DNA barcoding of museum specimens of Lymantriidae preserved in the Royal Museum for Central Africa

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Summary: Some preserved specimens of the lepidopteran collection of the Royal Museum for Central Africa were used in a pilot DNA barcoding study. They differed in age, the applied preservation methods and collection sites. Thereby, short and standard DNA barcode sequences were obtained, and species assignments based on neighbour-joining tree reconstruction were carried out and compared with morphological identification. Our results show that the DNA barcoding approach is able to distinguish lymantriid species on an efficient way. Furthermore, we show three examples where it also provides with novel information to taxonomic problems: we found a possibly undescribed species of *Leucoma*, recovered the possible synonymy of *Stracena promelaena* and *Stracena flavescens*, and found an unexpected genetic diversity of some *Porthesaroa species*.

Key words: DNA barcoding, Lymantriidae, COI, Afrotropic

Introduction

The entomological section of the Royal Museum for Central Africa (RMCA) is specialized in the study of Afrotropical insects and holds a collection of about six million specimens. The bulk of this collection was established in the first half of the last century and came mainly from the Democratic Republic of the Congo, Rwanda and Burundi. Later and also in recent years, the collections were enriched with specimens from other countries, mainly from West and East Africa.

The lepidopteran collection of the section holds about one million specimens, about half of them belong to butterflies, the other half to moths. The family Lymantriidae has a moderate size in terms of species number including about 2500 described species (Kitching *et. al.* 1998). This moth family is represented in the Afrotropical region by more than 1200 species (see the recently launched website of Afrotropical moths, De Prins & De Prins 2010). The collection of RMCA possess nearly 40% of the Afrotropical taxa. In 2007, the Joint Experimental Molecular Unit (JEMU) was founded as a collaborative project between the Royal Belgian Institute of Natural Sciences (RBINS) in Brussels and the Royal Museum for Central Africa (RMCA) in Tervuren. In a joint effort, JEMU assisted the researchers of the Lepidoptera collection in obtaining molecular genetic information based on dry preserved museum specimens. For comparison, also freshly collected material was used.

The usefulness of DNA barcoding in species identification of lepidopteran samples is well documented (e.g. Hebert *et al*. 2003, 2004, Vincent *et al*. 2009). This approach may assist in sorting unidentified samples into distinct genetic clades but also may lead to the discovery of undescribed taxa including the recovery of cryptic species (e.g. Hajibabaei *et al*. 2007). Our aim here was to perform a pilot DNA barcoding study of a hitherto underrepresented taxon, the family Lymantriidae, with a special focus on Afrotropical representatives.

Material and methods

Sampling Four series of lymantriid samples representing the dry collection were selected for molecular genetic analyses (Table 1). The specimens of the first series were collected in the Democratic Republic of Congo (Banza, Lubumbashi, Luena and Shaba River), Kenya (Kakamega Forest) and Ivory Coast (Lamto). They also differed in age and number of included representatives. Specimens of the second series came exclusively from South Africa (Drakensberg Park, Tswaing Meteorite Crater Res. and the Royal Natal National Park). Specimens of the third and fourth sample series came from the Nyungwe Forest, Rwanda, but differ in the treatment of leg removal. Specimens of the third series were already spread, i.e. they

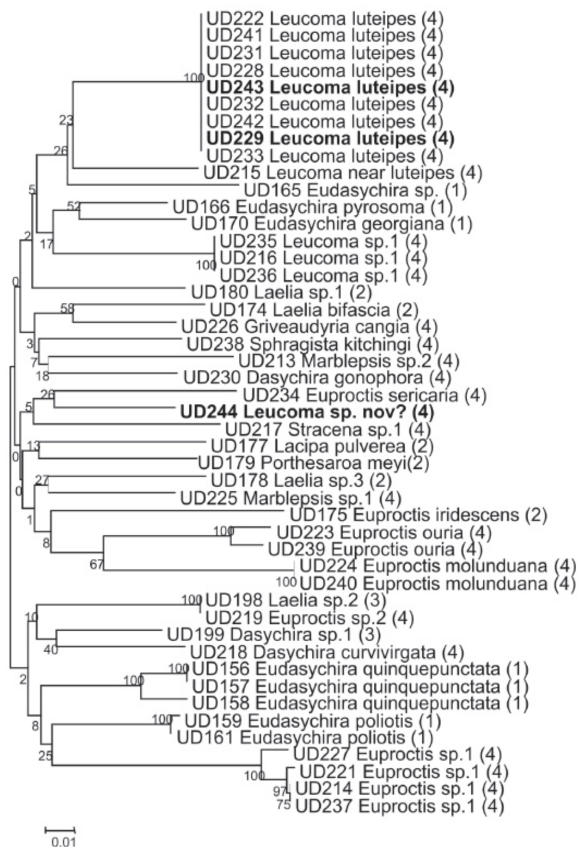

Fig. 1. Neighbour-joining tree based on partial COI sequences of the four series of dry preserved lymantriid samples. Species names are followed by the locality code (see details in Table 1) in parentheses. The three specimens marked in bold are photographed (Fig.2).

were relaxed, while in the fourth series the legs of the insects were taken off before relaxing.

Finally, a series consisted of fresh material collected in 2009 in the Bia Conservation Area, Ghana.

One or two legs of the selected moth specimens of the dry collection were removed for genetic investigations. Thereby, great care was taken to prevent any contamination (e.g. equipment was sterilized by flame and washed in methanol). Samples were stored dry in microtubes until processing them. In contrast, legs of the freshly collected specimens were preserved in absolute ethanol.

Genetic analyses Prior to DNA extraction, tissue samples were crushed and broken with forceps. Total genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's instructions. Lysis time was increased (overnight incubation at 56°C) to allow the tissue samples to dissolve completely. DNA was recovered from the membrane with 100µl elution buffer preheated at 70°C to increase yield. In order to avoid contamination, lab benches were regularly cleaned with DNA-removal agents (DNA-Away, VWR) and with ethanol. Furthermore, disposable tips with aerosol filters were used.

Polymerase chain reactions (PCRs) were carried out in 40µl end volume containing 1x PCR buffer, 5µl of DNA extract, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 units/µl of Taq DNA polymerase (Qiagen PCR Kit) and 0.4 µM of the forward and reverse primers. The primers Lep-HybLCO and Lep-HybHCO were used since this combination was successfully used to amplify "DNA barcoding" fragments of the cytochrome oxidase I (COI) gene in lepidopterans (Wahlberg & Wheat 2008).

The PCR profile started with an initial denaturation of 4 min at 94 °C, followed by 40 cycles of 60 s at 94 °C, 60 s at 46 °C and 60 s at 72 °C. The PCR ended with a final elongation step of 10 min at 72 °C. The PCR was evaluated on pre-cast 1.2% agarose gels (Invitrogen). PCR products were purified on NucleoFast PCR plates (Macherey-Nagel) using vacuum filtering. Cleaned PCR products were sequenced in both directions on an ABI 3130xl DNA sequencer and using the BigDye Terminator v1.1 chemistry (both Applied Biosystems).

Data evaluation DNA sequences were checked, assembled and aligned using the SeqScape v2.5 (Applied Biosystems) software. Basic phylogenetic analyses were conducted using MEGA v4 (Tamura *et al*. 2007). A simple neighbour-joining tree based on Kimura 2-parameter (K2P) distances was used to compare and visualize the obtained sequences. Non-parametric bootstrapping was used to infer branch supports.

Results and discussion

The utility of dry preserved material Table 1 shows that sample series had different ages. The first and oldest series included *Eudasychira* specimens, this genus was recently revised (Dall'Asta 2009). Unfortunately, DNA quality was low which was reflected in the relatively low success rate of the PCRs. Furthermore, only "mini-barcodes" of ca. 300bp were obtained, although these still allowed species distinction.

The more recent material from South Africa performed significantly better. DNA yield was still low but the amplification of (at least) the same short fragment was possible in all cases.

Using a third and fourth series of samples, a comparison was possible between similar samples collected in the same region (Nyungwe Forest, Rwanda). These were collected recently (in 2007), the main difference between both series was related to the re-

Fig. 2. Specimens identified as *Leucoma luteipes* (Walker, 1855). COI data show that the specimen at the bottom probably represents a different species than the two specimens on the top.

laxation of the specimens in wet environment, a common practice in lepidopteran collections. Relaxed specimens performed insufficiently, only degraded DNA coupled with very low PCR success rate was obtained. In contrast, the majority of the unrelaxed material gave positive results. These observations clearly show the negative effect of relaxation on DNA preservation.

In total, 47 COI sequences were obtained out of 89 samples (Table 1). These sequences of the four sample series were analysed in a combined manner, a neighbour-joining tree is presented in Fig. 1. Species are separated by long branches, high support values are only found at species level where species are represented by multiple specimens. Short sequence markers are obviously not applicable to infer any phylogenetic relationships. In the current study, we used the COI marker for specific assignment. For a phylogenetic study within the family, further genetic markers coupled with an appropriate taxon sampling would be needed.

Example for the discovery of a possible new taxon The moth genus *Leucoma* is represented in the Afrotropical region by more than thirty species, many of them are very similar in appearance. Nearly half of them have white fore- and hind-wings. Collenette

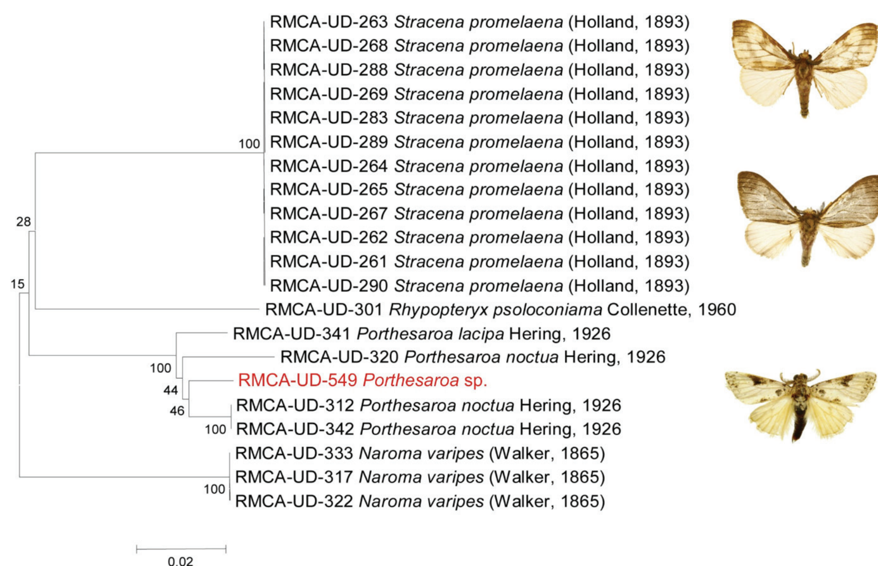

Fig. 3. Part of the neighbour-joining tree based on partial COI sequences of some freshly collected lymantriid samples. See explanation in text.

Table 1. Series of lymantriid samples used in molecular genetic analyses.

Sample Group	Locality	Collection date	Type of preservation	Number of samples / successful results	PCR success	Fragment length
(1)	DRC, Ivory Coast & Kenya	1979-1999	Relaxed specimens	18/8	44%	307bp
(2)	South Africa	10/2003	Relaxed specimens	6/6	100%	643bp/307bp
(3)	Nyungwe Forest (Rwanda)	02/2007	Relaxed specimens	32/2	6 %	643bp
(4)	Nyungwe Forest (Rwanda)	16/04-18/08/2007	Not relaxed specimens	33/31	94 %	643bp
(5)	Bia Conservation Area, Ghana	01/2009	Absolute ethanol	88/88	100 %	658bp

(1960) discovered that these are best identified based on their genital morphology.

A series of *Leucoma* specimens was used in the molecular study, specimens were collected in the Nyungwe Forest (Rwanda) between April and August 2007. Most specimens were identified as *Leucoma luteipes* (Walker, 1855) or remained unidentified on the species level as *Leucoma* sp. 1. *Leucoma luteipes* typically has a peculiar shining white hue in the wings and very thin dark margin on the fore and outer side of the fore-wing (Fig. 2). The specimen RMCA-UD-244 appeared slightly different in external morphology, and had a completely different COI sequence (Fig. 1), and thus possible represents an undescribed species.

Fresh material used for DNA barcoding Tissue samples were taken from recently collected specimens in the field in the Bia Conservation Area (around 3°00 W, 6°30 N), Ghana. An initial set of this fresh material (see Table 1) was selected for a second analysis. A neighbour-joining tree was estimated based on COI sequences and presented in Fig. 3. Here, two particular results are highlighted.

***Stracena promelaena* (Walker, 1855)** Between 1993-1997, a series of five expeditions were organized in the 'Forêt classée de la Bossematié', a semi-deciduous tropical forest in Ivory Coast, situated about 200 km to the north of Abidjan (at around the same latitude as the Bia Conservation Area in Ghana). Moths were collected, and two species of the genus *Stracena* were found to be the most common lymantriids. The first one was identified as *Stracena promelaena* (Walker, 1855). The identification of this species is simple, based on numerous rounded yellow spots on the fore wing between the veins on a brownish background. The second one was identified as *Stracena flavescens* (Aurivillius, 1925) in which the yellow spots are absent. That means that the colour of the fore wing is more or less uniformly brown (see the top two pictures on the tree in Fig. 3). Both putative species were equally abundant and no notable intermediately coloured specimens were caught. Interestingly, no significant difference was found in the structure of their genitalia, although the valvae had a very intricate structure.

In the Bia conservation area, situated about 80 km to the east of the Bossematié Forest, the same two *Stracena* species were found to be the most abundant lymantriids. Specimens of both *promelaena* and *flavescens* were collected for molecular genetic analyses. Interestingly, all specimens possess the same COI sequence, and thus probably belong to the same species. It means that molecular data and genital morphology agree while these observations contradict the external morphology (i.e. colour) making colour in this case less suitable for identification and species delimitation.

The *Porthesaroa* diversity The genus *Porthesaroa* Hering, 1926 was described including a Malagasy type species (*P. aureopsis*) and two species from Cameroon, *P. lacipa* and *P. noctua*. The latter ones were often found in West Africa and were also collected during the Bia expedition. Besides these two, one putative unknown species was also caught, readily recognizable by a large black area in the middle part of the forewing (Fig. 3, photo at the bottom). This specimen (RMCA-UD-549) was also included in the molecular study, and its COI sequence represents a unique haplotype (Fig. 3). Interestingly, the specimen

RMCA-UD-320 originally identified as *Porthesaroa noctua* also shows a different haplotype compared to the other *noctua* specimens, although divergences are rather low. Also in this case, more samples from different localities will yield more information on (phylo)geographic differences, and an in-depth morphological study of the genitalia will be used to confirm or refute the molecular findings.

DNA degradation As soon as an organism dies, DNA starts decaying. Under certain conditions, such as high salinity and very low temperatures, DNA may be preserved for a considerable longer time. They all have in common that water is removed from the cells or immobilised. As a consequence the nucleases that cause the DNA to break down are inactivated (Lindahl 1993, Wandeler *et al*. 2003, Pääbo *et al*. 2004, Nicholls 2005). The particular details of taxonomic collection and specimen mounting and preservation practices make lepidopteran collections more vulnerable to rapid degradation. The common practice is to air-dry specimens for a certain period and then resoak them in water to be able to spread the wings for display in the collection. Because of slow removal of water from the tissues by air-drying and because of re-soaking the tissues both practices are very detrimental to the size and quality of the DNA molecules.

DNA barcoding in the identification process It is well known that DNA barcoding can assist with identifying species in a quick way as well as in finding new or possible cryptic species (Hebert *et al*. 2004, Laguerre & Rougerie 2009) once an appropriate reference database is available. Our aim is to contribute to this reference database by investigating catalogued, traceable specimens of lymantriid moths. In the present case, DNA barcoding provided a good hint towards detecting possible areas of interest for taxonomists. Also, using short sequences ("mini-barcodes") we were able to assign specimens correctly (see also Hajibabaei *et al*. 2006). However, the retrieved sequences are not suitable to be used to infer phylogenetic relationships.

Conclusions

Molecular genetic analyses of lymantriid specimens represent a promising tool for the study of the diversity, taxonomy and phylogeny of the family. This analysis performs less efficiently on museum specimens than it does on freshly collected material. Even in recently collected specimens, DNA can degrade quickly through relaxing before spreading. If molecular information is needed of dry specimens, tissue samples should be taken before spreading the specimens. Taking samples for molecular analysis ideally should become part of every sampling program in the Afrotropical region because of the promising prospectives in finding taxonomic novelties.

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