Journal of Zoological Systematics and Evolutionary Research

WILEY

RESEARCH ARTICLE OPEN ACCESS

Molecular Identification of Immature Longhorn Beetles (Cerambycidae and Disteniidae) From South Korea

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Received: 6 January 2026 | **Revised:** 24 February 2026 | **Accepted:** 25 February 2026

Academic Editor: Carmelo Peter Bonsignore

Keywords: Cerambycidae | cytochrome c oxidase 1 | DNA barcode | immature stages | molecular identification

ABSTRACT

The morphology of larval and pupal stages of longhorn beetles is of critical importance in both evolutionary and applied contexts, often providing diagnostic characters that are inaccessible in adults. Nevertheless, the systematic utility of immature-stage morphology has been limited by challenges in accurate species-level identification, largely due to insufficient comparative material and the lack of standardized identification frameworks. In this study, we analyzed a total of 1057 cytochrome oxidase I (COI) sequences, including 520 newly generated sequences (161 larvae, 109 pupae, and 250 adults) and 537 publicly available sequences retrieved from GenBank. Of the 270 preliminarily identified immature specimens, 143 (53.0%) were confirmed, 64 (23.7%) were newly identified to the species level, and 18 (6.7%) were reidentified after misidentification. The remaining 45 specimens (16.7%) remained unidentified at the species level. Based on these results, we discuss the microstructural characteristics of immature stages, present preliminary cases of morphological misidentification, and demonstrate how molecularly validated immature morphology can clarify the systematic placement of problematic taxa. The barcode data generated in this study provide a robust foundation for an integrated DNA barcode library and highlight the essential role of accurately identified immature stages in longhorn beetle systematics.

1 | Introduction

Insect systematics seeks to classify and organize insect diversity based on evolutionary relationships, and morphology remains a crucial component of this process even in the genomic era [1]. In particular, larval and pupal morphology can provide unique and informative characters absent in adults, thereby offering critical insights into taxonomy and phylogeny [2–4]. However, immature stages often occupy different microhabitats than adults and are considerably more difficult to obtain, especially in wood-boring beetles, which develop inside woody tissues. This ecological constraint has long hindered comprehensive morphological studies, leaving immature characters insufficiently documented and inconsistently integrated into systematics. Moreover, several recent studies have emphasized that larval and pupal microstructures can provide phylogenetically informative characters that have been underestimated in long-horned beetle systematics [5, 6].

East Asian cerambycids are comparatively better documented than other wood-boring lineages, primarily referenced through some major works, including Cherepanov's [7–12] multivolume series and Ohbayashi et al. [13]. Cherepanov provided extensive ecological and distributional data across the Eastern Palaearctic, with valuable notes on larval feeding, pupal cell location, and

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oviposition biology. However, his keys relied on ambiguous morphological characters and omitted critical structures such as larval head capsules. By contrast, Ohbayashi et al. [13] concentrated on head capsule characters and made important contributions to larval morphology, but lacked descriptions of body form, pronotal shields, and setal arrangements. Both works are complementary yet incomplete, and neither provides a morphological dataset comprehensive enough for unambiguous species-level identification of larvae and pupae. This incompleteness frequently leads to misidentification, even by experts [14], and underscores the necessity of integrating DNA barcoding as an independent and confirmatory tool. In particular, the coexistence of multiple species in a single host plant and the morphological convergence of unrelated taxa make reliance on morphology alone problematic.

The previous study on molecular identification of immature South Korean long-horned beetles focused on Cerambycinae [6], which is the second most species-rich subfamily and includes some economically important pests (e.g., *Aromia bungii*) in South Korea. By constructing a curated cytochrome oxidase I (COI) barcode reference library from morphologically verified adults, Lee and Lee [6] demonstrated that over 90% of larvae and pupae could be identified to the species level through DNA barcoding. Many misidentifications were found in preliminary identifications based on morphology and ecological evidence (host plant, feeding traces, and distribution). Lee and Lee [6] highlighted not only the value of molecular data in connecting immature and adult stages but also emphasized the role of larval and pupal microstructures in long-horned beetle systematics. However, its scope was restricted to Cerambycinae, leaving other subfamilies in Korea without comparable molecular resources.

Therefore, the next step needs to extend this integrative approach to all remaining cerambycid subfamilies, in order to provide a comprehensive framework across the family level. The importance of such molecular tools extends beyond taxonomy to applied contexts. Among the cerambycids intercepted in North America, Cerambycinae represented only about 32% of the species and 29% of the specimens, with the remainder distributed among other subfamilies [15]. Korea's cerambycids are similarly represented, with only 114 species of Cerambycinae recorded, accounting for approximately 32% of the total 356 species [16]. These findings highlight that non-Cerambycinae subfamilies are equally, if not more, important. Indeed, several of them include invasive alien pests of forestry and agriculture, such as *Anoplophora horsfieldii*, *A. chinensis*, *A. glabripennis*, and *Psacothea hilaris* [6, 17–20]. A taxonomically curated DNA barcode library encompassing all major cerambycid subfamilies is indispensable for pest monitoring, invasive species detection, and biodiversity assessments. Such a resource would also provide immediate benefits for quarantine services and forestry management in Korea and East Asia, where early identification of larvae and pupae is essential for preventing the spread of invasive pests.

In this study, we extend the scope of molecular identification beyond Cerambycinae to include Lamiinae, Lepturinae (including Necydalini, see Nie et al. [21]), Spondylidinae, Prioninae, and the family Disteniidae in South Korea. Our objectives are to: (i) obtain COI sequences from accurately identified adult specimens across these groups, (ii) compile a curated and taxonomically reliable barcode reference library, and (iii) identify larvae and pupae to species level by matching their sequences against this library. By establishing a species-rich and taxonomically robust molecular library across all major cerambycid subfamilies, this study aims not only to improve species-level identification of immature forms but also to provide a critical resource for both systematic research and applied entomology, including forestry, agriculture, and biosecurity programs.

2 | Materials and Methods

2.1 | Specimen Collection, DNA Extraction, PCR Amplification, and Sequencing

All specimens were collected by the first author between 2015 and 2018. Sampling was primarily conducted during autumn and winter by bringing host trees into the laboratory, maintaining them indoors, and collecting larvae and pupae from within the plant material after adult emergence. This procedure allowed preliminary matching of immature stages with conspecific adults. In cases where multiple species emerged from the same tree, additional but preliminary morphological identification of larvae and pupae was carried out. All larvae and pupae were photographed using the high-resolution imaging protocol described in Lee and Lee [6], and preserved in 99% ethanol in the freezer of the Seoul National University Insect Biosystematics Laboratory.

In total, 683 individuals were examined, comprising 385 adults, 180 larvae, and 118 pupae. These included 554 specimens morphologically identified to species across five subfamilies, 105 genera, and 159 species of Cerambycidae and *Distenia gracilis* (Disteniidae), as well as 129 unidentified specimens. Among the identified material, there were 361 adults (two families, five subfamilies, 96 genera, and 147 species), 101 larvae (two families, five subfamilies, 52 genera, and 65 species), and 91 pupae (two families, five subfamilies, 47 genera, and 66 species). The unidentified specimens comprised 24 adults, 79 larvae, and 26 pupae. Identification data for each specimen followed the collection records of the Insect Biosystematics Laboratory (Supporting Information 1: Table S1).

Tissue samples for DNA extraction were taken from various body parts, including the right leg, wing, thoracic muscle, and abdominal segments of larvae. DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Inc., Germantown, MD, U.S.A.) following the manufacturer's instructions. We amplified approximately 658 bp of the mitochondrial COI gene using two primer sets: LCO1490 (5′-TGTAACAGCCCATGCATTCATCATAAT-3′) and HCO2198 (5′-GCTAAGACTACTCCTGTTAATCCTCCAACTG-3′), and CLepFolF (ATTCAACCAATCATAAAGATATTGG) and CLepFolR (TAAACTTCTGGATGTCCAAAAAATCA). LCO1490–HCO2198 was used first; samples failing to amplify were reattempted with CLepFolF–CLepFolR. PCR reactions employed BioNEER AccuPower PCR Pre-Mix under the following conditions: initial denaturation at 95 °C for 15 min; 40 cycles of denaturation at 95 °C for 1 min, annealing at 47–53 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 4 min. Amplified products were purified and sequenced by Bionics Inc. (Seoul, Korea) using Sanger sequencing. Some specimens underwent additional extraction and PCR to obtain more DNA. Voucher specimens were returned to freezer storage after extraction. Sequence chromatograms were checked for quality and assembled into contigs using SEQMAN PRO v.7.1.0 (DNASTAR, Inc., Madison, WI, U.S.A.). Alignments

were conducted in MEGA X [22] under the amino acid translation option.

2.2 | Public Data Compilation, Alignment, and Phylogenetic Analysis

To verify the morphological identifications of adult specimens and to obtain additional barcode data for molecular identification of larvae and pupae, we downloaded public sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for Cerambycidae. For each species represented in our private dataset, we preferentially retrieved conspecific public COI sequences. When no public sequence existed for a species, we downloaded sequences from congeners. Initially, 5956 COI sequences and 69 mitochondrial genomes were obtained. COI regions were trimmed and edited in MEGA X to match the target fragment.

Perfectly redundant sequences were removed using Jalview 2.11.2.7 [23] at a 100% identity threshold. For species with >20 sequences, redundancy was further reduced at a 99% threshold. Sequences <500 bp were removed with Seqkit v.2.5.1 [24], except in cases where only shorter sequences were available for a species (e.g., DQ222871.1 *Astathes episcopalis* and AF332940.1 *Sophronica obrioides*). Three COI sequences from Chrysomelidae were added as outgroups (Supporting Information 1: Table S2).

The combined dataset of edited public sequences and our private data was analyzed using IQ-TREE [25] under the maximum-likelihood method. The optimal substitution model was selected with ModelFinder [26] using the Bayesian information criterion, resulting in the GTR + F + I + G4 model. Nodal support was assessed with 1000 ultrafast bootstrap replicates. Phylogenetic trees were visualized in FigTree v1.4.4 to allow intuitive and species-level identification. Species-level identification was accepted when an immature sequence clustered within a monophyletic clade of conspecific reference sequences supported by ultrafast bootstrap values ≥90. Cases not meeting this criterion were treated conservatively and are discussed in detail in the following Sections 4.2.

3 | Results

The de novo dataset comprised 520 COI sequences: 250 adults, 161 larvae, and 109 pupae. Of these, 426 sequences were from preliminarily identified specimens (two families, five subfamilies, 87 genera, and 134 species): adults (245 sequences; two families, five subfamilies, 76 genera, and 112 species), larvae (94 sequences; five subfamilies, 49 genera, and 61 species), and pupae (87 sequences; five subfamilies, 44 genera, and 64 species). The remaining 94 sequences were from unidentified specimens (five adults, 67 larvae, and 22 pupae; Supporting Information 1: Table S1).

The public dataset consisted of 537 COI sequences: 534 Cerambycidae and three Chrysomelidae outgroups (Supporting Information 1: Table S2). In total, 1057 sequences (de novo + public) were used for phylogenetic analyses and species identification. Species identification was based on monophyly in the ML tree (Figure 1A), with reference to public sequences and morphologically identified adult sequences in the de novo dataset (Figure 1B). Lamiinae and Cerambycinae were recovered as monophyletic, whereas Lepturinae and Spondylidinae were not. Most species-level groups were monophyletic, facilitating molecular identification, with exceptions described below.

We identified 471 specimens to the species level, with the remainder identified to genus, tribe, or subfamily; five sequences remained unidentified. For larvae, 134 individuals were identified to 81 species (five subfamilies and 62 genera). Ten sequences were reidentified, and 47 previously unidentified larvae were assigned to species. Others were identified to genus (17), tribe (seven), or subfamily (one), with two sequences remaining unidentified. For pupae, 91 individuals were identified to 65 species (two families, five subfamilies, and 48 genera). Eight sequences were reidentified, 17 previously unidentified pupae were assigned to species, 12 were identified to genus, three were identified to tribe, and three sequences remained unidentified (Supporting Information 1: Table S1).

Many species-level identifications of immature specimens were supported by ultrafast bootstrap values ≥90, with following exceptions. Several clades contained mixtures of sequences from different nominal species, preventing confident species-level identification. Examples include *Menesia* (*M. albifrons*, *M. flavotecta* and *M. sulphurata*; Figure 1C), and *Saperda* (*S. octomaculata* and *S. subobliterata*; Supporting Information 2: Figure S1). In such cases, adults were identified to species morphologically, but larvae and pupae were conservatively identified at the genus level. Some sequences formed clades inconsistent with their original identifications, such as a specimen initially determined as *Monochamus guerryi* grouping with (*A. chinensis* + *A. glabripennis*; Supporting Information 2: Figure S1). These sequences were reassigned at the tribe level. Sequences lacking comparable public or adult reference data were identified to higher taxonomic ranks (genus, tribe, and subfamily) or left unidentified. *Sophronica koreana* sequences (adults + larvae) formed a monophyletic group but were distant from a public sequence labeled as its synonym, *So. obrioides* (Supporting Information 2: Figure S1). We treated larval sequences as *So. koreana* based on adult reference data. *Anoplophora chinensis* and *Dinoptera minuta* sequences formed a paraphyletic assemblage. *Anoplophora chinensis* larvae were identified to species using adult references, whereas a larval *D. minuta* sequence was identified based on conspecific adult sequences in our dataset and a matching public record (Supporting Information 2: Figure S1). *Pidonia puziloi* were divided into two clades, one containing the larval sequences was strongly supported, whereas internal nodes within the species showed relatively lower support.

4 | Discussion

4.1 | Molecular Species Identification

The phylogenetic tree showed nonmonophyly at deeper levels, including apparent polyphyly of major subfamilies such as Cerambycinae and Lepturinae (Figure 1). As expected, these relationships are inconsistent with those recovered by recent extensive phylogenetic studies based on multilocus or genomic data [21, 27]. Such low resolution in deeper relationships is a well-known limitation of COI-only phylogenetic analyses, largely attributable to saturation effects [28–31]. Nevertheless, because the primary focus of this study was species-level identification rather than deep phylogenetic inference, the COI dataset proved

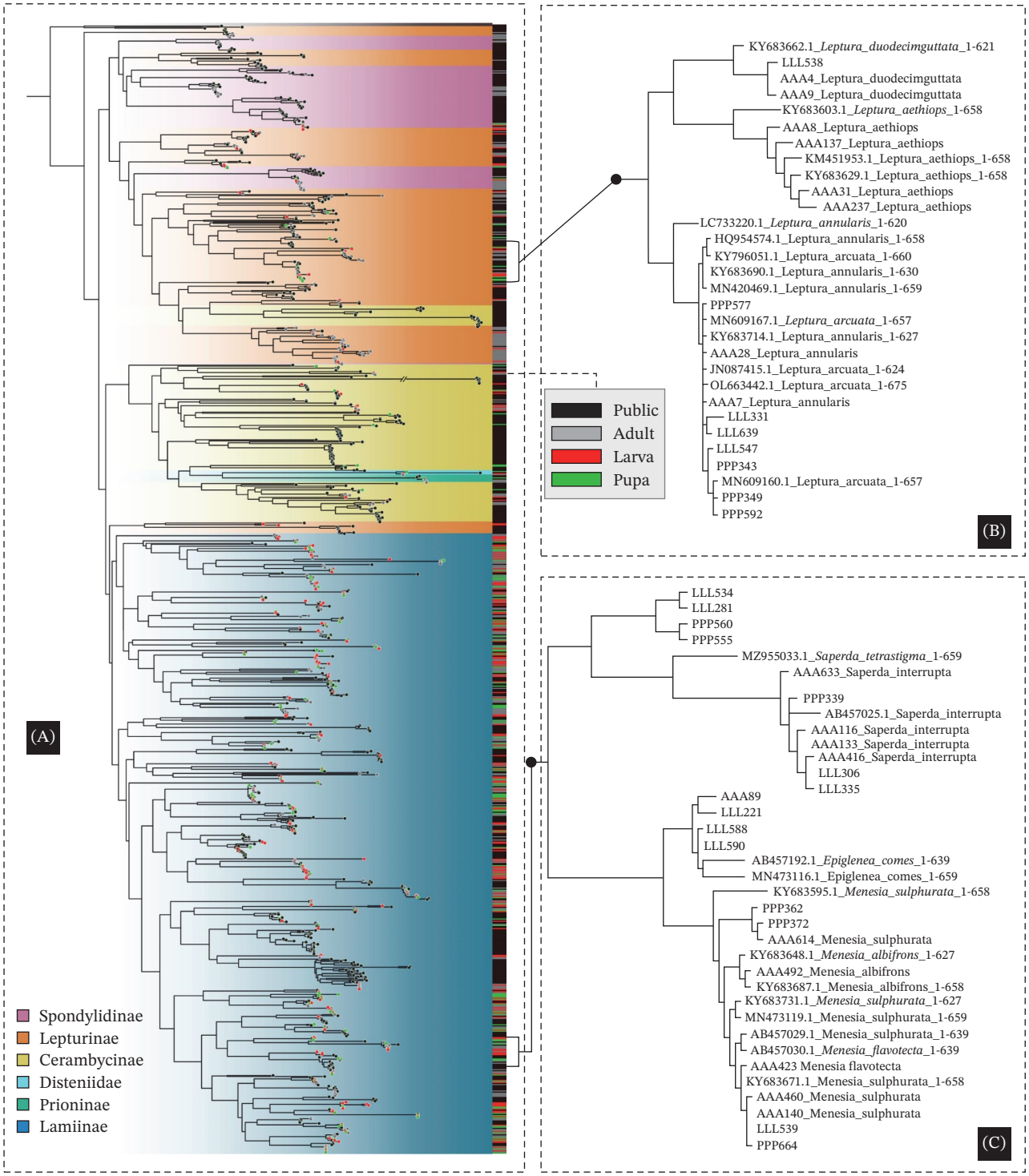

FIGURE 1 | Maximum-likelihood phylogenetic tree based on the mitochondrial COI gene. (A) Overall phylogenetic tree. Subfamilies are indicated in the lower left and life stages are shown in the center right. (B) Results for selected *Leptura* species. (C) Results for selected species of *Saperda* and *Menesia*.

effective for tip-level matching and enabled the identification of 83% of morphologically unidentified larvae and pupae.

This study also verified several cases of misidentification and expanded the reference dataset for non-Cerambycinae subfamilies in South Korea. As in many other insect groups, morphological information for immature stages of long-horned beetles is far less complete than for adults [5]. While adult identification is generally straightforward, larvae and pupae are often difficult to identify due to pronounced differences in morphology and ecology between life stages. Molecular species identification, applicable across life stages, is, therefore, particularly advantageous for identifying larvae and pupae [6, 15, 32, 33]. In this

FIGURE 2 | *Stictoleptura variicornis* and *Sachalinobia koltzei* occurring in the same host plant. (A) Host plant in which Lepturinae larvae were collected. Arrows indicate the positions where larvae were found. (B) Lepturinae larva collected from the same host, tentatively identified as *Sa. koltzei*. (C) Pupa of *St. variicornis* identified by molecular analysis. (D) Larva of *Sa. koltzei* identified by molecular analysis.

study, DNA barcoding enabled the detection of errors in previous morphological identifications and allowed a greater number of immature specimens to be identified to species level.

Even acknowledging the inherent uncertainty of preliminary identification, several misidentification cases were particularly striking. For example, during sampling for *Sachalinobia koltzei* from dead fir roots in high-altitude area several larvae with similar size, feeding galleries, and frass were collected. These were reared, and the emergence of *Sa. koltzei* adults led to the assignment of the associated larvae and pupae to that species. However, barcoding revealed that while the larva belonged to *Sa. koltzei*, the single pupa was actually *Stictoleptura variicornis* (Figure 2). Although this could be considered a premature identification by the first author, since pupal morphology for *Sa. koltzei* and *St. variicornis* are available [7], it is a type of error that can easily occur in cases where such descriptions are lacking. This misassignment, made despite the availability of pupal morphological data, would have gone unnoticed without molecular evidence, and similar errors are likely to occur in other morphological studies of immature stages.

Certain larval morphotypes, when combined with ecological traits, can be reliably distinguished, in some cases even at the genus level. For example, C-shaped larvae of *Agapanthia* (Figure 3A) associated with living herbaceous hosts, or larvae of *Apriona* characterized by distinct pronotal markings, conspicuous oviposition pits, and wet frass extruded through ventilation holes, are readily recognizable (Figure 3B, also see [16]). Some morphological characters alone are also informative at higher taxonomic levels. In Lamiinae, the presence or absence of so-called pronotal markings allows an initial separation between lineages such as Acathocinini, Monochamini, and Saperdini, and markingless taxa including *Ropica*, *Mesosa*, and *Pterolophia* (Figure 3C–E). However, even within the pronotal markings bearing lineages appear similar externally but differ in microstructure: For example, Saperdini shields bear clusters of small, dark, and raised tubercles, whereas Monochamini markings consist of elevated areas with distinct depressions [19]. Thus, while provisional identification based on larval morphology is often feasible, reliable discrimination frequently requires detailed examination using microscopy or high-resolution photomacrography. Molecular confirmation is, therefore, important not only for resolving subtle morphological differences but also for minimizing errors arising from specimen mislabeling or transcription mistakes.

4.2 | Limitations of Molecular Identification and Some Undetermined Results

Despite its advantages, COI–based molecular identification has limitations. Public sequence data may be incomplete, unreliable, or of low quality [34, 35]. Some taxa lack species-level public COI sequences (e.g., *Pidonia gibbicolis*, *P. amurensis*, and *Ropica coreana*), and in some cases even genus-level sequences are absent (e.g., *Miaenia* and *Atimia*). Even where sequences exist, some are misidentified, as seen in public records of *Clytus arietis*, *C. rhamni*, and *Monochamus rosenmuelleri*, which do not cluster with conspecific sequences. Low-quality sequences are also present, as shown in *Palimna liturata* (MW982108 and MW984203).

Species-level misidentifications can also arise from biological factors such as cryptic species, subspecies, and geographic variation, which may produce high intraspecific genetic divergence [36, 37]. In our dataset, *Parechthistatus gibber*, *Exocentrus fisheri*, *Pidonia puziloi*, *Stictoleptura rubra*, and *Rhagium inquisitor* formed monophyletic groups with multiple internal clades. For example, *Pa. gibber* occurs across multiple Japanese islands and is subdivided into several subspecies [38], and its phylogenetic relationships closely reflect the largely geographic signals of these subspecies (Supporting Information 2: Figure S1). Weak internal support and the presence of two distinct molecular

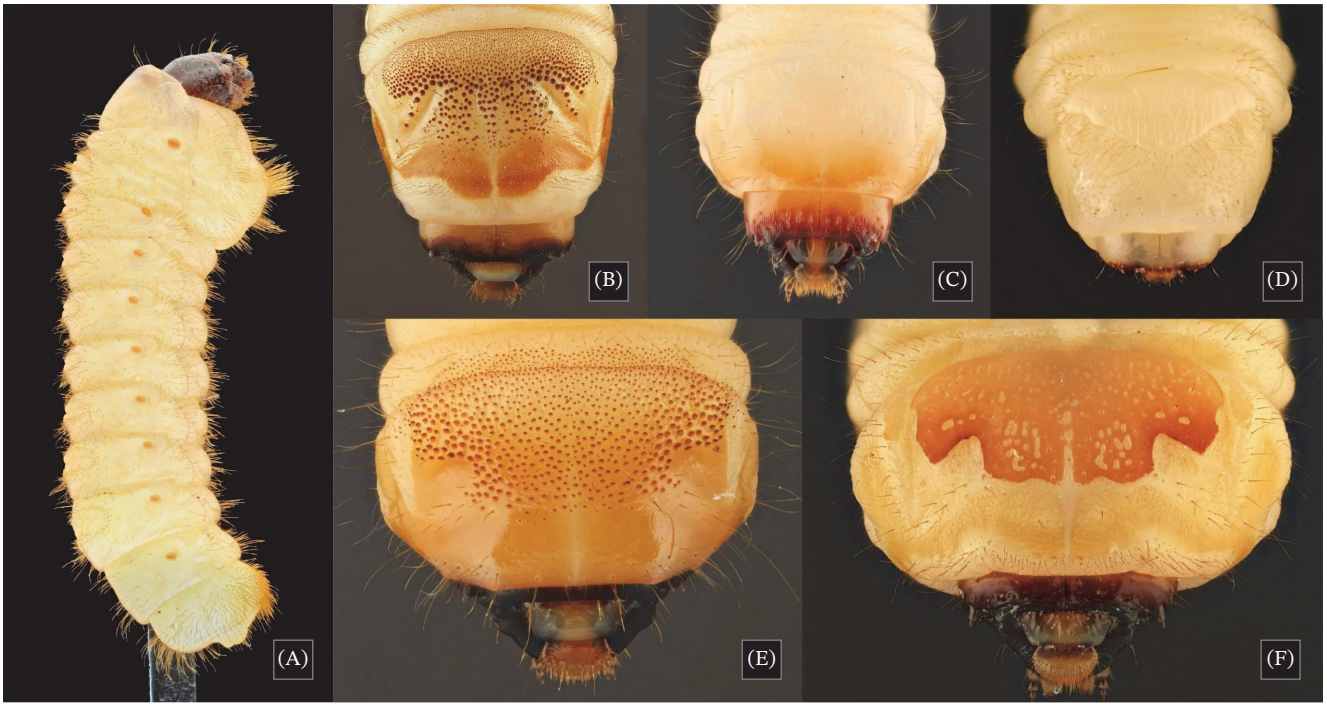

FIGURE 3 | Photomacrographs of larvae of several *Lamiinae* species. (A) Lateral view of *Agapanthia amurensis* larva. (B) Head and pronotum of *Apriona germari* larva. (C) Head and pronotum of *Mesosa hirsuta* larva. (D) Head and pronotum of *Exocentrus lineatus* larva. (E) Head and pronotum of *Saperda alberti* larva. (F) Head and pronotum of *Anoplophora glabripennis* larva.

clades in *Pidonia puziloi* are more likely a taxonomic issue. Although currently treated as a single species in Korea, multiple similar-looking species from other countries [39] suggest the possibility of unrecognized or cryptic taxa within this species complex. Notably, the clade containing both adult and larval sequences was strongly supported (ultrafast bootstrap = 100), indicating that the larvae were reliably identified within the *P. puziloi* species complex, although further investigation of species boundaries is needed.

In certain closely related species pairs (e.g., *Menesia albifrons*, *M. flavotecta*, and *M. sulphurata*; *Saperda octomaculata* and *S. subobliterata*), COI sequences from both public and private sources formed mixed clades, preventing reliable identification of immatures (Figure 1C and Supporting Information 2: Figure S1). In fact, the three *Menesia* species were assigned to the same cluster in a previous study [40], showing that they are molecularly indistinguishable using COI. However, because these are morphologically robust species, it is also possible that they represent a barcode complex that cannot be reliably separated using COI. In such cases, identification may be feasible only to the genus level. Testing additional nuclear markers (e.g., CAD and wingless) could be an effective alternative approach [41, 42].

Larvae and pupae morphologically identified as *Saperda tetrastigma* formed a sister clade to public sequences of that species, suggesting that adult sequences from the same populations should be obtained to confirm identity (Figure 1C). Because the public sequences were generated from specimens collected somewhere in China, the geographic distance from South Korea could plausibly explain the observed divergence. Therefore, considering intraspecific genetic variation, these results indicate that matching larvae and adults collected from nearby (or the same) localities is substantially more reliable for accurate identification.

5 | Conclusions

In conclusion, this study demonstrates the high efficacy of COI DNA barcoding for identifying the immature stages of longhorn beetles in South Korea. By integrating newly generated data (2015–2018) with public GenBank sequences and constructing a molecular tree, we successfully identified 134 larvae and 91 pupae at the species level. This molecular approach not only resolved prior morphological misidentifications but also provided taxonomic clarity for previously unidentified specimens, establishing a valuable foundation for a comprehensive DNA barcode library of the Cerambycidae and Disteniidae in South Korea.

To further enhance the reliability of this identification system, some refinements are proposed: exploring supplementary diagnostic methods for taxonomically complex clades, securing additional adult reference sequences for underrepresented groups, and implementing systematic genetic distance calculations. Addressing these factors in conjunction with DNA-evidenced morphological data will facilitate a more precise and accessible identification framework for longhorn beetle larvae and pupae.

Importantly, although we emphasize the significance of morphological characters in immature stages, we do not advocate species-level identification based solely on immature morphology. In the contemporary academic environment, DNA barcoding has become widely accessible and routinely applied and is now almost indispensable for accurate species delimitation. Accordingly, the most practical approach is to conduct preliminary identification to the genus or tribe level based on morphology, which remains both efficient and informative, and to achieve final species-level assignment through molecular confirmation.

Beyond preliminary identification, immature morphological characters retain clear phylogenetic significance and may also

reflect evolutionary adaptations to specific larval microhabitats and behaviors. Furthermore, individual-level variation in immature morphology may provide additional evidence for detecting cryptic diversity and refining species delimitation. Therefore, an integrative approach combining morphology, ecology, and molecular data from immature stages provides an independent and complementary line of evidence for species identification, stage association, and understanding evolutionary patterns.

Author Contributions

Seunghyun Lee: conceptualization, methodology, investigation, formal analysis, data curation, writing – original draft, visualization, validation. **Sunggum Sul**: writing – original draft, data curation, formal analysis. **Seunghwan Lee**: writing – review and editing, supervision, project administration.

Acknowledgments

The first author thanks Hyunkyu Jang and Woong Choi for their assistance with fieldwork and for sharing their expertise on cerambycid beetles.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea government (Grants RS-2024-00405751 and RS-2024-00452022) and the R&D Program for Forest Science Technology (Project Number RS-2024-00397614) provided by the Korea 160 Forest Service (Korea Forestry Promotion Institute).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in NCBI GenBank at https://www.ncbi.nlm.nih.gov/genbank/, Reference Number PP990798-PP991317.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. Table S1 and S2: List of supporting information tables of private COI sequences and public COI sequences, consisting of identification and GenBank accession numbers.

Supporting Information 2. Figure S1: Whole phylogenetic tree of this study.