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DNA barcodes: Evaluating the potential of COI to diffentiate closely related species of *Elachista* (Lepidoptera: Gelechioidea: Elachistidae) from Australia

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Abstract

We compared DNA barcoding to "traditional" taxonomic tools in clarifying relationships in complexes of closely related, putative "species" of Elachistinae moths (Gelechioidea: Elachistidae) occurring in Australia. A 705 bp fragment of the 3'-end of cytochrome c oxidase subunit I gene (COI) was used. This mtDNA fragment did not differentiate between all species-level taxa that could be defined by morphological and/or ecological differences. Different evolutionary rates of COI among closely related lineages were observed. Although our findings are based on the variability of the 3' end of the COI gene and not the 5' end barcode fragment, we are convinced that thorough exploration of traditional morphology and ecology is a prerequisite for exploring insufficiently known taxonomies by the barcode approach. The sole use of COI barcoding, whether considering COI-5' or COI-3' fragment, may fail to recognize closely related species. Our results discourage this approach for delimitation of closely related species, but its use is encouraged as an additional tool for exploring little known taxonomies or as an identification tool for previously thoroughly studied species complexes.

Key words: barcode, COI-3', taxonomy, *Elachista*, identification, delimitation of species, morphology

Introduction

DNA barcoding proposes the use of DNA sequences to identify and classify an organism. The potential of a 650 bp fragment of the 5'-end of mitochondrial cytochrome c oxidase subunit I (COI)-based species identification system was proposed and partially demonstrated by Hebert *et al*. (2003b). Among the benefits of this particular gene is its ease of acquisition and alignment, in addition to the fundamental criterion, a high level of

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diversity. Hence, the COI-5' fragment has been proposed to serve as the core of a global bio-identification system for animals (Hebert *et al*. 2003a). The performance of this system remains unclear, however, when applied to species that by traditional taxonomy are classified as very closely related (e.g. Prendini 2005). As Blaxter (2003) and Hebert *et al*. (2003a) have discussed, a major unresolved issue is how closely the molecular taxa correspond to what traditional biologists recognize as species, i.e., species defined by DNA barcodes might not always correspond with species recognized by traditional ecological and morphological criteria. The question remains as how to delimit the species in question. The sole use of barcoding could incorrectly identify two members of a species as separate species, or two separate species as the same. Moreover, the conceptual issue of definition of species remains intact: if a genomic integrity of a species is assumed, how is it defined or characterized (see Sperling 2003 for discussion)?

In this paper we present examples from Australian complexes of Elachistinae moths that presently are unresolved species, and demonstrate discrepancies in delimitation of species using 'traditional' criteria (i.e., ecological and morphological) versus the mitochondrial COI-3' fragment. We also demonstrate problems in applying the use of this genomic fragment as a DNA barcode of species.

Taxonomy

Our focal taxon is *Elachista*, a large genus of Lepidoptera (Gelechioidea: Elachistidae). *Elachista* comprises 550 named and two hundred discovered, yet unnamed species worldwide (L. Kaila, unpublished). The larvae of *Elachista* are leaf-miners specialising on monocotyledenous plants, especially Poaceae and Cyperaceae. Their phylogeny was examined previously in a morphological analysis that used 131 characters with 171 informative character states derived from adult and pupal morphology and larval ecology (Kaila 1999a), and later revisited by a more extensive morphology-based data set by Kaila & Sugisima (2003, and in preparation).

The taxonomic knowledge of *Elachista* has a long history in Europe where highly skilled amateurs have contributed detailed knowledge on the life histories and identification of species. *Elachista* species are generally morphologically rather uniform and thus difficult to identify based on external examination (Traugott-Olsen & Schmidt Nielsen 1977 and references therein). During the past two decades the taxonomy of the genus has been under revision worldwide by LK, resulting in nineteen revisionary works (Albrecht & Kaila 1997, Huemer & Kaila 2003, Kaila 1992, 1996, 1997, 1998ab, 1999abc, 2005, Kaila *et al.* 2001, Kaila & Jalava 1994, Kaila & Junnilainen 2002, Kaila & Karsholt 2002, Kaila *et al.* 2003, Kaila & Sugisima 2003, Kaila & Varalda 2004, Sugisima & Kaila 2005). The taxa examined in the present paper are included in an ongoing revision of the fauna of Australia (L. Kaila, in preparation).

The aim of the present study is to elucidate the taxonomic utility of a sequence fragment, the 3' end of the COI gene, for a group of very closely related, putative species of the genus *Elachista*. The taxa were selected for the present study from complexes of closely related species or populations which show differences in host plant selection, larval mine architecture, external appearance of the adults, and to some extent, morphology of the adult genitalia. The morphological differences, however, are slight, sometimes overlapping, and frequently only one sex can be identified by genital characteristics. Based on this kind of evidence the specimens were preliminarily classified to "species," which are assumed to be cohesive genealogical lineages. This practice is in accordance with the current (traditional) concept of delineating species in the Elachistinae, which, however, should not be equated with the "correct" way in our opinion. It is here used as a starting point towards a more integrative taxonomic approach (cf. Dayrat 2005). The "species" are grouped into three informal species complexes, referred below to as "yellow" (name derived from the characteristic yellowish wing colour), "*Ficinia*" (name derived from the host plant genus), and "zigzagger" (name derived from the peculiar zigzagging larval mine) complexes.

Material and methods

Molecular study

The specimens used for molecular analysis are listed in Table 1. All of them were collected as larvae by LK thus confirming the correct host plant association. The larvae were reared in laboratory conditions using standard methods. The samples cover a modest subset of the taxonomic diversity of Australian representatives of *Elachista* subgenus *Elachista* that could be obtained for the molecular study. Presently at least 140 *Elachista* species are recorded from Australia (L. Kaila, unpublished), of which fourteen are treated here.

DNA was extracted usually from legs or head+thorax of single individuals from dry, pinned specimens (Table 1). They are preserved as DNA voucher specimens in the Zoological Museum of the Finnish Museum of Natural History (MZH) DNA voucher specimen collection, and labeled as listed in Table 1. DNA was extracted using the Nucleospin Tissue Kit (Machery-Nagel, Düren, Germany) according to manufacturer's protocols, and re-suspended in 50 µl of ultra-pure water.

PCR's were carried out in 25 µl reactions containing 1–2 µl DNA extract, 1 µl of each primer (at 10 pmol/µl), 0.25 µl of Amplitaq DNA polymerase (5U / µl), 2 µl 2.5 mM $MgCl_2$, 2.5 µl 10X Buffer II (Applied Biosystems) and 4 µl 200 mM dNTP (GeneAmp) and water. Thermocycler conditions were initial denaturing at 95°C 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s annealing at 49°C, 2 min extension at 72°C, followed by a final

extension of 8 min at 72°C. PCR products were purified using the GFX PCR Purification Kit (Amersham Biotech) and then sequenced (using the PCR primers) in both directions using the Big Dye Terminator Cycle Sequencing Kit vs. 1.1 (Applied Biosystems) at one-fourth of the recommended volumes on ABI PRISM 377 DNA sequencer. The primers used for amplifying and sequencing the COI-3' were C1-J-2183 (alias "Jerry", 5' CAACATTTATTTTGATTTTTTGG 3') and tl2-n-3014 (alias "Pat", 5' TCCAATGCACTAATCTGCCATATTA 3') (Simon *et al.* 1994).

Sequences were assembled and edited using Sequence Navigator™ (version 1.01). The alignment of the protein-coding COI was straightforward. Phylogenetic relationships of included terminals were estimated (using equal weights) using the parsimony program NoNa vs. 2.0 (Goloboff 1999) using the command line "hold 100000; hold*; hold/50, mult*100; max*;". Bremer (Bremer 1988, 1994) values were estimated using NoNa and Jackknife support values using WinClada (Nixon 2002).

Morphological study

Adult specimens and their pupal exuviae were examined externally using a stereomicroscope, in order to evaluate possible differences in their colouration and wing shape. Extensive series, whenever available, were dissected using standard procedure (Robinson 1976). The genital morphology was examined using a Wild M10 stereomicroscope (maximum magnification 512x and Leitz Diaplan phase contrast compound microscope (maximum magnification 1560x). The terminology of anatomy follows Traugott-Olsen & Schmidt Nielsen (1977).

Results

Molecular study

We obtained 705 nucleotides of the 3' end of the COI gene spanning nucleotide positions 2239 to 2944 in COI (numbering is based on *Drosophila yakuba* sequence; Clary and Wolstenholme 1985) for 47 ingroup specimens belonging to 14 putative morphospecies and two outgroup taxa. The mean AT was 72.2 %.

The number of parsimony informative sites was 120. Parsimony analysis using NoNa found two equally parsimonious trees (length 290 steps). Their strict consensus cladogram with Bremer support values and Jackknife support values is shown in Fig. 1. COI resolves species groups well, but is invariant in four cases of putative recently diverged species within the 'zigzagger 'complex, and within the two sections of "zigzaggers" at most three bp differences are observed. For species in the 'yellow' clade complex we obtained intraspecifically identical sequences that support morphological taxonomy. On the other hand, the amount of the intraspecific variation of included taxa varied considerably between the complexes.

TABLE 1. Collecting data, DNA voucher and GenBank accession numbers of the specimens examined.

Taxon	Collecting data	DNA voucher MZH	Accession number GenBank
Perittia obscurepunctella Stainton	Finland U Vantaa, Keimola, 18.V.1998 L. Kaila	**LK 10**	**AY800290**
Elachista adscitella Stainton	Finland Al Finström, emg. 1991 ex *Milium effusum* J.-P. Kaitila	L007	**AY800291**
Elachista sp._Cleland	Australia SA Cleland Cons. Pk., emg. 1999 ex *Gahnia* sp. L. Kaila	**LK 4**	**AY800298**
Elachista Ficinia complex 1	Australia SA Moana Sands Cons. Pk., emg. 1999 ex *Ficinia nodosa* L. Kaila	**LK 2**	**AY800292**
Elachista Ficinia complex 1	Australia NSW Long Beach nr. Bateman's Bay, emg. 1999 ex *Ficinia nodosa* L. Kaila	**LK 3**	**AY800293**
Elachista Ficinia complex 1	Australia NSW Burrewarra Point, emg. 2001 ex *Ficinia nodosa* E. Edwards & L. Kaila	**LK 25**	**AY800294**
Elachista Ficinia complex 1	Australia NSW Burrewarra Point, emg. 2001 ex *Ficinia nodosa* E. Edwards & L. Kaila	**LK 35**	**AY800295**
Elachista Ficinia complex 2	Australia WA Myalup Beach, emg. 2001 ex *Ficinia nodosa* L. Kaila	**LK 26**	**AY800296**
Elachista Ficinia complex 2	Australia WA Myalup Beach, emg. 2001 ex *Ficinia nodosa* L. Kaila	**LK 36**	**AY800297**
Elachista yellow complex 1	Australia WA Hamelin Bay, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 41**	**AY800299**
Elachista yellow complex 1	Australia WA Yanchep NP., emg. 2001 ex *Lepidosperma ?elatius* L. Kaila	**LK 43**	**AY800300**
Elachista yellow complex 1	Australia WA Porongurup, emg. ex *Lepidosperma ?elatius* L. Kaila	**LK 44**	**AY800301**
Elachista yellow complex 2	Australia WA Porongurup, emg. 2001 ex *Lepidosperma ?elatius* L. Kaila	**LK 22**	**AY800302**
Elachista zigzagger complex A1	Australia NSW Burrewarra Point, emg. 2001 ex *Lepidosperma concavum* E. Edwards & L. Kaila	**LK 28**	**AY800303**
Elachista zigzagger complex A1	Australia NSW Burrewarra Point, emg. 2001 ex *Lepidosperma concavum* E. Edwards & L. Kaila	**LK 46**	**AY800306**
Elachista zigzagger complex A2	Australia NSW Morton NP., emg. 1999 ex *Lepidosperma viscidum/concavum* L. Kaila	**LK 69**	**AY800329**
Elachista zigzagger complex A3	Australia SA Aldinga Scrub Cons. Pk., emg. 2001 ex *Lepidosperma sp. nr. laterale* L. Kaila	**LK 70**	**AY800330**
Elachista zigzagger complex A3	Australia SA Aldinga Scrub Cons. Pk., emg. 2001 ex *Lepidosperma sp. nr. laterale* L. Kaila	**LK 71**	**AY800331**
Elachista zigzagger complex A4	Australia WA Kalamunda, emg. 2001 ex "cylindrical *Lepidosperma*" L. Kaila	**LK 72**	**AY800332**
Elachista zigzagger complex A5	Australia WA Kalamunda, emg. 2001 ex *Lepidosperma longitudinale* L. Kaila	**LK 73**	**AY800333**
Elachista zigzagger complex A6	Australia SA Cox's Scrub Cons. Pk., emg. 2001 ex *Lepidosperma longitudinale* L. Kaila	**LK 75**	**AY800334**
Elachista zigzagger complex A6	Australia SA Cox's Scrub Cons. Pk., emg. 2001 ex *Lepidosperma longitudinale* L. Kaila	**LK 76**	**AY800335**
Elachista zigzagger complex B1	Australia WA Warren NP., emg. 2001 ex *Lepidosperma effusum* L. Kaila	**LK 78**	**AY800336**
Elachista zigzagger complex B1	Australia WA Margaret River, emg. 2001 ex "striated *Lepidosperma*" L. Kaila	**LK 79**	**AY800337**
Elachista zigzagger complex B1	Australia WA Margaret River, emg. 2001 ex "striated *Lepidosperma*" L. Kaila	**LK 80**	**AY800338**
Elachista zigzagger complex B1	Australia WA Cervantes, L. Thetys, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 29**	**AY800304**

TABLE 1 (continued).

Taxon	Collecting data	DNA voucher MZH	Accession number GenBank
Elachista zigzagger complex B1	Australia WA Myalup, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 30**	**AY800305**
Elachista zigzagger complex B1	Australia WA Cervantes, L. Thetys, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 47**	**AY800307**
Elachista zigzagger complex B1	Australia WA Myalup, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 48**	**AY800308**
Elachista zigzagger complex B1	Australia WA Jewel Cave, emg. 2001 ex "striated *Lepidosperma* sp." L. Kaila	**LK 49**	**AY800309**
Elachista zigzagger complex B1	Australia WA 10 km WSW Albany, emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 50**	**AY800310**
Elachista zigzagger complex B1	Australia WA Jewel Cave, emg. 2001 ex "striated *Lepidosperma* sp." L. Kaila	**LK 51**	**AY800311**
Elachista zigzagger complex B1	Australia WA Jewel Cave, emg. 2001 ex "striated *Lepidosperma* sp." L. Kaila	**LK 52**	**AY800312**
Elachista zigzagger complex B1	Australia WA Myalup Beach, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 57**	**AY800317**
Elachista zigzagger complex B1	Australia WA Myalup Beach, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 58**	**AY800318**
Elachista zigzagger complex B1	Australia WA Yalgorup NP., emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 61**	**AY800321**
Elachista zigzagger complex B1	Australia WA Yalgorup NP., emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 62**	**AY800322**
Elachista zigzagger complex B1	Australia WA Torndirrup NP., emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 63**	**AY800323**
Elachista zigzagger complex B1	Australia WA: Torndirrup NP., emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 64**	**AY800324**
Elachista zigzagger complex B1	Australia WA 10 km WSW Albany, emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 65**	**AY800325**
Elachista zigzagger complex B1	Australia WA 10 km WSW Albany, emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 66**	**AY800326**
Elachista zigzagger complex B1	Australia WA Torndirrup, emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 67**	**AY800327**
Elachista zigzagger complex B1	Australia WA Torndirrup, emg. 2001 ex "small *Lepidosperma* sp." L. Kaila	**LK 68**	**AY800328**
Elachista zigzagger complex B2	Australia WA Myalup Beach, emg. 2001 ex "spongy *Lepidosperma* sp." L. Kaila	**LK 55**	**AY800315**
Elachista zigzagger complex B2	Australia WA Myalup Beach, emg. 2001 ex "spongy *Lepidosperma* sp." L. Kaila	**LK 56**	**AY800316**
Elachista zigzagger complex B2	Australia WA Yanchep NP., emg. 2001 ex "spongy *Lepidosperma* sp." L. Kaila	**LK 59**	**AY800319**
Elachista zigzagger complex B2	Australia WA Yanchep NP., emg. 2001 ex "spongy *Lepidosperma* sp." L. Kaila	**LK 60**	**AY800320**
Elachista zigzagger complex B3	Australia WA Esperance, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 53**	**AY800313**
Elachista zigzagger complex B3	Australia WA Esperance, emg. 2001 ex *Lepidosperma gladiatum* L. Kaila	**LK 54**	**AY800314**

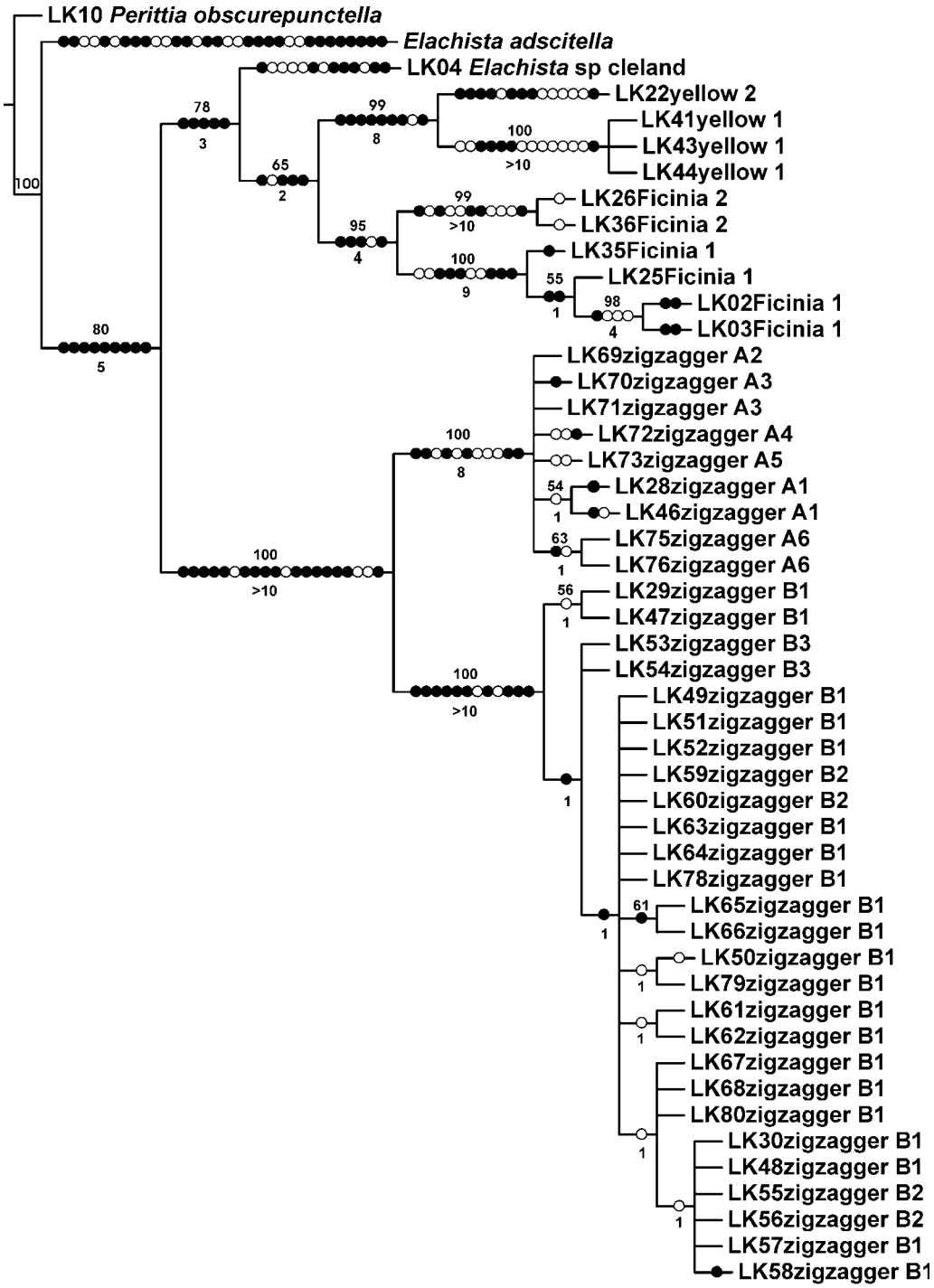

FIGURE 1. Strict consensus cladogram (length 293 steps, CI 0.75, RI 0.92) of the two equally parsimonious cladograms based on parsimony analysis of 705 nt 3' end of the COI gene for 47 ingroup specimens belonging to 14 morphospecies and two outgroup taxa. Bremer support values are given below, and Jackknife values above the branches. Black dots indicate unique synapomorphies, open circles homoplastic synapomorphies.

Uncorrected interspecific pairwise divergence between yellow 1 and yellow 2 was 4.54%, and that between *Ficinia* 1 and *Ficinia* 2 samples ranged from 3.69% to 4.40%. Between the taxa of the A section of the zigzagger complex these value ranged from 0.0% to 0.85%. Between the taxa of the B section of the zigzagger complex they ranged from 0.0% to 0.71%. The divergence between the taxa of A and B sections ranged from 3.55% to 4.11%. The divergence between the yellow and *Ficinia* complexes (yellow 1 vs. *Ficinia* 1) ranged between 5.96%–6.95%, and divergences between yellow and *Ficinia* complex taxa vs. zigzagger complex's taxa were of the same magnitude.

Uncorrected intraspecific pairwise divergences of the yellow 1 was 0.0%, within *Ficinia*-group complex taxa the values ranged from 0.28–1.56%. Within the A section of the zigzagger complex values ranged from 0.0% to 0.43%, and within the B section values were 0.0% to 0.85%, respectively.

The division of the zigzagger complex into A and B sections, based on the presence/ absence of the female signum (for definition see Scoble 1992), was supported by the sequence data. Intra- and interspecific uncorrected pairwise divergences between specimens and hypothetical taxa of the zigzagger complex were almost completely overlapping, with intraspecific divergences greater than interspecific in some cases. Therefore, within the zigzagger B complex of species the morphospecies B1 appears paraphyletic with respect to two other species, and B2 appears as polyphyletic.

The intraspecific divergence ranges for zigzagger A and B complexes overlap with the interspecific ranges between taxa of these complexes; hence, intraspecific divergences are bigger than interspecific values in some instances.

Study of morphology and ecology

External appearance is similar between *Ficinia* complex species 1 and 2 (Figs. 2 A–F). The taxa exhibit differences in genitalia as follows. Males: The uncus lobes are smaller in sp. 1 than in sp. 2, the valva is medially narrowed in sp. 2, not in sp. 1, and the digitate process of sp. 1 is distally curved, blunt-tipped in sp. 2 (Figs. 3 A, B). Females: Sp. 1: basal dilation of the ductus seminalis is naked, and its colliculum is slightly longer than antrum, occupying half of the length of caudal part of ductus bursae. Sp. 2: basal dilation of the ductus seminalis is sparsely covered with small sclerotised internal granules; the colliculum of sp. 2 is almost twice as long as the antrum, occupying 2/3 of the length of the caudal part of ductus bursae (Figs. 4 A, B). The biologies of *Ficinia* 1 and 2 are similar - both species tunnel under the epidermis of the culm of *Ficinia nodosa*, making a straight yellowish mine.

External appearance is similar between yellow complex species 1 and 2. (Figs. 2 C, D). The taxa exhibit differences in genitalia as follows. Males: the distal opening of the phallus is dorsally extended as a small and indistinctly delimited rounded–triangular extension in sp. 1; it bears a small but distinctive dorsolaterally directed triangular lobe in sp. 2 (Fig. 3 C, D). Females: the ostium bursae of sp. 2. is narrower than that of sp. 1. In

sp.1 it occupies about 1/3 of the distance between apophyses anteriores, in sp. 2 about 1/4 of the distance between apophyses anteriores. The lateral margin of the antrum is somewhat convex in sp. 1, straight in sp. 2 (Figs. 4 D, E). The biologies of yellow complex 1 and 2 are similar. Both species make a straight mine on the leaf of *Lepidosperma ?elatius* [nomenclature and taxonomy of the plant genus *Lepidosperma* (Cyperaceae) is somewhat unclear (J. Bruhl, personal communication)].

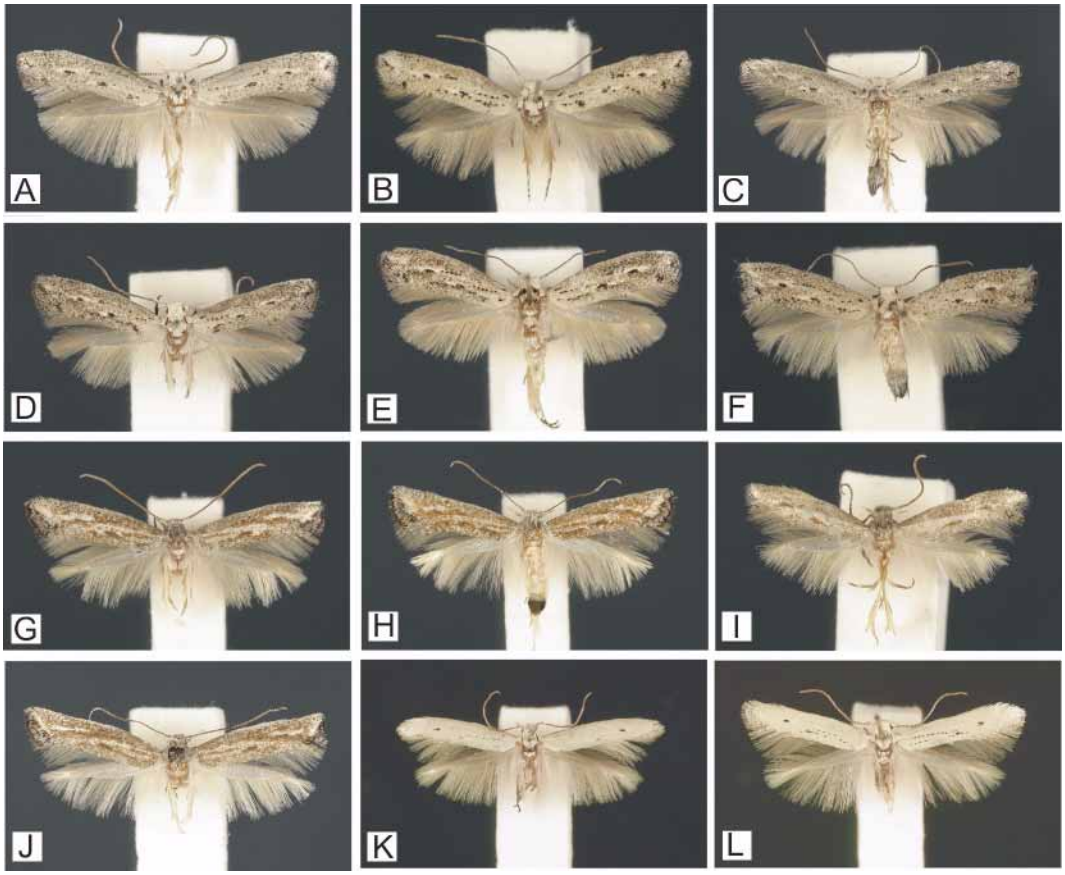

FIGURE 2. External appearance of *Elachista* spp. A: *Elachista Ficinia* complex 1 male (Australia: SA Adelaide); B: *Elachista Ficinia* complex 1 male (Australia: NSW Burrewarra Point); C: *E lachista Ficinia* complex 1 female (Australia: SA: Normanville); D and E: *Elachista Ficinia* complex 2 male (Australia: WA Myalup Beach); F: *Elachista Ficinia* complex 2 female (Australia: WA Myalup Beach); G: *Elachista* yellow complex 1 male; H: *Elachista* yellow complex 1 female; I: *Elachista* yellow complex 2 male; J: *Elachista* yellow complex 2 female; K: *Elachista* Cleland sp. male; L: *Elachista* Cleland sp. female.

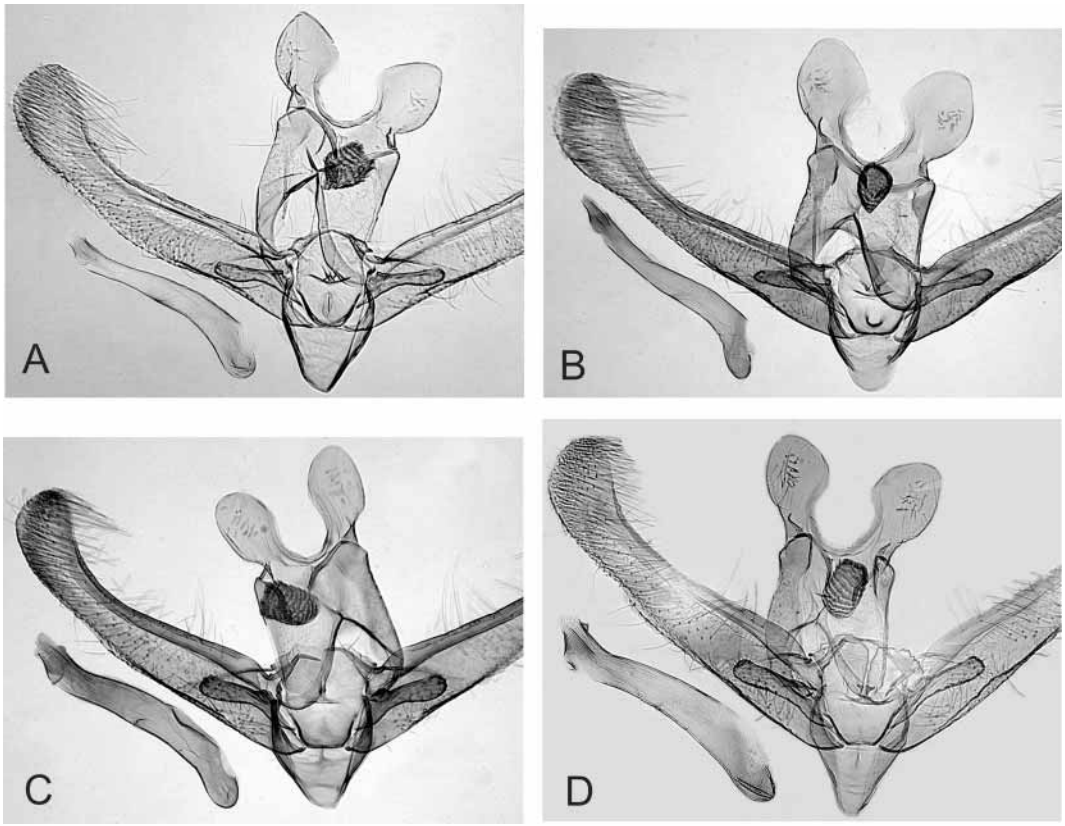

FIGURE 3. Male genitalia of *Elachista* taxa, in ventral view. A: *Elachista Ficinia* complex 1; B: *Elachista Ficinia* complex 2; C: *Elachista* yellow complex 1; D: *Elachista* yellow complex 2.

External appearance differs to some extent among the taxa of the zigzagger complex (Fig. 5). A2 and A6 are the largest, and A4 is the smallest and most narrow-winged. A1, A3, or A5 can hardly be distinguished from each other on the basis of their appearance only. All taxa of the A section are greyer than those of B section whose forewings are powdered with brownish scales as well. B1, B2, and B3 are similar to each other. Examination of a large number of samples of the B1–B3 taxa implies, however, the following trends: B2 tends to be the darkest, brownest, and most broad-winged of these taxa; and B3 has quite bright black markings in the distal part of its forewing.

The male genitalia of taxa of the zigzagger complex are shown in Figs. 6 and 7. A2 seems consistently distinguishable from others by the thin basal half of the phallus (Fig. 6 B). The cucullus of the valva is more expanded in A6 than in the other species. The juxta lobes of A5 differ in their shape from the other species (Fig. 6 E). Although the shape of the spinose knob of the gnathos is somewhat variable within the taxa, it nevertheless distinguishes A2 and B3 from others, as being basally broader in these taxa than in others.

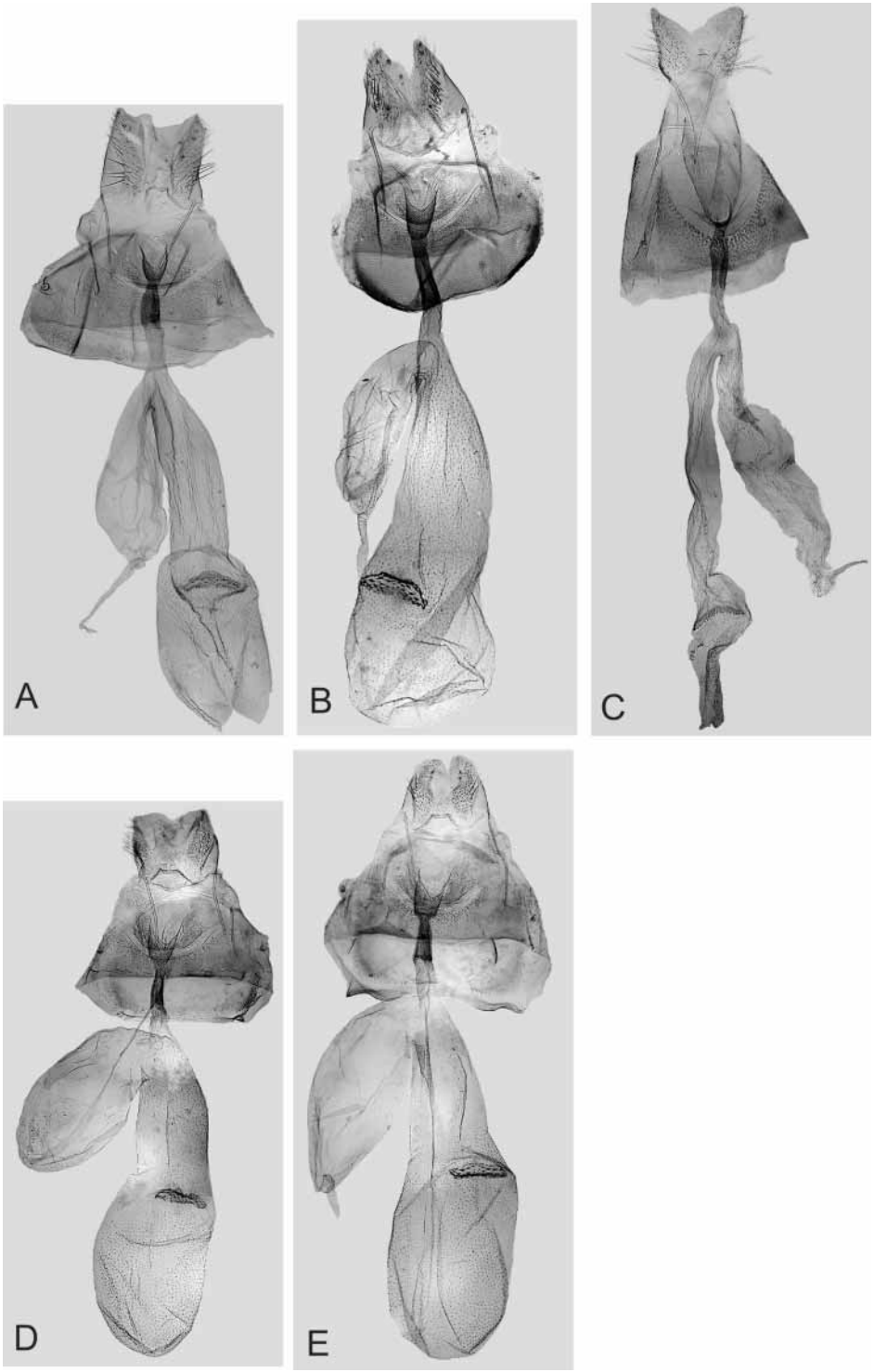

FIGURE 4. Female genitalia of *Elachista* spp. A: *Elachista Ficinia* complex 1; B: *Elachista Ficinia* complex 2; C: *Elachista* Cleland sp.; D: *Elachista* yellow complex 1; E: *Elachista* yellow complex 2.

A summary of the further male genital characters of the taxa of the zigzagger complex is given in Table 3. The females of this groupcomplex (Figs. 8–10) are keyed here:

1. Signum present (Fig. 8) 2
- Signum absent (Fig. 9) 7
2. Between sclerotised spinose area that surrounds ostium and invagination of integument between sternum 7 and 8 a membranous area that is wider than invagination of integument between sternum 7 and 8 (Fig. 10 F)..... A6
- Sclerotised spinose area that surrounds ostium and invagination of integument between sternum 7 and 8 without membranous area, or membranous area narrower than invagination of integument between sternum 7 and 8 8 (Fig. 10 A–E) 3
3. Width of ostium bursae half of width of invagination of integument between sternum 7 and 8 (Fig. 10D) A4
- Width of ostium bursae less than half of width of invagination of integument between sternum 7 and 8 4
4. Caudal longitudinal, and cephalic transverse sclerotisations of colliculum separate from each other (Figs. 10 B, E)..... 5
- Sclerotisations of colliculum fused to each other (Figs. 10 A, C) 6
5. Cephalic transverse sclerotisation of colliculum a simple evenly sclerotised bent band (Fig. 10 E) A5
- Cephalic transverse sclerotisation of colliculum asymmetric, with one end strongly sclerotised and sickle-shaped (Fig. 10 B)..... A2
6. Ductus bursae constricted at caudal end of colliculum (Fig. 10 A) A1
- Ductus bursae not constricted at caudal end of colliculum (Fig. 10 C) A3

7. Females of *Elachista* zigzagger complex B1, B2 and B3 only identifiable from immature stages.

There appear to be no differences in the immature stages or the life histories among yellow complex species or among the *Ficinia* complex species. The pupal exuviae of the taxa of the zigzagger complex are characterised by the dark brownish grey mesial area of the ventral side (Fig. 11). There appear to be constant differences in this character among some taxa of this complex: A2 has most expanded dark area with forewing veins visible as pale only laterally; the A taxa have in general larger dark area than B taxa. The larval mines of the zigzagger complex taxa A1, A2, and B2 are shown in Fig. 12. The biological traits of the taxa of the zigzagger complex are summarised here. Some differentiating traits are summarised in Table 3.

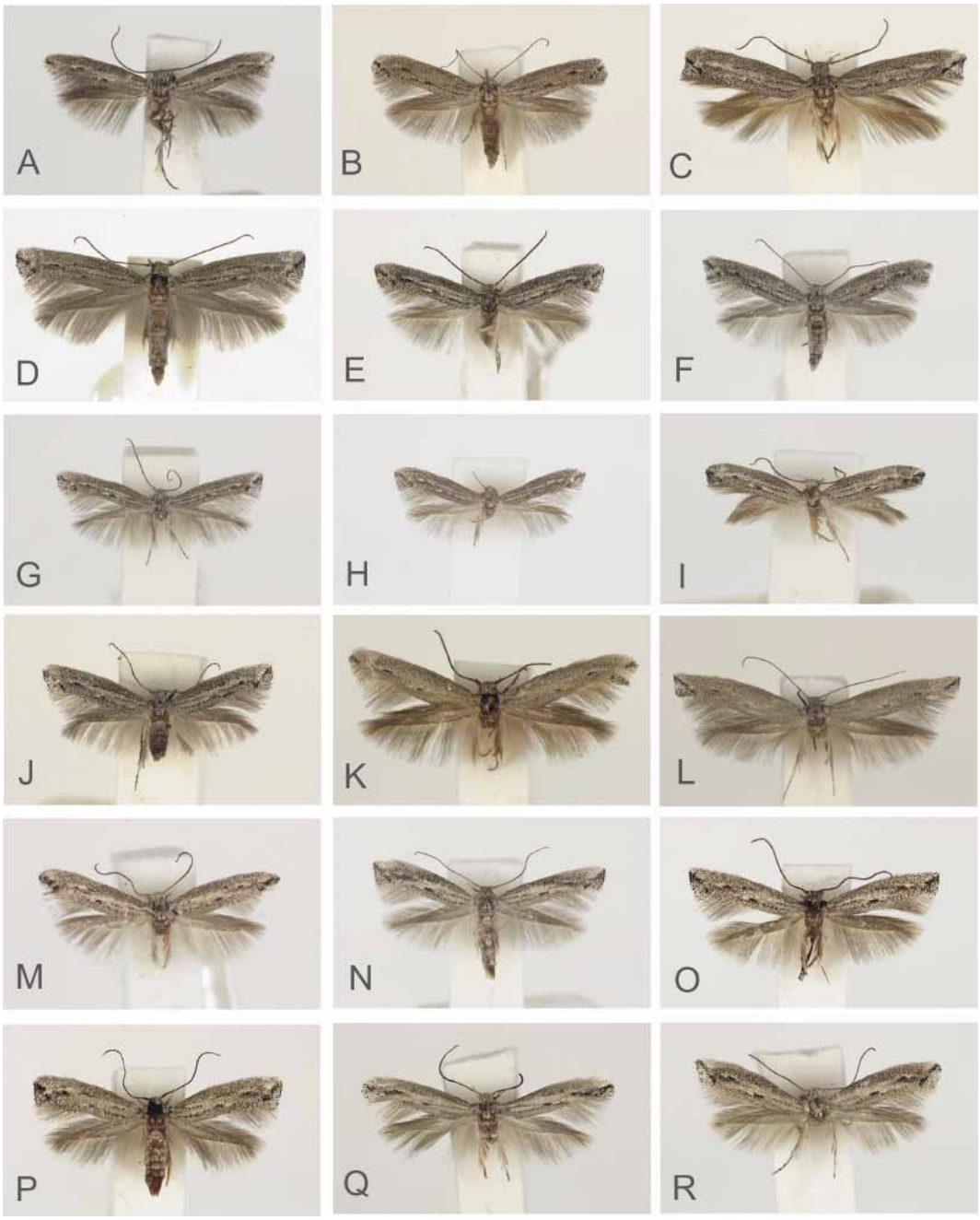

FIGURE 5. External appearance of the taxa of the *Elachista* zigzagger complex. A: A1 male; B: A1 female; C: A2 male; D: A2 female; E: A3 male; F: A3 female; G: A4 male; H: A4 female; I: A5 male; J: A5 female; K: A6 male; L: A6 female; M: B1 male; N: B1 female; O: B2 male; P: B2 female; Q: B3 male; R: B3 female.

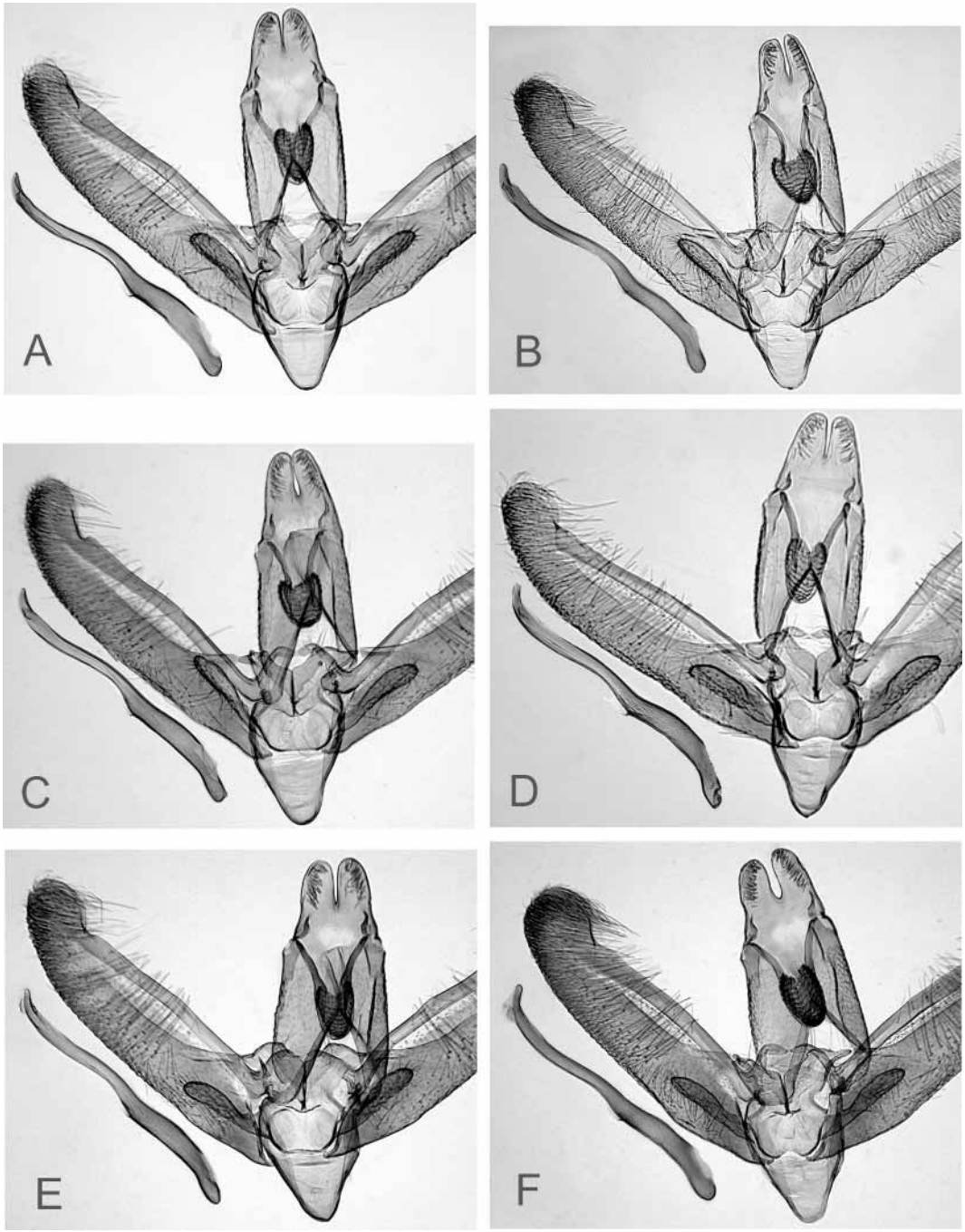

FIGURE 6. Male genitalia of the taxa of the *Elachista* zigzagger complex, in ventral view. A: A1; B: A2; C: A3; D: A4; E: A5; F: A6.

The taxon A1 inhabits shaded or half-open dry sites where its host plants, *Lepidosperma concavum* and *L. laterale*, grow. The egg is laid near base of the leaf near

the leaf margin. The larva starts mining during summer and completes development in late autumn–early winter. The full-grown larva hibernates in the mine until August. The initial stage of the mine is narrow, about 15 cm long and visible on both sides of the leaf, with a characteristic pattern. The mine first runs about 4 cm straight upwards along the margin, then makes a 100° angle and moves to the other edge where it runs along the edge another centimetre, and again moves back in 100° angle. It makes 4–6 such turns, after which the larva turns to mine downwards. Then the mine abruptly broadens, filling the whole width of the leaf forming a 3 cm long swollen chamber. The lower part of the chamber is yellowish, the upper part green. If the small larva is parasitised, the sharp angles of the mine are more wavy and undulating. The larva exits the mine during August. Over a hundred mines were examined.

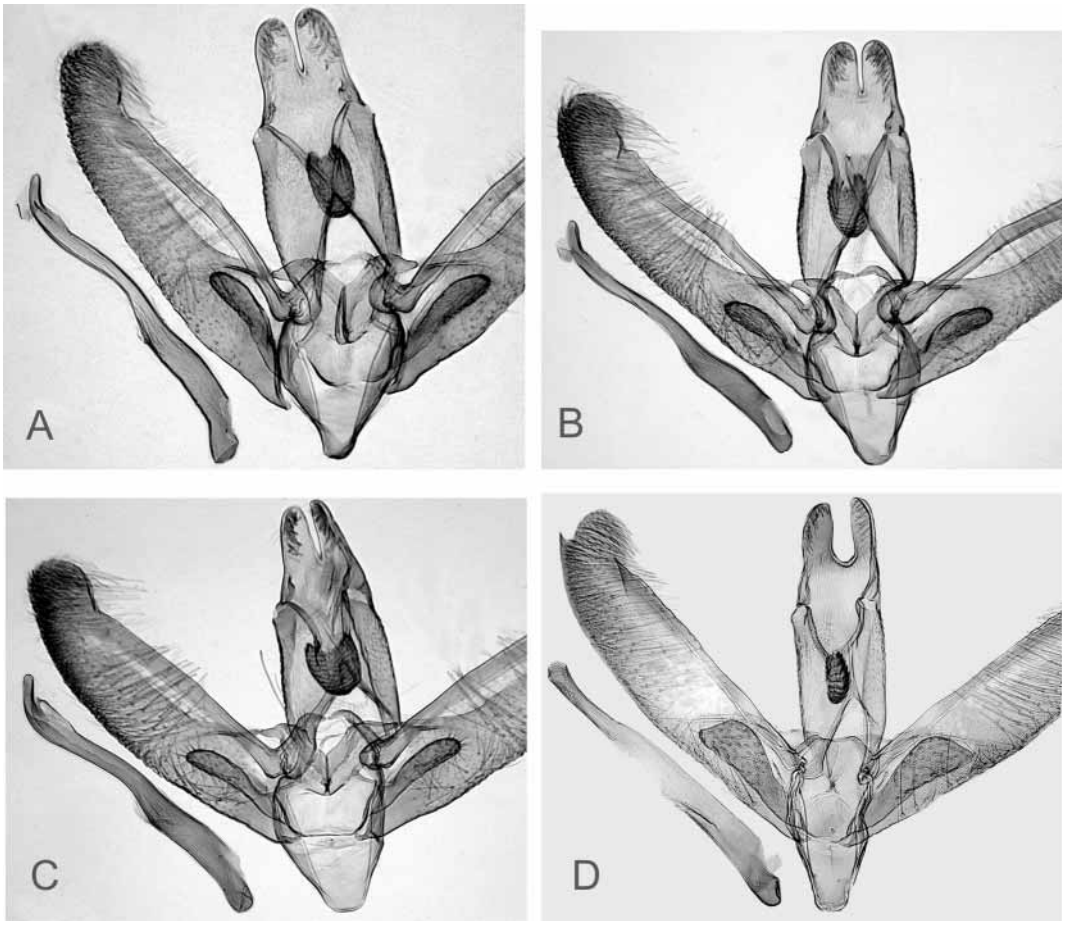

FIGURE 7. Male genitalia of *Elachista* taxa, in ventral view. A: *Elachista* zigzagger complex B1; B: *Elachista* zigzagger complex B2; C: *Elachista* zigzagger complex B3: D: *Elachista* 'Cleland sp.'.

Larvae of A2 were found in moist sandstone sites with open stone surfaces surrounded by dense bush vegetation and small eucalypts. *Lepidosperma concavum* and *L. viscidum* have been recorded as the host plants. The egg is laid near base of the leaf near margin. The initial stage of the mine is narrow, about 20 cm long, otherwise similar to that of A3, but the intervals of the nearly right-angled turns are about 10–15 mm from each other, usually not quite reaching the margin of the leaf on one side. Every other across-leaf section of the mine is visible only on the upper side of the leaf, while the remaining are visible only on the lower side. Then the mine abruptly broadens encompassing nearly the entire width of the leaf, and the larva mines downwards making a 5–7 cm long swollen chamber. The larva feeds during autumn and early winter, and appears to hibernate as a full-grown larva within the mine. The larva exits the mine during August. The initial mines of two larvae have been observed in the same leaf, but under such conditions apparently only one of them is able to survive. About 50 mines were examined.

TABLE 2. Male genital traits of the taxa of the *Elachista* zigzagger group complex..

	Scales of juxta lobes	Length of aedeagus as % of length of valva	Length of valva in relation to width of valva
complex A1	as group on truncate lobe	80 %	4.5 x length of v.
complex A2	in row along distal margin	85–92 %	4.5 x length of v.
complex A3	as group on truncate lobe	85 %	4.5 x length of v.
complex A4	as group on truncate lobe	75 %	4.5 x length of v.
complex A5	as group near convex dist. margin	78–80 %	4.5 x length of v.
complex A6	as group near convex dist. margin	85 %	4 x length of v.
complex B1	as group in convex dist. margin	82–84 %	4.5 x length of v.
complex B2	as group in convex dist. margin	82 %	4.5 x length of v.
complex B3	as group in convex dist. margin	80–83 %	4.5 x length of v.

Larvae of A3 were found in shaded and open forest in rather dry sites where its host plants, *Lepidosperma curtisiae* and *Lepidosperma* sp. nr. *laterale*, grow. The egg is laid near the base of the leaf near the margin. The initial mine is narrow, 5–10 cm long in *L. curtisiae*, 15 cm in *L.* sp., similar to that of A2. The mine runs, however, along margins of the leaf. The intervals of the nearly right-angled turns are about 5 mm from each other, and every second across-leaf section of the mine is visible only on the upper, every second on the lower side of the leaf. Then the mine abruptly broadens filling the whole width of the leaf, and the larva mines downwards making a 2–5 cm long swollen chamber. In *L.*

curtisiae the leaf soon withers above the mine. The larva feeds during autumn and early winter, and appears to hibernate as a full-grown larva within the mine. It exits the mine during August. About 50 mines were examined.

TABLE 3. Mine traits of the taxa of the *Elachista* zigzagger-group complex.

	Angle of turns	reaching of leaf edge	Visibility on one/both sides of the leaf
complex A1	100°	y	both
complex A2	90°	n	one
complex A3	90°	y	one
complex A4	90°	?	?
complex A5	90°	y	one
complex A6	90°	y	one
complex B1	100°	y/n	one
complex B2	110°	y	(both)
complex B3	100°	n	one

Larvae of A4 were found in an exposed site where its host plant, *Lepidosperma* sp., grows. The unidentified host plant species is characterised by its cylindrical culm (flower stem) transsection. The initial mine is narrow, about 10 cm long. The initial mine resembles that of the other species in the zigzagger complex, making turns at right angle at intervals of 5 mm on its way upwards the culm. Finally the mine abruptly broadens, and the larva mines downwards making a 4 cm long chamber which occupies the entire space of the culm. The larva feeds during winter and exits the mine during July. Fifteen mines were examined.

Larvae of A5 were found in a shaded site where its host plant, *Lepidosperma longitudinale*, grows. The egg is laid near base of the leaf near margin. The initial mine is narrow, about 15 cm long. The mine first runs 1 cm straight upwards along the margin, then makes a right angle and moves to the other edge where it runs along the edge 7 mm, and again moves back in a right angle. The intervals of the right-angled turns are regular, 7 mm from each other, and every other across-leaf section of the mine is visible only on the upper side of the leaf, the remaining only on lower side. Then the mine abruptly broadens, and the larva mines downwards making a 4 cm long swollen chamber, which occupies two-thirds of the width of the leaf. The larva feeds during winter and larva exits the mine during July. Thirty mines were examined.

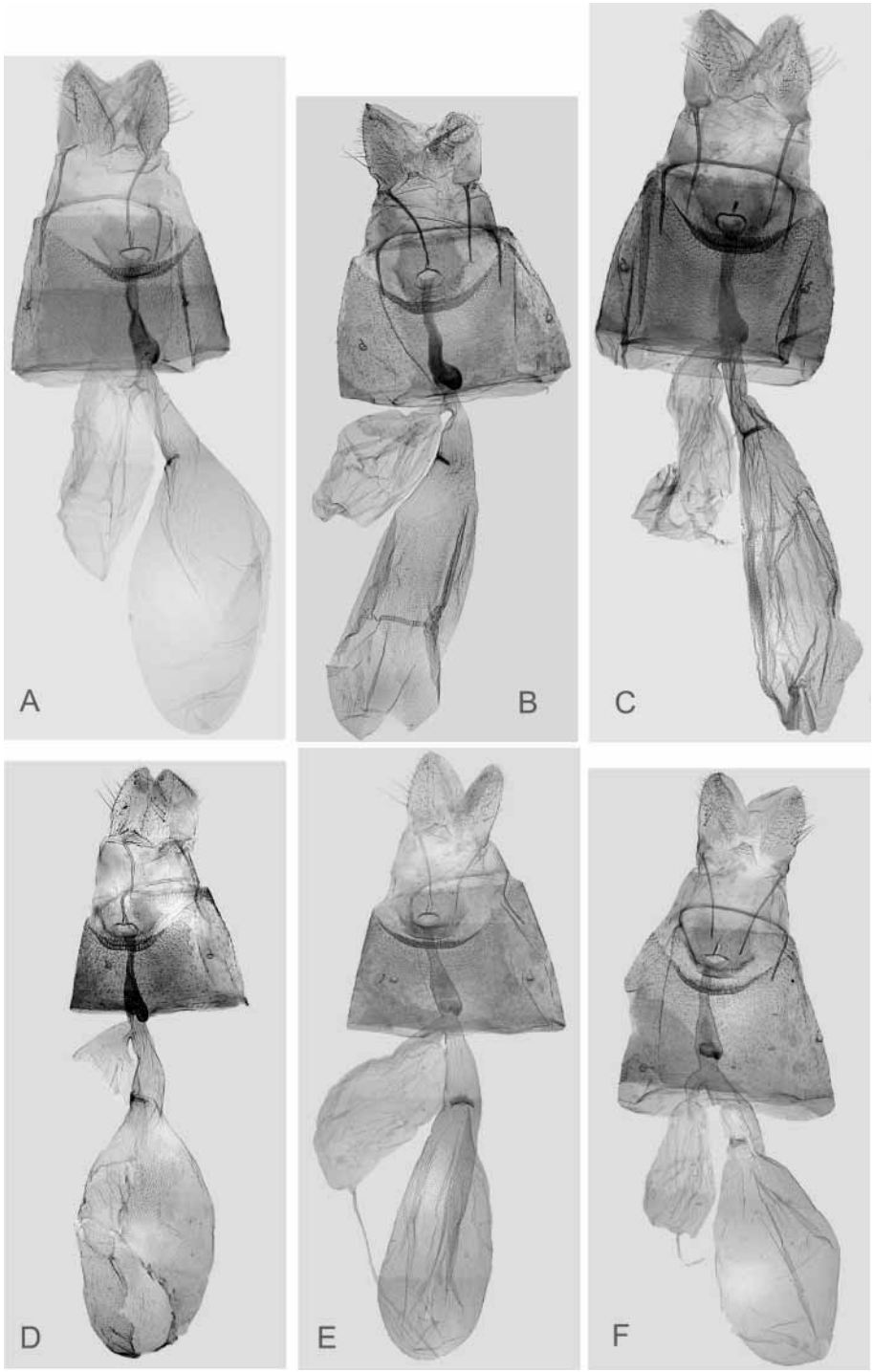

FIGURE 8. Female genitalia of the taxa of the *Elachista* zigzagger complex. A: A1; B: A2; C: A3; D: A4; E: A5; F: A6.

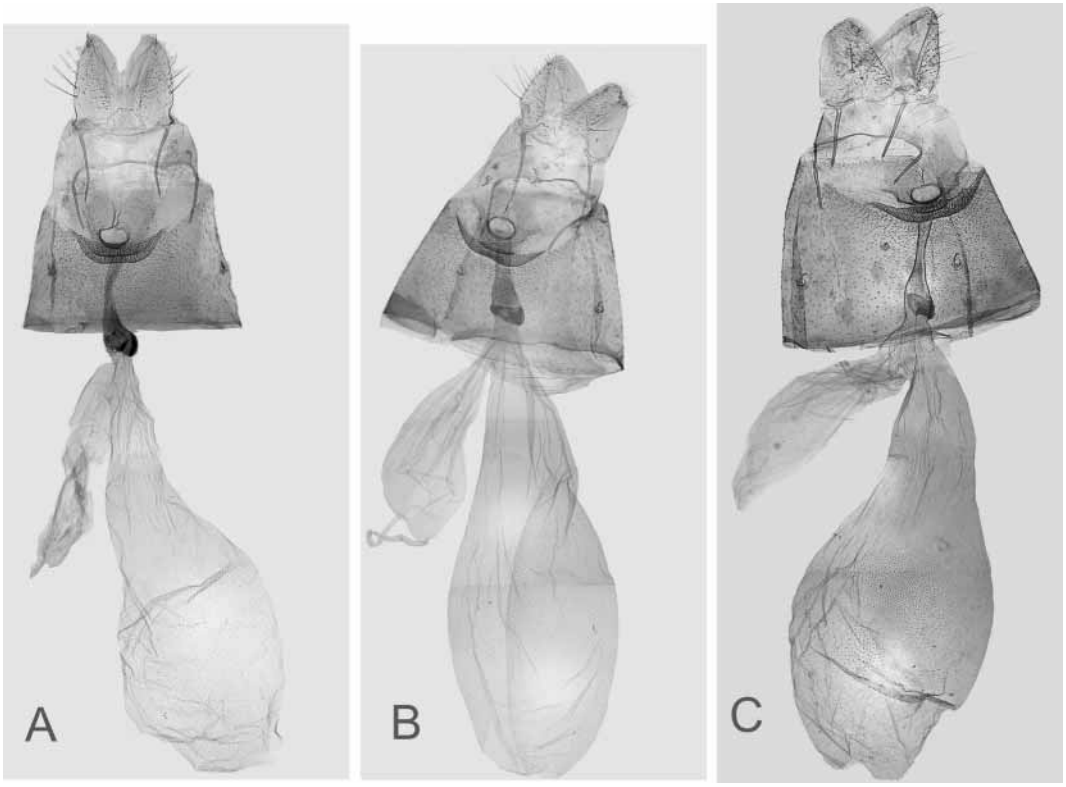

FIGURE 9. Female genitalia of the taxa of the *Elachista* zigzagger complex. A: B1; B: B2; C: B3.

Larvae of A6 were found in an open, moist site in a sparse stand of its host plant, *Lepidosperma longitudinale*. The egg is laid near base of the leaf near margin. The initial mine is narrow, about 25 cm long. The mine first runs 6 cm straight upwards along the margin, then makes a right angle and moves to the other edge where it runs along the edge 1 cm, and again moves back in a right angle. The intervals of the right-angled turns are regular, 1 cm from each other, and every second across-leaf section of the mine is visible only on upper, every second on lower side of the leaf. Then the mine abruptly broadens filling the whole width of the leaf, and the larva mines downwards making a 5 cm long swollen chamber which occupies most of the width of the leaf. The larva feeds during winter and exits the mine during August. Fifteen mines were examined.

Larvae of B1 have been found in open and shaded sites mining leaves of various *Lepidosperma* species. The egg is laid near base of the leaf near margin. The initial mine is narrow, about 25 cm long. The mine first runs some centimetres straight upwards near the margin, then makes a 90–100° angle and moves to the other edge where it runs along the edge 0.5 cm, and again moves back in a similar angle. In narrow-leaved host plants this is repeated up to 10 times. In *L. gladiatum* the shape of the mine is different: the larva does not reach the edge of the leaf but mines within the median 2/3 of

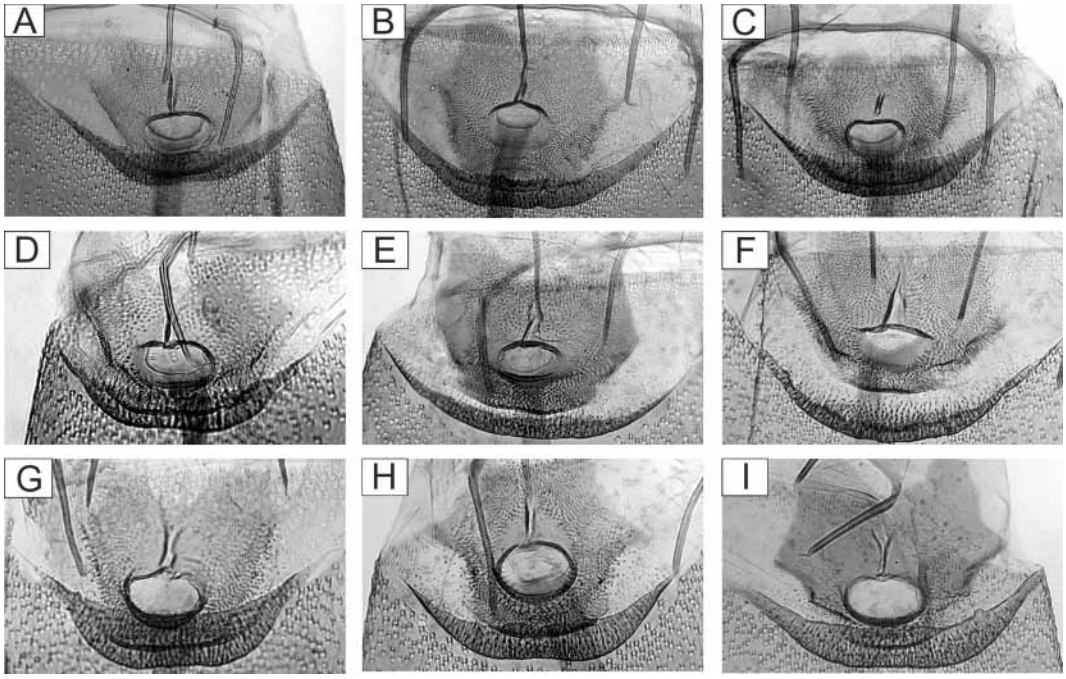

FIGURE 10. Ostium bursae of the female of the taxa of the *Elachista* zigzagger complex. A: ‘ A1; B: ‘ A2; C: A3; D: A4; E: A5; F: A6; G: B1; H: B2; I: B3.

the leaf width. It makes a few turns of 100°, after which the turns become more frequent. The larva makes 100° angle turns in every 2–4 mm, after which the mine continues as irregularly undulating for some centimetres. Every second across-leaf section of the mine is visible only on upper, every second on lower side of the leaf. Finally the mine abruptly broadens and the larva mines downwards making a 5 cm long swollen chamber which occupies half of the width of the leaf. The larva feeds during winter and exits the mine during July-August. Over two hundred mines were examined.

Larvae of B2 were found in shady lakeshore sites mining leaves of an unidentified very large *Lepidosperma* species characterised by the spongy matrix of its leaves. The initial mine is narrow, about 25 cm long. The mine first runs 5 cm straight upwards near the margin, then makes a 100° angle and moves to the other edge where it runs along the edge 5 millimetres, and again moves back in a 100° angle. The larva makes a few such turns, after which the turns become more frequent, the larva mining parallel to the leaf edge 2 mm, and makes 110° angle turns whenever it encounters the leaf margin. This is repeated about five times. The mine runs within the epidermis, and is equally faintly visible in both sides of the leaf as a reddish brown line. Finally the mine abruptly broadens and the larva mines downwards making a 5 cm long swollen chamber which occupies half of the width of the leaf. The larva feeds during winter and exits the mine during July-August. Sixty mines were examined.

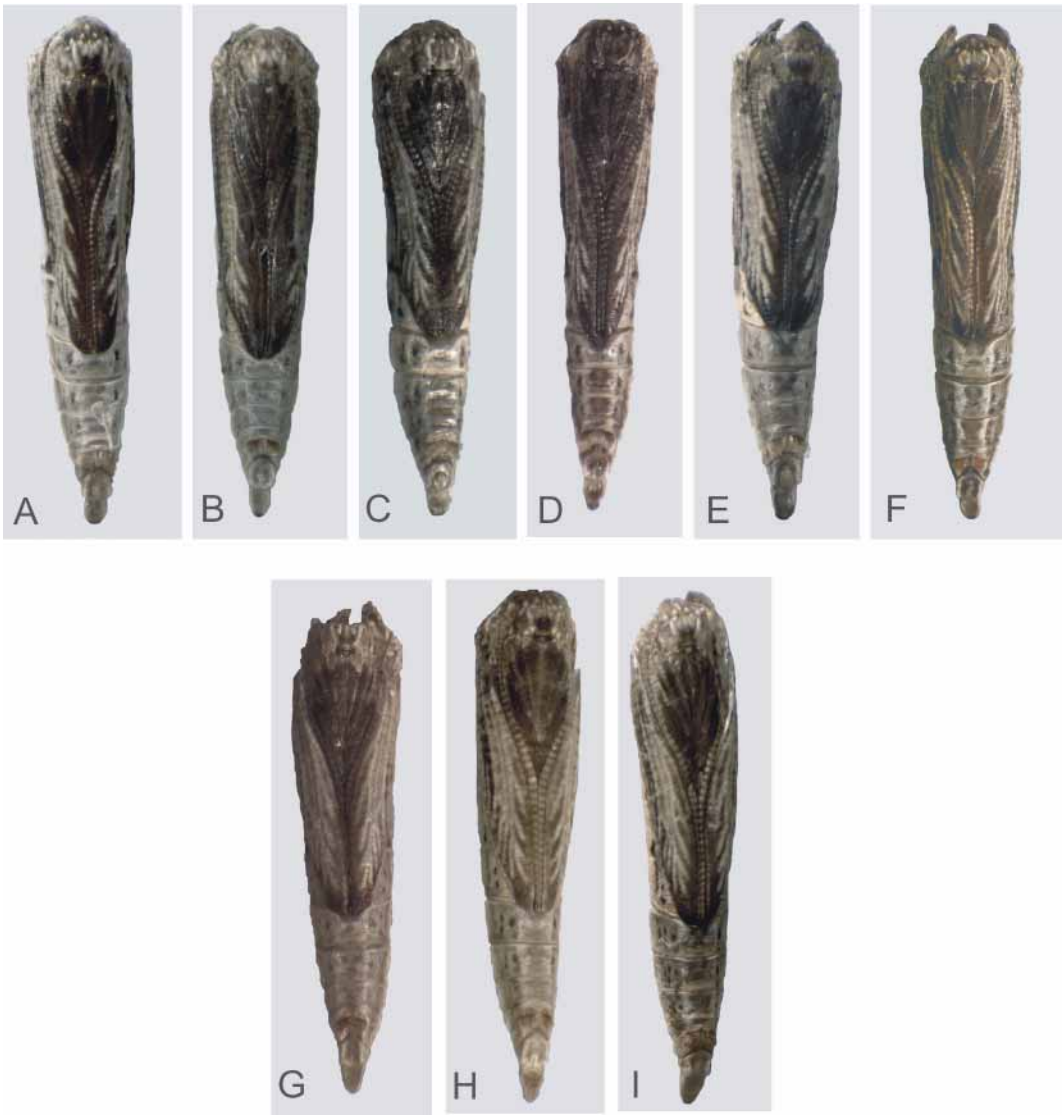

FIGURE 11. Pupal exuviae of the taxa of the *Elachista* zigzagger complex, in ventral view. A: A1; B: A2; C: A3; D: A4; E: A5; F: A6; G: B1; H: B2; I: B3.

Larvae of B3 were found in open sandy site mining leaves of *Lepidosperma gladiatum*. The egg is laid near base of the leaf near margin. The initial mine is narrow, about 25 cm long. The mine first runs 10 cm straight upwards near the margin, then makes a 100° angle and moves to the other edge where it runs along the edge 1 cm, and again moves back in a 100° angle. The larva makes a few such turns, after which the turns become more frequent, the larva not mining parallel to the leaf edge at all, but makes 100° angle turns whenever it encounters the leaf margin. This is repeated about five times. Then the larva again changes the style as continuing near midrib of the leaf, the mine continuing as irregularly undulating for about five centimetres. Every other across-leaf section of the

mine is visible only on the upper surface of the leaf, the remainder only on lower surface. Finally the mine abruptly broadens and the larva mines downwards making a 5 cm long swollen chamber which occupies half of the width of the leaf. The larva feeds during winter and exits the mine during July-August. Thirty mines were examined.

Taxonomic conclusions

Integrating the evidence from the 3' end of CO1 mitochondrial DNA sequences, biological traits of larvae, and pupal and adult morphology, we conclude the following. Yellow 1 and yellow 2 are distinct species supported by MtDNA and genital morphology. *Ficinia* 1 and 2 are distinct species based on similar reasoning. The A and B sections of the zigzagger complex are distinct, monophyletic entities.

A1 merits species rank based on its characteristic larval mine and its female genital morphology; the genomic data neither strongly support nor contradict this conclusion.

A2 and A3 are distinct from other taxa based on their characteristic larval mines and their male and female genital morphology; the mitochondrial data neither strongly support nor contradict this conclusion. The two species cannot be distinguished from each other based on mtDNA data (uncorrected pairwise divergence 0.0%). Characteristics of their adult external appearance, as well as both their male and female genitalia, suggest that they belong to separate cohesive genealogical units, i.e., are distinct species.

A4 merits species rank owing to its life history, adult external appearance, and female genitalia; the mtDNA data neither strongly support nor contradict this conclusion.

A5 merits species rank owing to its life history and male and female genitalia; the mtDNA data neither strongly support nor contradict this conclusion.

A6 merits species rank owing to its life history, adult external appearance and male and female genitalia; the mtDNA data neither strongly support nor contradict this conclusion.

As here delimited, B1 appears paraphyletic with respect to B2 and B3 on the basis of the mtDNA data. B2 appears polyphyletic. The samples LK 29 and 47, resolved as a separate clade and as sister clade of the remaining B section, are allopatric to the other B1 samples and also somewhat externally different. Their status as representing B1 could (and perhaps should) be questioned. If they were excluded, B3 would be pulled off from the remaining B clade, though without molecular synapomorphies. B3 is distinct from others according to the male genitalia. B1 cannot be differentiated from B2 by morphology either. The sole difference among these taxa seems to be the characteristic mine structure of B2. It is, however, noteworthy that among B1 samples (as here delimited) there is more variation in this trait than in the other taxa. Therefore, the present evidence is not sufficient to delineate B1; the status of B2 is unclear. B3 is distinct, allopatric taxon in relation of the other B samples; its rank is a matter of opinion and should be consistent with the ranking of other similar taxa of *Elachista*.

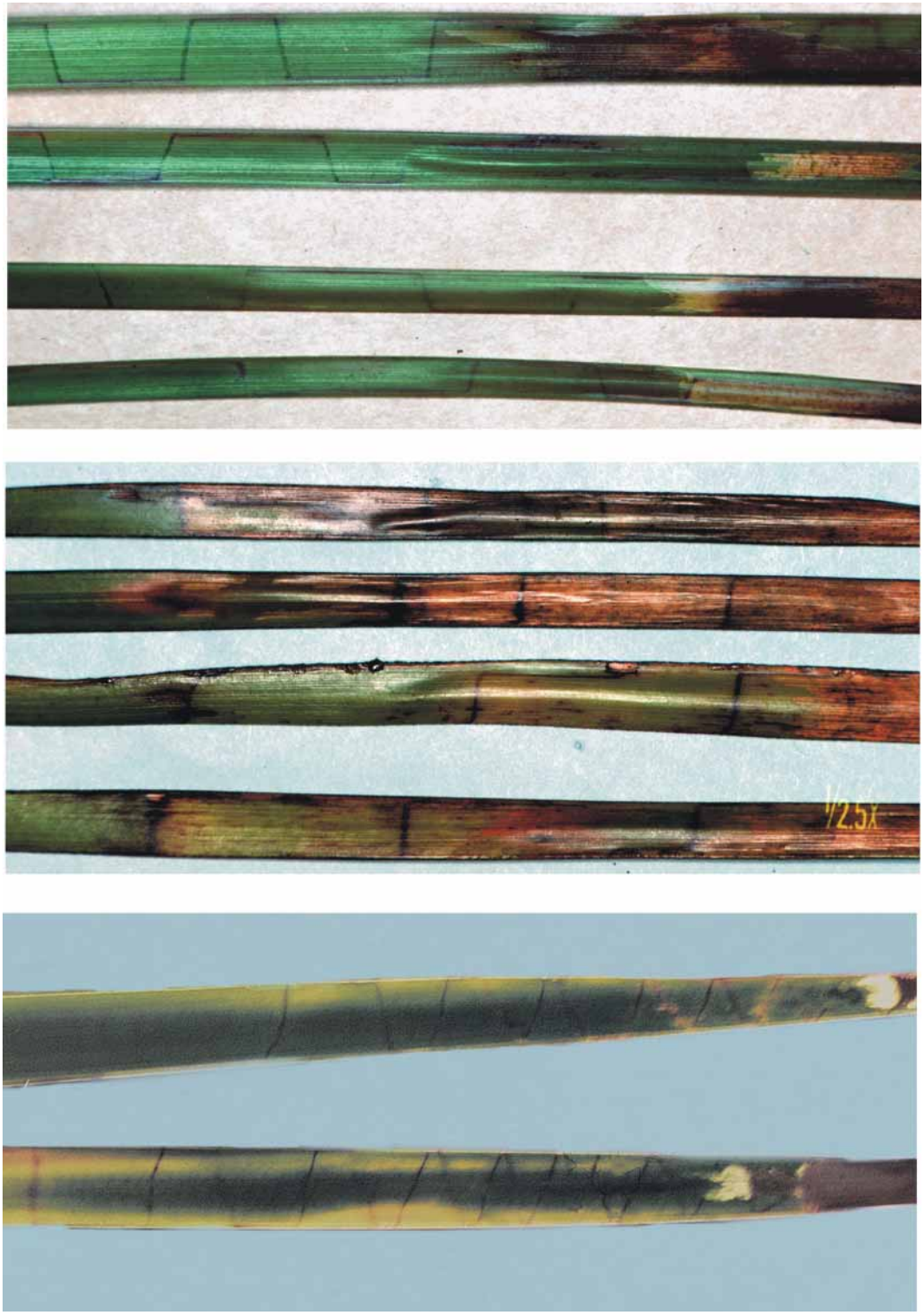

FIGURE 12. Larval mines of the taxa of the *Elachista* zigzagger complex. A: A1; B: A2; C: B2.

Discussion

Barcoding as a method for recognizing taxa is independent of questions as to whether individual taxa are species, what species are or should be, and where they fit in a unified tree of life (Besansky *et al*. 2003, Sperling 2003). We agree with this. The delimitation of species in problematic situations should be based on maximal evidence derived from different sources, including morphological and ecological data and molecular evidence from more than one genomic fragment as already suggested by Sperling (2003, see also Dayrat 2005). We suggest that the use of COI barcoding in species identification should be restricted to previously thoroughly studied specific cases, such as the pest ermine moths of the genus *Yponomeuta* (Yponomeutidae) (e.g. Sperling *et al*. 1995).

Our results support previous studies on the uninformativeness of the barcoding gene in providing information in cases with recently diverged species. Of the nine putative species in the complex of Australian zigzagger species, only two groups (A and B) were recovered with consistent robust molecular support. Based on morphological and biological evidence, both of these groups segregate into several species. The taxon zigzagger B2 displays a distinctive shape in its larval mine architecture, which supports its status as a distinct species; however, samples representing it (LK 55, 56, 59 and 60) were not nested together in the COI tree (Fig. 1). Another species group, the *Ficinia*-complex of taxa, showed more intraspecific variation in their COI sequence within one putatively conspecific population (all larvae sampled from a single tussock of the host plant) than did putatively different species of the zigzagger species complex. These complexes are (very) closely related to each other, yet they show distinctly different rates of differentiation. This finding argues against generalisations about the utility of this single gene fragment in species delimitation, unless supported by other kinds of evidence.

Besansky et al. (2003) argued that DNA barcoding is not an end in itself, but may aid research and boost the rate of discovery. Our experience gives credit to the potential utility of routine barcoding as a one source of taxonomic information, as even our small sample detected one species (yellow complex 2) morphologically so close to others that it had remained unrecognised. After characterised by the COI sequence, diagnostic morphological features subsequently were recognised. Barcoding of a COI fragment is certainly a useful source of information, among others sources, to explore the taxonomy of insufficiently known organisms. Nevertheless, caution should be taken when interpreting the patterns observed from a single gene sequence. We emphasize that DNA taxonomy should be firmly anchored with morphological taxonomy and take into account biological traits of the organisms under study. Only in this way can pitfalls caused by features such as genetic polymorphisms older than the divergences of the species in question (Wahlberg *et al*. 2003, Hebert *et al*. 2004) be avoided.

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References

Albrecht, A. & Kaila, L. (1997) Variation of wing venation in Elachistidae (Lepidoptera, Gelechioidea): methodology and implications to systematics. *Systematic Entomology*, 22, 185–198.

Besansky, N.J., Severson, D.W. & Ferdig, M.T. (2003) DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends in Parasitology*, 19, 545–546.

Blaxter, M. (2003) Molecular systematics: Counting angels with DNA. *Nature*, 421, 122–124.

Bremer, K. (1988) The limits of amino acid sequence data in angiosperms phylogenetic reconstruction. *Evolution***,** 42, 795–803.

Bremer, K. (1994) Branch support and tree stability. *Cladistics*, 10**,** 295–304.

Clary, D.O. & Wolstenholme, D.R. (1985) The mitochondrial DNA molecule of *Drosophila yakuba*. Nucleotide sequence, gene organization and geneticcode. *Journal of Molecular Evolution*, 22, 252–271.

Dayrat, B. (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85, 407–415.

Goloboff, P. (1999) NONA, vs. 2.0, parsimony program and documentation.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, series B*, 270, 313–321.

Hebert, P.D.N., Ratnasingham, D. & de Waard, J.R. (2003b) Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. *Proceedings of the Royal Society of London, series B (Suppl.)* DOI 10.1098/rsbl.2003.0025.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H. & Hallwachs, W. (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator. Proceedings of the National Academy of Sciences of the United States of America*, 101, 14812–14827.

Huemer, P. & Kaila, L. (2003) *Elachista (Elachista) morandinii* sp. n., a new species from Central Europe (Lepidoptera, Elachistidae). *Gortania*, 24 (2002), 211–220.

Kaila, L. (1992) The Elachistidae of southern Siberia and Central Asia, with descriptions of five new species (Lepidoptera). *Entomologica Fennica*, 3, 177–194.

Kaila, L. (1996) A revision of the Nearctic *Elachista* s. l. I. The *tetragonella* group (Lepidoptera, E lachistidae). *Entomologica scandinavica*, 27, 217–238.

Kaila, L. (1997) A revision of the Nearctic *Elachista* s. l. II. The *argentella* group (Lepidoptera, E lachistidae). *Acta Zoologica Fennica*, 206, 1–93.

Kaila, L. (1998a) Redescriptions of three Meyrick's Asiatic elachistid species, with descriptions of three new species (Lepidoptera, Gelechioidea). *Entomologica Fennica*, 9, 53–63.

Kaila, L. (1998b) Two new *Elachista* species (Lepidoptera, Elachistidae) from the Polar Urals region, Russia. *Entomologica Fennica*, 8, 219–223.

Kaila, L., (1999a) Phylogeny and classification of the Elachistidae *s. s.* (Lepidoptera, Gelechioidea). *Systematic Entomology*, 24, 139–169.

Kaila, L., (1999b) A revision of the Nearctic species of the *Elachista* s. l. III. The *bifasciella*, *prae-*

lineata, *saccharella* and *freyerella* groups (Lepidoptera, Elachistidae). *Acta Zoologica Fennica*, 211, 1–235.

Kaila, L., (1999c) A review of the South-American Elachistidae s. str. (Lepidoptera, Gelechioidea) with descriptions of 15 new species. *Steenstrupia*, 25, 159–193.

Kaila, L. (2005) A review of *Dibrachia* Sinev & Sruoga, 1992, a subgenus of *Elachista* (E lachistidae: Elachistinae). *Nota lepidopterologica*, 28, 139–155.

Kaila, L., Bengtsson, B.Å., Šulcs, I. & Junnilainen, J. (2001) A revision of the *Elachista regificella* Sircom –complex (Lepidoptera, Elachistidae). *Entomologica Fennica*, 12, 153–168.

Kaila, L. & Jalava, J. (1994) *Elachista adelpha* sp. n., *E. coeneni titanella* ssp. n., and other Elachistidae (Lepidoptera) from North Caucasus. *Entomologica Fennica*, 5, 97–102.

Kaila, L. & Junnilainen, J. (2002) Taxonomy and identification of *Elachista cingillella* (H. S.) and its close relatives (Lepidoptera, Elachistidae), with descriptions of two new species. *Entomologica Fennica*, 13, 167–188.

Kaila, L. & Karsholt, O. (2002) Contribution to the Lepidoptera fauna of the Madeira Islands 3. E lachistidae. *Beiträge zur Entomologie*, 52, 225–233.

Kaila, L., Nupponen, K., Junnilainen, J., Nupponen, T., Kaitila, J.-P. & Olschwang, V. (2003) Contribution to the fauna of Elachistidae (Lepidoptera) of the Southern Ural Mountains. *Entomologica Fennica*, 14, 65–90.

Kaila, L. & Sugisima, K. (2003) Phylogeny of Elachistinae (Lepidoptera: Gelechioidea) revisited. *Cladistics*, 19, 154–155.

Kaila, L. & Varalda, P. (2004) The *Elachista juliensis* complex revisited (Elachistidae). *Nota lepidopterologica*, 27, 217–237.

Nixon, K. (2002) WinClada., Version 1.00.08. Published by the author, Ithaca, New York.

Prendini, L. (205) Comment on "identifying spiders through DNA barcodes". *Canadian Journal of Zoology*, 83. 498–504.

Robinson, G.S. (1976) The preparation of slides of Lepidoptera genitalia with special reference to the Microlepidoptera. *Entomologist's Gazette*, 27, 127–132.

Scoble, M.J. (1992) *The Lepidoptera. Form, function and diversity.* Oxford University Press, 404 pp.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. *Annals of the Entomological Society of America*, 87, 651–701.

Sperling, F.A.H. (2003) Butterfly molecular systematics: From species definitions to higher-level phylogenies. *In*: Boggs, C. L., Ehlrich, P. R. & Watt, W. B., (E d.), Butterflies: Ecology and E volution Taking Flight: Butterflies as Model Study Systems. University of Chicago Press, Chapter 20, pp. 431–458.

Sperling, F.A, Landry, J.-F. & Hickey, D.A. (1995) DNA-based identification of introduced ermine moth species in North America (Lepidoptera: Yponomeutidae). *Annals of the Entomological Society of America*, 88, 155–162.

Sugisima K. & Kaila, L. (2005) Japanese *Elachista* (Lepidoptera, Elachistidae *s. str.*) mining on the leaf of woody Poaceae. *Entomologica Fennica*, 16, 83–102.

Traugott-Olsen, E. & Schmidt Nielsen, E. (1977) The Elachistidae (Lepidoptera) of Fennoscandia and Denmark. *Fauna Enomologica Scandinavica*, 6, 1–299.

Wahlberg, N., Oliveira, R. & Scott, J.A. (2003) Phylogenetic relationships of *Phyciodes* butterfly species (Lepidoptera: Nymphalidae): complex mtDNA variation and species delimitations. *Systematic Entomology*, 28, 257–273.

