A procedure for combined genitalia dissection and DNA extraction in Lepidoptera

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Extraction of DNA from Lepidoptera is a destructive procedure and curators are often reluctant to provide museum specimens for molecular investigations. On the other hand, dissection of abdomens and genitalia is a standard procedure for description and identification of species and generally accepted even for type material. We present a method that combines the investigation of morphological traits in genitalia with the analysis of DNA sequence information by modifying the dissection protocol. Maceration of abdomens in potassium hydroxide is replaced by enzymatic digestion of soft tissue followed by DNA extraction. DNA extracted from abdomens is suitable for sequencing, as shown for the mitochondrial COI gene appropriate for species identification. Enzymatically treated abdomens proved to be sufficient for preservation of morphological traits. Recommendations are given for appropriate treatment of collected specimens and for routine use of enzymatic digestion.

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Introduction

Dissection of genitalia, the origin of which dates back to the 1850s (Zeiller 1855), has been established as a standard method for morphology-based taxonomy of Lepidoptera (Pierce 1909; Warnecke 1938; Scoble 1995). Since then, techniques have improved and suggestions for standardisation have been made (e.g., Clarke 1941; Robinson 1976; Huemer 1987; Dang 1993). Today it is almost indispensable for the description of species, and in many cases necessary for the identification of specimens.

In most laboratories the abdomen is macerated in boiling potassium hydroxide (KOH). Both cold maceration in KOH and more sophisticated enzymatic methods have also been described, allowing the preservation of delicate structures of the abdomen which may be destroyed by boiling in KOH (e.g., Wahl 1984, 1989; Fernandez-Rubio 1986; Kudrna, pers. comm.). This method preserves only sclerotised structures of the abdomen and genitalia, while soft tissue is discarded during the preparation, leading to a loss of considerable amounts of DNA.

Over the last decades, molecular methods such as DNA sequence analysis have gained increasing importance and have started to "complement (...) comparative morphology as the basis of classification" (Whitfield & Cameron 1994), thus contributing to a better understanding of taxonomy, phylogeny and evolution of insects (for review, see Caterino et al. 2000; Tautz et al. 2003; Blaxter 2003). In general, however, extraction of DNA is invasive and causes damage to the specimen, although efforts have been made to minimize destruction by using only part of the body like the thorax, legs, small portions of the wing or the anterior part of the abdomen (e.g., Rose et al. 1994; Mitchell et al. 1997; DeVerno et al. 1998; Schneider et al. 1999).
Nondestructive extraction methods have been described by some authors (e.g. Phillips & Simon 1995; Cruickshank 2002). However, the method of Cruickshank (2002) is destructive as the specimens are to be decapitated or cut in half, whereas the procedure of Phillips & Simon (1995) must be considered semi-destructive as abdomens of the specimens are perforated several times by using insect pins. Phillips & Simon’s protocol of submerging a specimen partly or entirely in extraction fluid, and subsequently washing off the remaining solution is also not acceptable for Lepidoptera, owing to the delicacy of their wings and scales. As lepidopteran specimens suffer from all the procedures mentioned above, type material, in particular, is rarely made available for molecular analysis. Here we report a method for extracting DNA that is suitable for sequence analysis as a by-product of genitalia dissection of fresh, alcohol-preserved and dry Lepidoptera. Using a maceration mix, both sclerotised abdominal structures and DNA can be preserved out of the same reaction vial, requiring no further damage to the specimen.

Material and methods

Organisms

DNA was extracted and genitalia preparations were obtained from a total of 81 lepidopteran specimens, belonging to 36 species of the families Geometridae and Pyralidae. All material had been killed in cyanide. For further information on preservation of specimens, see Table 1.

Table 1: Preservation of specimens and suitability for DNA extraction and amplification

<table>
<thead>
<tr>
<th>Preservation method</th>
<th>No. of specimens</th>
<th>No. yielding ≥200 ng DNA</th>
<th>No. yielding PCR product &gt;300 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>alcohol</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>dried, not relaxed</td>
<td>39</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td>set, possibly relaxed</td>
<td>35</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>kept in relaxing jar for &gt;=2 days before setting</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>74</td>
<td>55</td>
</tr>
</tbody>
</table>

Dissection of macerated abdomens

Dissection was performed in 10% isopropanol according to the standard protocol of Robinson (1976), with further details added by Brown (1997). If genitalia, particularly those of larger specimens, were still too rigid to be dissected easily, they were further macerated in KOH before dissection (cold; overnight; hot; for a few minutes) (Fernandez-Rubio 1986). Genitalia were stained with Mercurychrom and embedded in Euparal following recommendations of the standard protocols cited above.

Preparation of legs and thoraces for extraction

Legs and thoraces were individually put in Eppendorf tubes and crushed with a pestle while dry. Lysis buffer and proteinase K (DNeasy tissue kit, QIAGEN) were added and subsequently incubated as recommended in the manufacturer’s protocol for animal tissues. Remaining bodyparts of the specimens were kept as vouchers in gelatine capsules and small paper envelopes.

DNA extraction

DNA extraction and purification was continued using the DNeasy tissue kit (QIAGEN) following the manufacturer’s protocol for animal tissues.
After extraction, concentration of DNA was measured using a fluorometer (BioRad, Versa-Fluo\textsuperscript{TM}) and adjusted to 50 ng/μl with molecular biology grade water (Eppendorf).

**Amplification and sequencing**

Extracted DNA was subjected to polymerase chain reaction (PCR) using the following primers (Simon et al. 1994): TY-J-1460, C1-J-1718, C1-J-1751, C1-J-2183, C1-N-2191, C1-J-2195, C1-N-2329, and TL2-N-3014. These target various regions of the mitochondrial COI gene and produce fragment lengths up to 831 bp. The COI gene is known to resolve genetic diversity down to the species level in various groups of insects and was recommended as a standard for molecular systematics in insects (Caterino et al. 2000, 2001; Rand et al. 2000; Wahlberg & Zimmermann 2000; Artiss et al. 2001; Goto & Kimura 2001; Kruse & Sperling, 2001, 2002; Maus et al. 2001; Monteiro & Pierce 2001; Hebert et al. 2003).

Amplification was performed by using a PTC 220 DYAD thermocycler (MJ Research) in a total of 25 μl reaction volume using the Expand PCR system (Roche Diagnostics) with 25 pmol of each primer, 20 pmol of dNTPs, 12.5 pmol MgCl\textsubscript{2} and 0.88 units of Taq polymerase. PCR parameters were 94°C for 4 min, 45 cycles with 94°C for 1.5 min, 48°C for 1 min, 72°C for 1.5 min, followed by a final elongation at 72°C for 3 min. PCR results were visualized using ethidium bromide stained agarose gels under UV light.

PCR products were purified using the MinElute Purification Kit (QIAGEN) following the manufacturer’s protocols, measured and adjusted to a final concentration of 100 ng/μl. Cycle sequencing was done using the same primers as in PCR and BigDye V.2 master mix (Applied Biosystems), with 200 ng of PCR product as template in a 10 μl reaction volume. Sequencing was performed on an ABI Prism 377 DNA Sequencer.

Alignment of sequences was performed by using the GenTool 1.0 software (Biotools, Edmonton, Canada), BioEdit 5.0.9 (Hall 1999) and ClustalX 1.81 (Thompson et al. 1997).

**Results**

**Suitability of DNA from abdomens for molecular taxonomy**

By using the protocol described above, we successfully extracted DNA from abdomens of 74 out of 81 specimens (yield of DNA: ≥ 200 ng) (table 1).

In general, we observed that the younger the specimens the better the results obtained (data not shown).

It turned out that best extraction and amplification results were obtained from material kept permanently dry after killing, and from alcoholized specimens (table 1).

Weak or negative results were repeatedly found with set museum specimens, of which the history of treatment by the collector was unknown.

Three specimens that failed completely had been kept in a relaxing jar for several days by the collector (table 1). Since these specimens were otherwise in perfect condition and less than one year old, the negative result possibly suggests that relaxing might enhance DNA degradation. Similar problems with DNA extraction/amplification from relaxed specimens have been reported by other researchers as well, but systematic investigations are yet lacking (N. Wahlberg, personal communication).

For a few specimens, we also compared DNA yield from abdomens with yields obtained from legs and thoraces and found total amount of DNA from abdomens to be highest in all but one cases.
Table 3: Compilation of the subset of totally sequenced specimens:

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Coll. Date</th>
<th>Age (yrs)</th>
<th>Preserv.</th>
<th>DNA-TAX no.</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyralidae</td>
<td>Hypochalaeta ahenella ([Denis &amp; Schiffermüller, 1775])</td>
<td>2002</td>
<td>1</td>
<td>a</td>
<td>01583</td>
<td>AJ 868573</td>
</tr>
<tr>
<td>Pyralidae</td>
<td>Dioryctria juniperella Yamanaka, 1990</td>
<td>2002</td>
<td>1</td>
<td>b</td>
<td>01848</td>
<td>AJ 868569</td>
</tr>
<tr>
<td>Pyralidae</td>
<td>Dioryctria schuetzezella Fuchs, 1899</td>
<td>2002</td>
<td>1</td>
<td>a</td>
<td>02089</td>
<td>AJ 868570</td>
</tr>
<tr>
<td>Pyralidae</td>
<td>Dioryctria okui Mutuura, 1958</td>
<td>2002</td>
<td>1</td>
<td>b</td>
<td>01844</td>
<td>AJ 868571</td>
</tr>
<tr>
<td>Pyralidae</td>
<td>Dioryctria abietella ([Denis &amp; Schiffermüller, 1775])</td>
<td>2002</td>
<td>1</td>
<td>a</td>
<td>01585</td>
<td>AJ 868572</td>
</tr>
<tr>
<td>Pyralidae</td>
<td>Dioryctria resiniphila Segerer &amp; Pröse, 1997</td>
<td>2002</td>
<td>1</td>
<td>a</td>
<td>01845</td>
<td>AJ 871091</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Rhodometra sacaria (Linnaeus, 1767)</td>
<td>1994</td>
<td>9</td>
<td>a</td>
<td>01654</td>
<td>AJ 870398</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Cyclophora suppuratrix (Zeller, 1847)</td>
<td>1994</td>
<td>9</td>
<td>a</td>
<td>01662</td>
<td>AJ 870399</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Iadae sericeata (Hübner, [1813])</td>
<td>1994</td>
<td>9</td>
<td>a</td>
<td>01668</td>
<td>AJ 870400</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Prolepsis ocellata (Butler, 1886)</td>
<td>1995</td>
<td>9</td>
<td>a</td>
<td>01672</td>
<td>AJ 870401</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa porphyra (Zerny, 1936)</td>
<td>1996</td>
<td>7</td>
<td>a</td>
<td>02180</td>
<td>AJ 870402</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa porphyra (Zerny, 1936)</td>
<td>1996</td>
<td>7</td>
<td>a</td>
<td>02181</td>
<td>AJ 870403</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa porphyra (Zerny, 1936)</td>
<td>1997</td>
<td>7</td>
<td>a</td>
<td>02182</td>
<td>AJ 870404</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa mucicaria (Hübner, [1799])</td>
<td>1997</td>
<td>7</td>
<td>a</td>
<td>02187</td>
<td>AJ 870405</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa mucicaria (Hübner, [1799])</td>
<td>1997</td>
<td>7</td>
<td>a</td>
<td>02187</td>
<td>AJ 870406</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa pfefferi (Wehrli, 1926)</td>
<td>1999</td>
<td>7</td>
<td>a</td>
<td>02192</td>
<td>AJ 870407</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa cremulata (Staudinger, 1871)</td>
<td>1998</td>
<td>5</td>
<td>a</td>
<td>02193</td>
<td>AJ 870408</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa cremulata (Staudinger, 1871)</td>
<td>1998</td>
<td>5</td>
<td>a</td>
<td>02194</td>
<td>AJ 870409</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Charissa eiscaucasia (Rioavo, 1964)</td>
<td>2001</td>
<td>2</td>
<td>a</td>
<td>02200</td>
<td>AJ 870410</td>
</tr>
</tbody>
</table>

*Preservation of specimens: a = kept permanently dry after killing; b = alcohol-preserved

The specimen DNATAX-02082 (collected in 2002) was only dried, never relaxed, and yielded the highest total amount of DNA of 63312 ng (table 2).

A systematic comparison of DNA yield for the different body parts and the effects of previous treatment of the specimens was, however, not the scope of this study.

PCR success of extracted samples was defined by yielding at least one fragment of >300 bp. Different primer combinations were used in order to rule out the possibility of primer mismatch causing a failure of PCR.

For about two thirds of the samples we were able to get at least one fragment product of around 300 bp (table 1), in ~50% of these cases even longer than 800 bp.

In order to test if suitable sequence can be produced from abdominal DNA, a subset of samples that gave best PCR results were subjected to cycle sequencing (table 3). Resulting electropherograms could be interpreted unambiguously. They did not show doublepeaks for any same baseposition. The identity of the COI gene was confirmed by alignment with Bombyx mori COI sequence (GenBank access number NC 002355).

Genitalia slides

The characteristics of the enzymatically treated abdomens were as suitable for dissection, descaling, and staining as if macerated using traditional methods, allowing the preservation of even delicate structures, e.g. bristles present on sternite I/II of male Dioryctria spp. (figs. 1-2).

Discussion

The approach described above combines the extraction of DNA with dissection of genitalia, a routine method used by most lepidopterists. It was shown to be suitable for obtaining sequences of mitochondrial COI gene from museum specimens. Curators and many lepidopterists have often been reluctant to provide material from their collections for molecular studies, since destructive extraction methods will potentially lead to deterioration of the specimen and to loss of significant morphological information. Abdomens may carry taxonomically important traits (abdominal plates, bristles, tympanons) which are likely to be partly or entirely destroyed if the abdomen is cut into pieces or ground. The probability of severe damage is especially high in tiny Lepidoptera like leaf miners.
Fig. 1: Example of a pyralid moth subjected to the combined procedure

a) *Diorystria schuetzeella* Fuchs, 1903; male; DNA-TAX-02089; Germany, Bayern, Kelheim, Sippensaur Moor, 29.VI.1997, leg. A. Segerer; scale bar, 1 cm

b) Sternite I/II after dissection, showing delicate bristles (arrow); scale bar, 1 mm

c) Male genitalia (Gen. Prep. No. M3469 AHS, ZSM); scale bar, 1 mm

d) Aedeagus with everted vesica (same data as fig. 1c); scale bar, 1 mm
As any taxonomic study and any identification of a specimen ultimately refer to the type specimen as the link between name and species, the problem becomes even more serious. Tautz et al. (2003) argue that "one should be prepared to accept damage or destruction of specimens for DNA taxonomy. ... there will also be specimens that have to be fully destroyed to extract sufficient DNA". This proposal was followed by vehement reactions, e.g. Seberg et al. (2003) state that "destructive sampling of type specimens for any characters has always been a severe problem and such procedures are accepted only as a last resort. To destroy a sample to extract its DNA might be inadvisable in the light of future needs."

Notably, the use of abdomens for genitalia dis-
section (and, hence, destruction of abdominal soft tissue) is broadly accepted by lepidopterists for the purposes of taxonomy and identification. Our combined procedure offers the following advantages under these circumstances:

(a) When specimens are chosen for dissection, extraction of DNA from the maceration mix implies neither the need for an extra procedural step nor further damage to the specimen.

(b) It applies well to microlepidoptera, many of which cannot be identified by external features and therefore genitalia dissection is necessary.

(c) As both morphological and molecular information can be obtained and crosslinked for the same specimen out of the same reaction vial, this approach appears to be especially useful for unique and valuable specimens, e.g., types.

(d) Dissection of genitalia is performed by almost all lepidopterists all over the world and adopting this method of preparation would prevent loss of DNA, preserving many informative characters.

(e) By using the combined procedure, DNA can be easily and cheaply preserved by any morphologist, even if the equipment for molecular investigation is not available. DNA-containing maceration fluid can be stored frozen and made accessible for molecular studies (see recommendations for treatment of specimens below).

Like any other protocol published so far, our procedure critically depends on adequately preserved material. Owing to the fact that most type material is too old to yield sufficient sequence information, it seems advisable to preserve substitutional DNA from more recent material, as long as it is possible. DNA should always be preserved when specimens are designated to become types.

It should be noted that the bursa copulatrix of females often contains spermatophores. Although the bursa is not severed in course of the extraction process, the possibility of contamination of the sample with male DNA cannot be ruled out. Though we did not find any problems when using the method for our purposes (reconstruction of phylogeny based on COI total sequences), this fact might potentially become critical under some circumstances (e.g., in intraspecific phylogeographic studies). In such cases, the use of female abdomens as source of DNA should be thoroughly evaluated.

**Recommendations for the treatment of specimens**

It is well known that DNA degrades over time due to "radiation (mainly UV), temperature, moisture, pH, oxidative agents and mechanical stress..." (Poinar et al. 1994). Hence, treatment of specimens has a significant impact on their suitability for molecular analyses.

Although a detailed analysis of factors influencing PCR results was not aim of this study, we got some preliminary evidence that relaxing of specimens can be deleterious (table 1, 2). Collectors commonly use humid containers in order to keep lepidopteran specimens relaxed and ready for setting for many days. Although to our knowledge detailed studies addressing this topic are still missing, it seems likely that humid conditions and microbial activity accelerate breakdown of DNA (N. Wahlberg, personal communication). Thus, also relaxing of dry specimens, as necessary before setting, could have negative effects on DNA.

Therefore, our recommendations for proper treatment of specimens as valuable vouchers for future molecular studies are:

1. a. Freshly collected material should be set immediately without secondarily relaxing the specimens in humid containers.

   b. If specimens cannot be set immediately, they should be pinned, the wings preliminarily spread (if necessary for recognition of traits), and dried as quickly as possible.

   c. If proper treatment of all specimens is not possible under field conditions, at least a representative subset of the sample should be treated this way.

2. Properly treated museum specimens should be clearly labeled with the method of killing and preservation, in order to indicate their suitability for molecular analyses ‘at a glance’.

3. Especially in case of type specimens, lepidopterists should routinely use enzymatic maceration for genitalia dissection and preserve the DNA-containing fluid for future molecular investigations. The whereabouts of DNA-deposition should be stated in each publication.

4. In general, DNA is saved from enzymatic degradation and hydrolysis more effectively when purified and stored frozen or dried (Lindahl 1993). Usually, professional equipment for long-term storage of DNA is not available for amateurs. Therefore, one may adjust the DNA-containing maceration fluid to 0.2 M NaCl, add
2 volumes of absolute ethanol, and preliminarily store at \(-20^\circ C\) after gentle mixing. This will preserve DNA samples for at least a few months, and it is recommended to send them to scientific institutions maintaining DNA collections as soon as possible (e.g., Zoologische Staatssammlung München, contact: Andreas.Segerer@zsm.mwn.de).

Thus, with very little extra effort, a global network of highly valuable DNA collections could be established by the community of lepidopterists. In fact this method might be useful for any other insects where maceration of abdomens is routinely used for identification.

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