ* (dorsal view); **J)** protruded and attached pupal shell (arrow) on pupal cocoon fold of an abaxial leaf mine; **K)** opened pupal cocoon fold on adaxial mine showing *Ageniaspis* cocoons *in situ*.

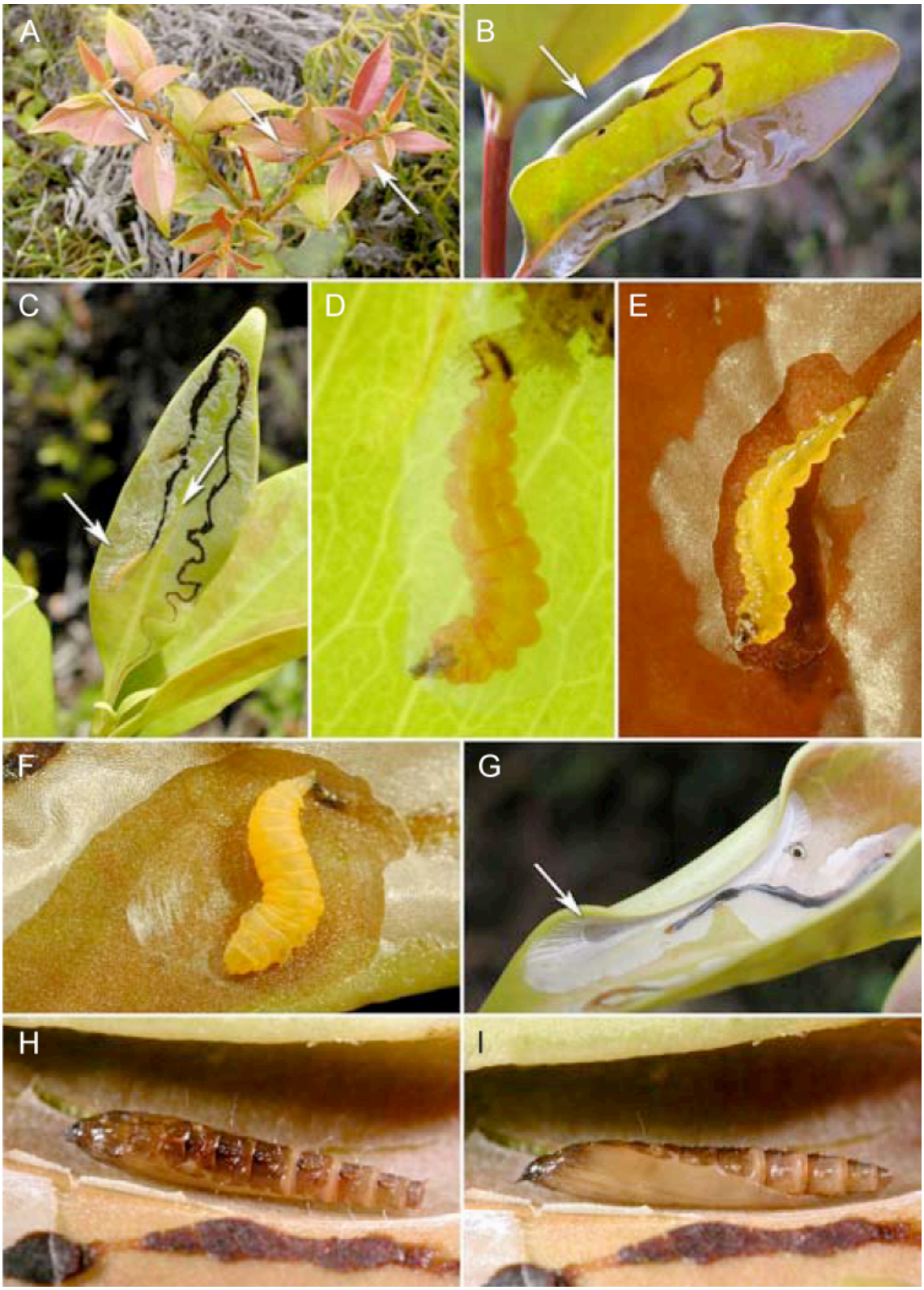


Fig. 5.11. (See legend on following page).

Fig. 5.11. Life history of *Phyllocnistis maxberryi*. **A)** Leaf mines (arrows) on young growing *Gaiadendron* shoot; **B)** mature mine with pupal cocoon fold (arrow); **C)** nearly mature mine and mature sap-feeding larva (left arrow), and oviposition location (right arrow); **D)** close-up view of mature sap-feeding larva; **E)** opened mine showing mature sap-feeding larva *in situ*; **F)** opened young pupal cocoon fold showing cocoon-spinning larva *in situ*; **G)** pupal cocoon fold, arrow pointing at thinner pupal exit; **H)** opened pupal cocoon fold showing pupa *in situ*, dorsal view; **I)** pupa *in situ*, lateral view.

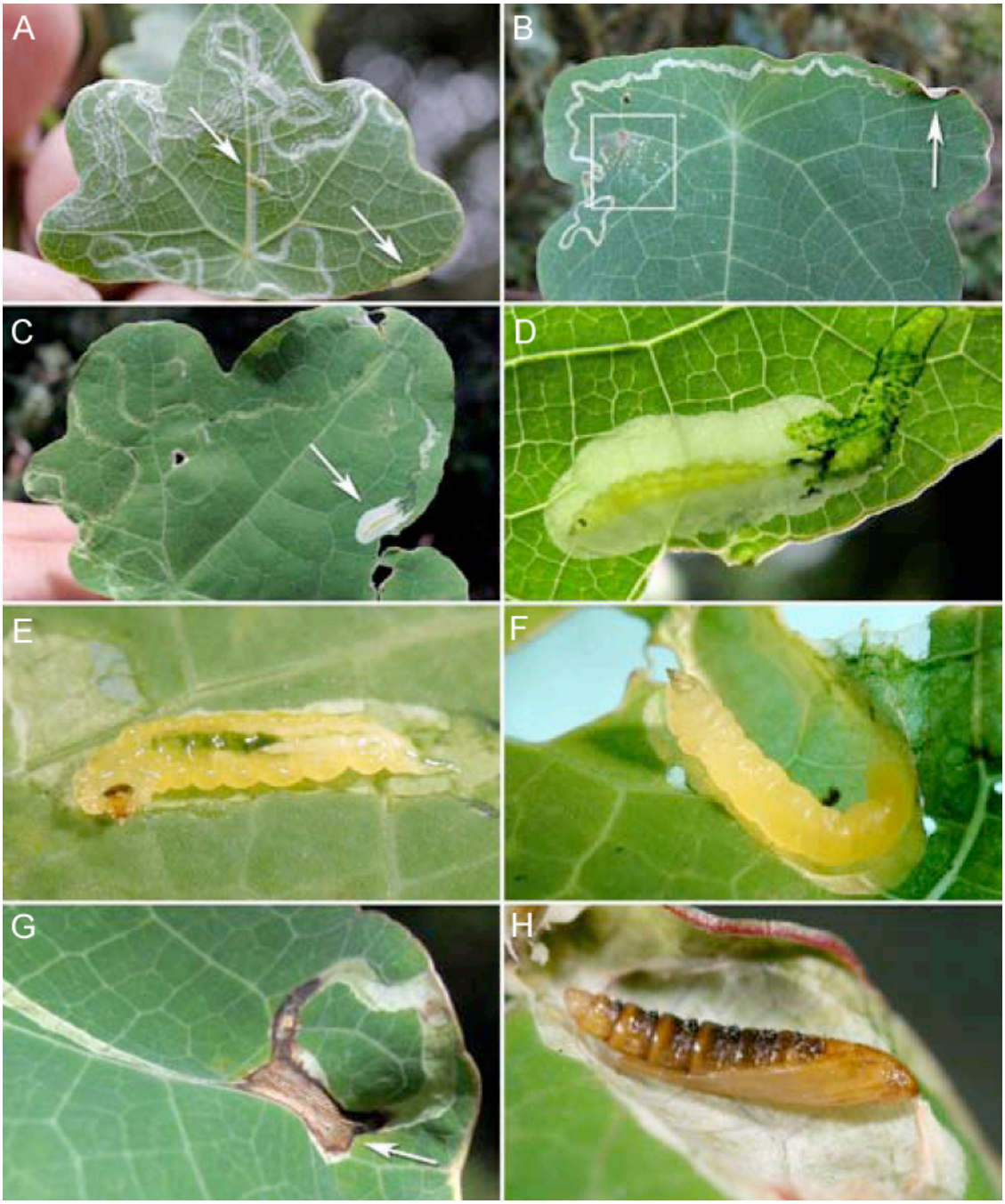


Fig. 5.12. Life history of *Phyllocnistis tropaeolicola* . **A)** Leaf mines on a young leaf, arrows pointing at young to middle instar larvae; **B)** mature leaf mine with pupal cocoon fold (arrow), white square enclosing early mine; **C)** mature sap-feeding larva in pre-cocoon chamber; **D)** detailed view of figure C; **E)** opened mine showing nearly mature sap-feeding larva *in situ*; **F)** opened young pupal cocoon fold showing cocoon-spinning instar *in situ*; **G)** pupal cocoon fold, arrow pointing to the more slender exit; **H)** opened pupal cocoon fold showing pupa *in situ* (lateral view).

CHAPTER 6

Five species of Gracillariidae new to Korea

Abstract

Five species of Gracillariidae: *Calybites securinella* (Ermolaev, 1986), *Epicephala relictella* Kuznetzov, 1979, *Parornix alni* Kumata, 1965, *P. betulae* Stainton, 1854, and *Spulerina castaneae* Kumata & Kuroko, 1988, are recorded as new from Korea. *Epicephala* is a genus that is reported for the first time in the country. Photographs of adults and genitalia are provided along with a brief description of each species and a list of host plants.

Introduction

Gracillariidae include nearly 2,000 species of leaf-mining micro-moths distributed throughout the world (De Prins and De Prins, 2010). Among microlepidoptera, Gracillariidae include some of the most important economic pests. Some have been reported as invasive (e.g., *Cameraria ohridella*, Valade et al., 2009; Causton et al., 2006; Heppner, 1993; *Phyllocnistis citrella*, Heppner and Dixon, 1995), and there is a great need to document their hosts and distributions. While there has been a recent effort to describe life history and distributions of gracillariids within the last few years (e.g., Davis and Wagner, 2005; De Prins and De Prins, 2010; Kawahara et al., 2009; Vargas and Landry, 2005), many species still remain poorly

understood. A recent effort has been set to catalog the host information, distribution, for all gracillariid species worldwide (De Prins and De Prins, 2010).

A total of 46 species of Gracillariidae has been documented from Korea (Kumata et al., 1983; Park, 1983; Park and Han, 1986; Park and Lee, 2001; Shin et al., 1994; Sohn, 2007). However, we predict that this figure is far short of their true diversity in the country, given the high diversity in neighboring Japan, ca. 242 spp. (Jinbo, 1988). We predict that a comprehensive study of the Korean Gracillariidae will result in many new records for the country. We report five gracillariid species new to Korea: *Calybites securinella* (Ermolaev), *Epicephala relictella* Kuznetzov, *Parornix alni* Kumata, *P. betulae* Stainton, and *Spulerina castaneae* Kumata and Kuroko. *Epicephala* is a genus that is reported from Korea for the first time. Adult specimens are pinned and stored in the Department of Plant Medicine, Chungbuk National University and DNA tissue samples are preserved in the University of Maryland LepTree frozen tissue collection (College Park, Maryland, USA).

Systematic account

***Calybites securinella* (Ermolaev)**

Figs. 6.1, 6.5

Caloptilia securinella Ermolaev, 1986, Ento. Obozr. 65(4): 747-749 (type locality: Gornotaezhnoe, South Primorye, Russia).

Calybites securinella: Noreika, 1997, Key Ins. Russ. Far East 5 (1): 395.

Adult (Fig. 6.1). Forewing length 3.8 – 4.0 mm. Head light brown, antenna filiform in both sexes, thorax light brown. Forewing light brown with four white transverse bands. Hindwing slender, light brown.

Male genitalia (Fig. 6.5). Uncus absent; tegumen round distally, parallel-sided; tuba anales with a long, sclerotized section. Valva elongate with rounded apex, curved dorsally, with a broad lobe at the middle of ventral margin. Vinculum narrow, elongated-triangular distally. Saccus absent. Aedeagus narrow, as long as valva, with small coecum; cornuti absent.

Female genitalia. Not available.

Material examined. 1♂, Mt. Weolak-san (N36˚53′16.9″ E128˚08′56.8″), Jecheon, Chungbuk Province, Korea. 23.vii.2005 (coll. J.C. Sohn), geni. slide no. SJC-791; 2♂, 3♀, Saeseulmak, Changwon-ri, Yeungwol, Gangwon Province, Korea. 28.vii.2008 (coll. J.C. Sohn), 3 samples in 100% EtOH.

Distribution. Korea (new record) and Russia (Far East).

Host plant. Euphorbiaceae: *Securinega suffruticosa* (Ermolaev, 1986).

Remark. Another species, *Calybites phasianipennella* Hübner was recorded in Korea (Park, 1983). *C. securinella* is easily distinguished from *C. phasianipennella* by wing patterns: presence of transverse mid-fascia and subterminal fascia.

Korean name. Gwang-dae-ssa-ri-ga-neun-na-bang.

***Epicephala relictella* Kuznetzov**

Figs. 6.2, 6.6, 6.8

Epicephala relictella Kuznetzov, 1979, Ento. Obozr. 58(4): 854 (type locality: Gornotaezhnoe, South Primorye, Russia)

Adult (Fig. 6.2). Forewing length 4.5 – 5.0 mm. Head with tuft of long white scales, antenna filiform, long, slender and brown. Forewing brown, three narrow white costal strigulae bending distally towards circular black dot near apex. Hindwing narrow, light brown. Male genitalia (Fig. 6.6). Uncus absent; tegumen elongate and subpentagonal. Valva elongate, curving before apex, uniform in width; sacculus separated at distal end from valva. Vinculum wide, V-shaped. Aedeagus straight, as wide a sacculus. Cornutus separated into three patches composed of one or more spines.

Female genitalia (Fig. 6.8). Ovipositor lobes piercing; apophyses posteriores longer than apophyses anteriores. Lamella antivaginalis sclerotized, medially concave. Ostium bursa weakly trapezoidal. Ductus bursae sclerotized near ostium bursa, then becoming unsclerotized and granulated before bearing longitudinal wrinkles. Corpus bursae elongate, oval, one conical signum present.

Material examined. 1♂, Mt. Weolak-san (N36˚53′16.9″ E128˚08′56.8″), Jecheon, Chungbuk Province, Korea. 23.vii.2005 (coll. J.C. Sohn), geni. slide no. SJC-789; 2♂, 3♀, Saeseulmak, Changwon-ri, Yeongwol, Gangwon Province, Korea. 28.vii.2008 (coll. J.C. Sohn), geni. slide no. SJC-787 (♀), 3 samples in 100% EtOH.

Distribution. Korea (new record) and Russia (Far East).

Korean name. Song-got-ga-neun-na-bang.

***Parornix alni* Kumata**

Figs. 6.3, 6.9

Parornix alni Kumata, 1965, Ins. Matsum. 28(1): 64-66 (type locality: Teine, Hokkaido, Japan)

Adult (Fig. 6.3). Forewing length 3.6-3.9 mm. Head with long white scales, antenna filiform, light brown. Forewing white with slender brown markings along costal margin, black dot at apex; hindwing light brown. Throax white, abdomen light brown.

Male genitalia. Not available in this study. See Kumata (1965) based on the Japanese specimens.

Female genitalia (Fig. 6.9). Papillae anales short, caudal half setose. Apophyses anteriores 2x longer than apophyses posteriores. Ductus bursae tubular, narrow, 2x longer than corpus bursae, and granulated near base of corpus bursae. Corpus bursae ellipsoidal with two dense patches of scobular signa. For comparison, we have included an image of the female genitalia of *P. multimaculata* (Fig. 6.7).

Material examined. 4♀, Saeseulmak, Changwon-ri, Yeongwol, Gangwon Province, Korea. 28.vii.2008 (coll. J.C. Sohn), 3 samples in 100% EtOH. Genitalia slide number SJC 788.

Distribution. Korea (new record), Japan and Russia (Far East).

Host plant. Betulaceae: *Alnus hirsuta* (Kumata, 1965).

Remark. The species as well as *P. betulae* are very similar superficially to *Parornix multimaculata* (Matsumura) which have been known in Korea since Park

(1983). However, close examination of hindwing venation and genital features reveals significant differences of *P. multimaculata* from them (see Kumata, 1965 for detailed comparison). It is noteworthy that three *Parornix* species co-exist in a collecting site, which may call for reexamining all previous records of *P. multimaculata* in Korea.

Korean name. Mul-o-ri-ga-neun-na-bang.

***Parornix betulae* (Stainton, 1854)**

Fig. 6.10

Ornix betulae Stainton, 1854, Insecta Britannica 3: 205-206 (type locality: [United Kingdom]).

Ornix scutulatella Stainton, 1854, Insecta Britannica 3: 206.

Ornix betulella: Herrich-Schäffer, 1855, Syst. Bearb. Schmett. Eur.: 297.

Ornix betulaevorella: Doubleday, 1859, Syn. List Brit. Lep. (2nd ed.): 33.

Ornix (Parornix) betulae: Spuler, 1910, Schmett. Europas 2: 44.

Parornix betulae: Pierce & Metcalfe, 1935, Genit. Tin. Brit.: 79.

Adult. Similar to *Parornix alni* Kumata.

Male genitalia. Not available in this study. See Kumata (1965) based on the Japanese specimens.

Female genitalia (Fig. 6.10). Papillae anales slightly prolonged, caudal half setose. Apophyses anteriores and posteriores short, both equal in length. Lamella antivginalis digitate. Ductus bursae tubular, its width nearly as wide as lamella

antivaginalis. Corpus bursae globular, signa present as dense patches of scobs. Further details can be found in Kumata (1965).

Material examined. 1♂, Saeseulmak, Changwon-ri, Yeongwol, Gangwon Province, Korea. 28.vii.2008 (coll. J.C. Sohn), geni. slide no. SJC-802, in 100% EtOH.

Distribution. Korea (new record), Japan, Far Eastern Russia. to Europe

Host plant. Betulaceae: *Betula alba, B. ermanii, B. humilis, B. lutea, B. mandschurica, B. nana, B. pendula, B. platyphylla, B. pubescens, B. utilis, B. verrucosa* (Buhr, 1935; Buszko, 1990; Ermolaev, 1981; Hartig, 1964; Kumata, 1965; Osthelder, 1951; Roüast, 1884; Stainton, 1854a).

Remark. The species is distinguished from the prior species by apical segment of labial palpus with a black ring or blotch (entirely white in *P. alni*).

Korean name. Bak-dal-ga-neun-na-bang.

***Spulerina castaneae* Kumata and Kuroko, 1988**

Figs. 6.4, 6.11

Spulerina castaneae Kumata and Kuroko, 1988, Ins. Matsum. N. S. 40: 81-83 (type locality: Morioka, Honshu, Japan)

Adult (Fig. 6.4). Forewing length 5.5-5.7 mm. Head shiny white, antenna filiform. Forewing white with five wide, yellow-brown transverse bands. Margin of apex dark brown. Hindwing slender, light brown. Thorax and abdomen yellow-brown.

Male genitalia. Not available in this study. See Kumata et al. (1988) for description.

Female genitalia (Fig. 6.11). Papillae anales short, setose. Apophyses anteriores and posteriores equal in length, 2x longer than antrum. Ductus bursae same in length as ellipsoidal corpus bursae. Signum with a heavily sclerotized, sharp, curved, and long median projection. Further details can be found in Kumata et al. (1988).

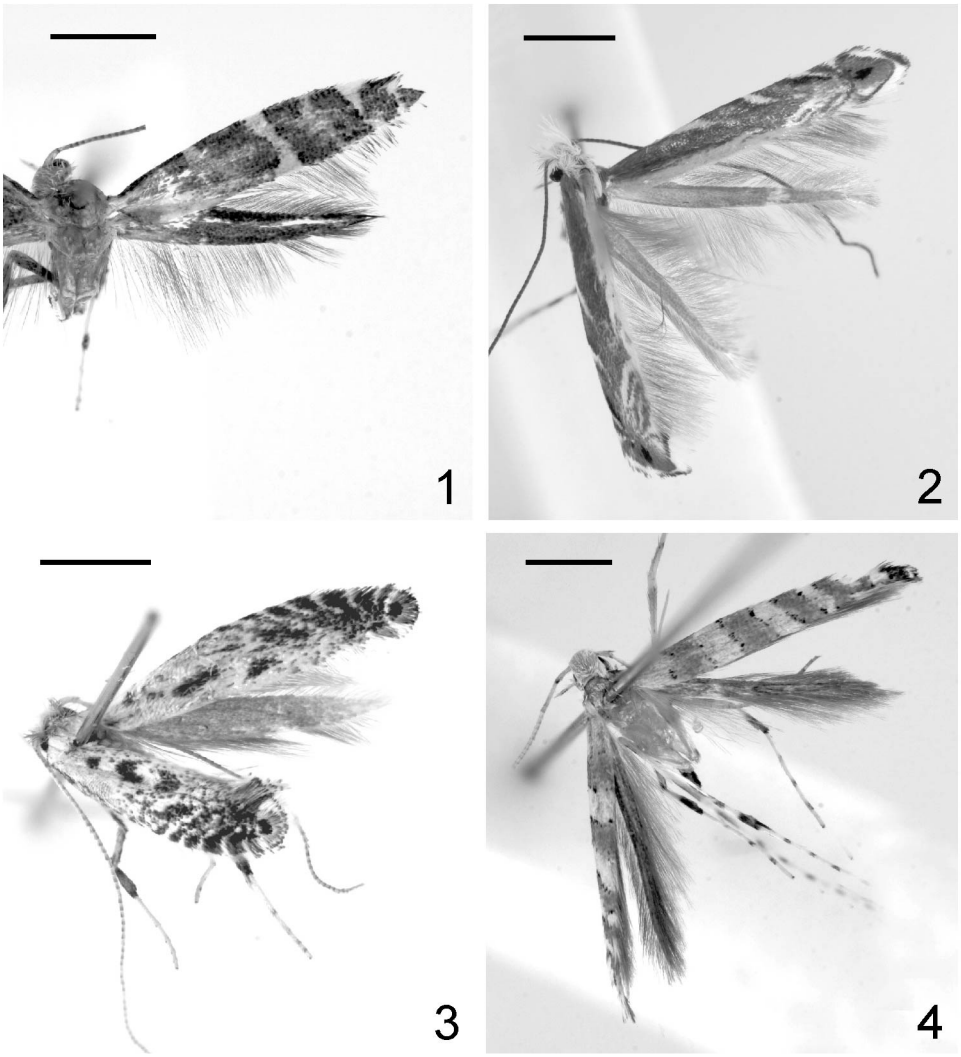
Material examined. 2♀, Hwayang Valley, Mt. Sokrisan, Boeun, Chungbuk Province, Korea. 26.v.2002 (coll. J.C. Sohn), geni. slide no. SJC-790.

Distribution. Korea (new record) and Japan.

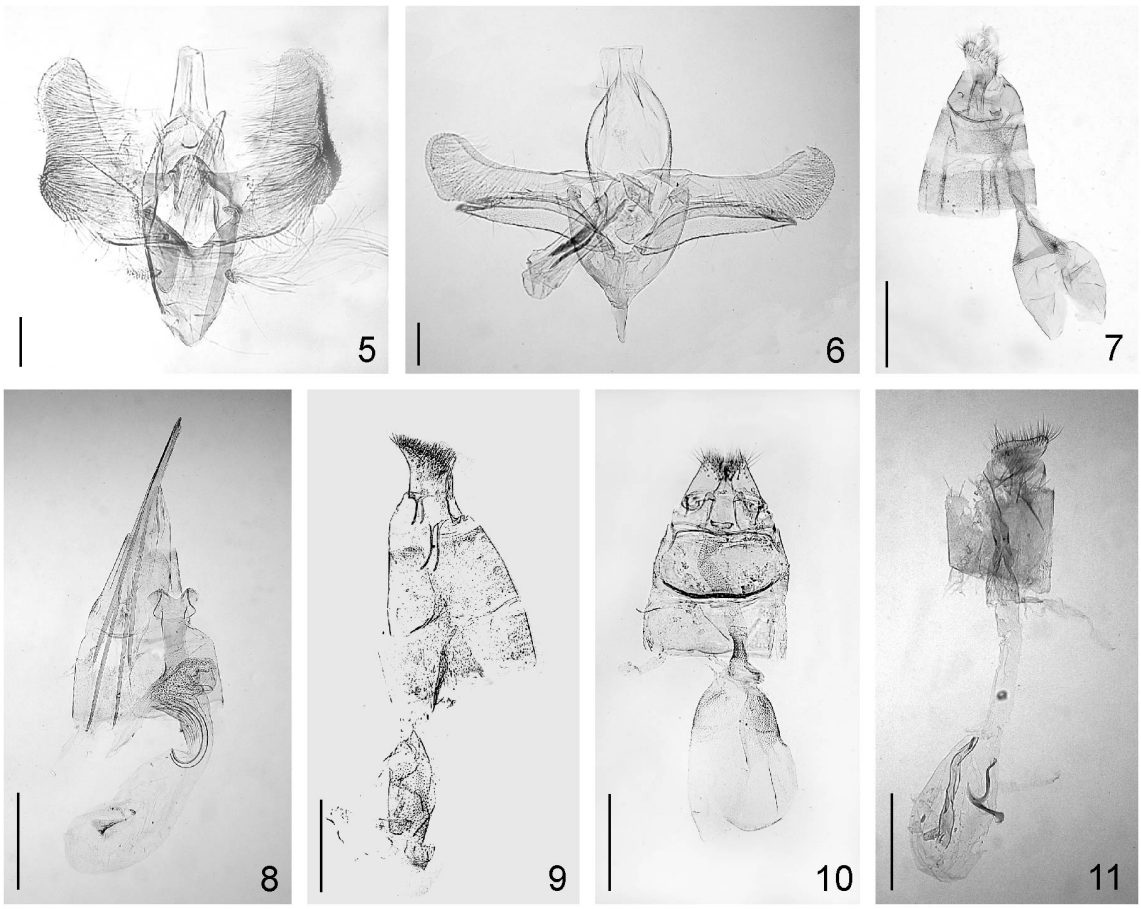
Remark. Two congeneric species, *S. astaurota (*Meyrick) and *S. dissotoma* (Meyrick), have been known from Korea (Park, 1983). The white fasciae as wide as brownish interspatial bands in *S. castaneae* and *S. astaurota* are distinguished from *S. dissotoma*. Discrimination of *S. castaneae* and *S. astaurota* can be done with checking subapical area of forewings: in the former, the area broadly darkened.

Host plant. Fagaceae: *Castanea crenata* and *Quercus* sp. (Kumata et al., 1988).

Korean name. Bam-jul-gi-ga-neun-na-bang.



Figs. 6.1-6.4. Adults of newly recorded gracillariid species from Korea. **1)** *Calybites saccurinella*, **2)** *Epicephala relictella*, **3)** *Parornix alni*. **4)** *Spulerina castaneae*. Scale bar = 1.0 mm.



Figs. 6.5-6.11. Genitalia of gracillariid species from Korea. **5-6)** Male genitalia. **5)** *C. saccurinella*, **6)** *E. relictella*. Scale bar = 0.1 mm. 7-11, Female genitalia. **7)** *Parornix multimaculata*, **8)** *E. relictella*, **9)** *P. alni*, **10)** *P. betulae*, **11)** *S. castaneae*. Scale bar = 0.5 mm.

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