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A Method for Improving DNA Yield from Century-plus Old Specimens of Large Lepidoptera while Minimizing Damage to External and Internal Abdominal Characters

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> Abstract

The external and internal genitalia of Lepidoptera have long provided a wealth of taxonomic and phylogenetic characters. However, traditional genitalia preparation techniques destroy both DNA, which is increasingly being used in Lepidoptera phylogenetics and species discrimination, and the scale pattern of the abdomen. In this paper, we describe a procedure for extracting both DNA for sequence analysis and genitalia from large Lepidoptera while retaining the surface scaling of the abdomen and, by permitting reattachment of the empty but still scaled abdomen, the general appearance of the specimens. Specimens both before and after the procedure has been undertaken are illustrated.

> Key words

Genitalia preparation, systematic characters, morphology, molecular, Sphingidae, *Hyles*.

1. Introduction

The genital morphology of Lepidoptera, both internal and external, has long provided a wealth of taxonomic characters and proven particularly useful for differentiating and diagnosing species and for elucidating phylogenetic relationships (SCOBLE 1992; HOLLOWAY et al. 2001; KITCHING 2002, 2003; SIMONSEN 2006). Traditionally, genitalia are prepared by maceration of all or the posterior part of the abdomen in an aqueous solution of potassium hydroxide (KOH) followed by removal the remnants of muscles, fat body, tracheae and ‘tubes’. This process thus destroys all the soft tissue, as well as a proportion of the more poorly sclerotized structures. In addition, in the case of Lepidoptera, the external scales of the abdomen, and thus all the characters associated with their colour, pattern and morpho-

logy, are also generally removed as part of the ‘cleaning’ process. Increasingly, however, questions of species delineation and phylogeny are being addressed by analysis of DNA sequences (e.g., HUNDSDOERFER et al. 2009; KEHLMAIER & ASSMANN 2010), which are most often obtained by sacrificing one or more legs (and generally their associated morphology). However, the amount of DNA recovered can be very low, especially from old museum specimens, which may be all that is available for certain species or which, as type material, is fundamental to the resolution of matters of species identity and the correct application of names. Furthermore, curators are often unwilling to permit the destruction of even small parts of such historically important specimens, especially when the yield of DNA

is small and unpredictable. Thus, traditional methods of genitalia preparation represent a significant wasted opportunity to acquire samples for molecular study, as relatively large amounts of DNA are destroyed by the KOH treatment. To remedy this, KNÖLKE et al. (2005) proposed a method for combining DNA extraction with genitalia preparation. Abdomens are first treated with a proteinase, which produces a macerate from which DNA can be recovered and sequenced. The remaining abdominal structures, including the genitalia, are then prepared by conventional techniques. However, they developed and applied their method to relatively small moths and species in which the abdominal colour pattern is not considered (at present) to be of importance, and thus these features were still lost.

We are currently undertaking a comprehensive study of the morphological and molecular phylogenetics of the hawkmoth genus, *Hyles* (Sphingidae). Although we have obtained fresh material of the majority of the species, some have proven elusive and others are unavailable due to the dangers associated with the places in which they occur (e.g. Afghanistan, Kashmir). Consequently, we have had to develop techniques to extract DNA ('ancient DNA', aDNA) from old specimens (collected more than about 50 years ago). As noted above, the yield from a single leg of such specimens is both low and unpredictable and thus extraction from the entire abdomen is currently necessary to obtain aDNA in sufficient amounts to provide adequate sequence data for phylogenetic reconstruction. However, our morphological studies have shown that there are important characters for phylogenetic analysis in the abdominal pattern and although high-resolution colour photography prior to aDNA extraction would preserve a record of the pattern, it would be preferable if as much of the pattern as possible could be retained for future direct examination and study.

Here, we describe a method of aDNA extraction, with optional genitalia preparation, that maintains the general appearance of the specimen, especially the abdomen, much better than do traditional methods of both morphological and molecular study.

2. Method

1. High-resolution digital photographs are taken of both the upperside and underside of the pinned moth.
2. The whole abdomens are carefully broken off. If any cutting is required, then a DNA-free scalpel or pair of scissors is used. Traces of DNA are destroyed on the instruments by dipping the part that will contact the tissue into 96% denatured ethanol and then holding it for a short time in a flame.
3. The abdomens are placed individually into tubes of sufficient size (generally 15 ml volume) with sufficient diluted lysis buffer (sbeadex forensic kit, Agowa, Berlin, Germany; ratio water: lysis buffer 3:2) to submerge the body (up to 4 ml for large female abdomen), even though the body may not be initially fully submerged due to surface tension and the water-repellent nature of the scales. The samples are incubated at 55°C, the duration of which differs between individual samples, depending on the amount of hard tissue that needs to be softened. Large female abdomens are left for at least 12 hours.
4. Once the tissue has softened sufficiently to allow liquid to reach the internal structures, the excess dilute lysis buffer is removed with a DNA-free pipette and the body parts submerged in a sufficient volume of undiluted lysis buffer. If necessary, the abdomens can be cut ventrally along one edge of the sternites using DNA-free scissors, as far as the seventh abdominal segment. This allows greater penetration of the proteinase solution. A one-tenth volume of proteinase K solution (20 mg/ml in water; Merck, Darmstadt, Germany) is then injected into or near the softened internal tissue (through the first abdominal segment of large females if this cannot yet be opened). So, if 3 ml of lysis buffer were required to submerge the abdomen, then 300 µl of proteinase K solution is injected. The samples should be incubated for at least 12 hours. To minimize damage to the surface scaling of the abdomen, the samples should not be vibrated more than is absolutely necessary. In particular, the tubes should not be shaken; otherwise, the abdominal scales may become detached and fall off, thus negating the point of the method. If abdomens are then still hard to open or some soft tissues remain undigested, the process should be repeated by adding a small amount of new proteinase K solution and re-digesting for a few hours.
5. Once digestion has proceeded to an adequate degree, the abdomen is transferred in its lysis buffer to a DNA-free dissection dish under a stereomicroscope. Using DNA-free forceps and scissors, the macerated tissue is carefully pushed or pulled out of the abdomen, avoiding both damage to the genitalia (particularly the delicate internal tubes) and disturbance to the abdominal scaling. Re-digestion in the same or fresh (if a large amount of macerated surrounding tissue has been removed) lysis buffer with new proteinase K may be necessary for particularly large specimens. The tissue macerate is then transferred to DNA-free tubes

and stored between -20 and -80°C. They are treated as undigested tissue for aDNA extraction in an aDNA-designated laboratory, to avoid contamination with DNA from fresh tissue.

6. If preparation of the genitalia is also to be undertaken, the abdominal wall can then be circumferentially cut between segments 7 and 8, taking care not to damage the internal genital structures, particularly the ductus bursae and corpus bursae in females, which often extend for a considerable distance anteriorly in the abdomen. It is important to wash and free the instruments of DNA between samples during this extraction phase. Subsequent preparation of the genitalia can then follow conventional procedures; for example, careful maceration in hot 10% aqueous potassium hydroxide solution for a short time followed by cleaning, for which DNA-free instruments are not necessary.

7. The empty abdomen is then carefully washed several times in water, followed by washing in 70% ethanol, and placed in an open tube to dry. Fine forceps and pipette tips are used to restore the abdomen to its original shape as far as is possible before it dries. The dried abdomen is reattached to the specimen using water-soluble insect glue.

If genitalia preparation is not required immediately, then the extraction procedure described in step 6 is skipped and the process proceeds directly to the washing and drying stage in step 7. The internal genitalia of large moths are robust enough to withstand drying without the tissue that normally surrounds them and they can be extracted later by removing and soaking the abdomen, then proceeding once again from step 6.

8. Finally, extraction of the aDNA with a kit follows the manufacturer's (Agowa, Berlin, Germany) protocol (including the first digestion step) in an aDNA laboratory, using an appropriate amount of the tissue macerate. The subsequent amplification of marker genes from the aDNA extracts for molecular phylogenetics is performed in numerous small, overlapping fragments using specially designed primers. The short sequences are then assembled in BioEdit (HALL 1999) to create the total fragment. Sequence analysis for phylogenetic reconstructions can then proceed.

3. Results

The procedure was tested using specimens of the hawkmoth species *Hyles salangensis* (Ebert, 1969),

H. calida hawaiiensis (Rothschild & Jordan, 1915) and *H. livornica tatsienluica* Oberthür, 1916, as part of a study into the phylogeny and evolution of this genus (cf. HUNDSDOERFER et al. 2009; A.K. Hundsdoerfer et al., unpublished data). The selected moths, from the collections of the Natural History Museum, London, U.K. (BMNH) and the Natural History Museum Vienna, Austria (NHMV), were at least 45 years old, and one was even more than 100 years old. Fig. 1 shows the uppersides and undersides of two moths before and after macerate extraction. The Hawaiian female *H. calida hawaiiensis* (Fig. 1A–D) is a bred moth that was accessioned into the BMNH in 1907. The year of capture of the male *H. livornica tatsienluica* from China is also not recorded but the specimen (Fig. 1E–H) was part of the L.W. Rothschild collection, which was bequeathed to the BMNH in 1939 (and the specimen may be older than 1903). The two studied paratypes of *H. salangensis* from Afghanistan (NHMV; not illustrated) were collected in 1965. Although there is a small amount of degradation of the vestiture, the main abdominal pattern characteristics are still clearly visible: the overall ground colour, the number and extent of the dorso-lateral black and white patches and the condition of the dorsal pale line. Furthermore, from the dorsal perspective, the general appearance of the moths as intact specimens is preserved. In ventral view, the lateral incision required for DNA extraction and the removed apex for genitalia preparation detracts somewhat from the aesthetics of the specimens, but we would argue that this is still preferable to the present practice in which the abdomen is permanently removed from the specimen during genitalia dissection. A method used by some lepidopterists, in which the abdomen is softened and the genital capsule gently pulled out for further preparation, is better at maintaining the appearance of the specimens, but does not allow for DNA extraction.

The sequence data targeted by PCR comprised 2,284 bp of three mitochondrial genes, the first two subunits of the cytochrome-c-oxidase (COI, COII) and the interposed gene of the ribosomal transfer RNA for leucine (tRNA-leu). Specific primers were designed to amplify this part of the mitochondrial genome in thirteen fragments of 110–280 bp in length.

The tissue macerates yielded amounts of sequence data (Tab. 1) that are long enough to place the specimens appropriately, and with little ambiguity, when subjected to phylogenetic analysis (A.K. Hundsdoerfer & I.J. Kitching, unpublished data).

The amounts of sequence data that can be obtained using the new technique are comparable to those obtained from younger specimens and, due to the larger amount of tissue available, considerably greater than those that can be obtained from just the legs of old specimens.



Fig. 1. Two of the *Hyles* moths used to test the method described. **A–D:** Paratype female *H. calida hawaiiensis*, Hawaii [Hawaii], Kau [Kau], BMNHE #271618 (BMNH sphingid genitalia preparation #3087). **E–H:** Male *H. livornica tatsienluica*, China [Sichuan], Ta-Tsien-Lu [Kangding], BMNHE #812379 (BMNH sphingid genitalia preparation #3039). A, B, E, F show the moths before DNA extraction and genitalia preparation; C, D, G, H show the same moths afterwards. The specimens are deposited in the Natural History Museum, London, U.K.

Tab. 1. Overview of the sequence data obtained from the tissue macerates. A length of 2,284 base pairs was attempted to be sequenced in all four specimens. Success rate: Amount [bp] and percentage (as related to 2,284 bp) of sequence data obtained; * see text.

Year of acquisition	No. of specimens	Success rate amount (percentage)
1965	2	1,992; 2,013 (87–88%)
pre 1939*	1	1,850 (81%)
1907	1	1,916 (84%)

4. Discussion

Although it is as invasive as the traditional KOH maceration technique for genitalia dissection and preparation, the method proposed here better preserves the appearance of the specimen as the abdominal segments can be reattached after DNA extraction (Fig. 1). If sufficient care has been taken, then minimal damage will have been inflicted upon the abdominal pattern characters, which are then available for future study. Such is not the case with traditional genitalia preparation where all the scales are removed from the abdominal skin prior to mounting it on the same microscope slide as the genitalia themselves. The present procedure is more time-consuming than the traditional KOH maceration technique but, in addition to the genitalia (if required), it does yield sufficient quantity and quality of aDNA for phylogenetic analysis. Thus, large amounts of tissue are retained for study that would otherwise be discarded. Some additional equipment is necessary, such as a water bath, tubes of adequate size, racks, pipettes used in molecular biology, and possibly small sterile Petri dishes to avoid having to free the same glass dish from DNA between samples. However, it is not necessary to grind up, and thus lose, any body parts, such as legs, that might bear characters of morphological and phylogenetic importance.

The methodology can also be used for fresh samples but with the lysis buffer from the kit used in this paper (which is optimised for aDNA extraction) substituted by the lysis buffer of the favoured kit for DNA extraction from fresh tissue. However, with large specimens, this will only be possible if the particular lysis buffer is available in larger volumes than is supplied with the kit itself. Alternatively, a custom-made lysis buffer will also serve the purpose of enabling the proteinase K digestion. We recommend a slightly more refined recipe than the 0.2 M NaCl suggested by KNÖLCKE et al. (2005), namely 75 mM NaCl, 25 mM EDTA and 10 mM Tris (pH 7.5 with NaOH; WINK 2007).

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