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DNA barcoding enables the identification of caterpillars feeding on native and alien oak

(Lepidoptera: Geometridae)

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Abstract

Insect ecologists have to deal with the identification of many species, e.g. when assessing the ecological consequences of the introduction of alien plant species. Insect identification is a big challenge, especially regarding larval stages, though caterpillars are important herbivores and their identification is crucial in biodiversity assessments. DNA barcoding is a promising method to solve this problem, but examinations with samples of biodiversity assessments are rare, so far. We tested if DNA barcoding is a forward-looking method for biodiversity assessments of caterpillars. Therefore we sampled caterpillars on native *Q. robur* and alien *Q. rubra* in southern Germany. For the five geometrid species recorded we showed that all species could be clearly separated and identified genetically. The success of our approach suggests that importance of DNA barcoding for future assessments of larval stages will considerably increase.

Introduction

Global loss of biodiversity has stimulated a large number of biodiversity assessment projects in the last decade. A leading cause of the biodiversity loss is the introduction of alien species (Sala et al. 2000), so understanding factors leading to success of invasives is of great importance. One factor that can lead to success of invasives is the lack of natural enemies (enemy release hypothesis; Keane & Crawley 2002). This can be tested by assessing herbivores on introduced and native species but this requires identification of all species. Identifying so many species is, however, a big challenge particularly for insect larval stages. For example, most Lepidoptera caterpillars, although very important herbivores in many ecosystems (Berryman 1996; Kendall et al. 1998; Myers 1988), cannot be identified reliably to species level by morphological characteristics. To overcome these difficulties DNA barcoding has become an increasingly important tool in biodiversity assessments (Caesar et al. 2006; Smith et al. 2005; Valentini et al. 2009). Apart from the identification problem, there is also the so-called 'taxonomic impediment' (de Carvalho et al. 2007), i.e. unresolved taxonomy and/or cryptic diversity which in many cases hampers the correct analysis of data. Looking at levels of intraspecific diversity may allow the identification of cryptic species.

The DNA Barcode of Life Data System (BOLD, http://www.boldsystems.org) has been developed since 2004 (Hebert et al. 2003) and was presented officially in 2007 (Ratnasingham & Hebert 2007). This program is entering a new phase in 2009 with the establishment of the international Barcode of Life initiative (iBOL) aiming to generate DNA barcodes for all animals (here basing on mtDNA COI gene 5' fragment, 658 bp), plants and fungi of our planet as a reference and re-identification tool for biodiversity assessments worldwide (iBOL 2009).

In Central European forests Red Oak (*Quercus rubra* L.) is the most economically important alien broad-leaved tree species (Knoerzer & Reif 2002). Studies on herbivores of this neophyte in Europe, however, are still rare. Previous empirical studies were based on trap samples (Gossner 2008; Gossner & Simon 2004; Gossner et al. 2009), analysis of acorn inhabiting species (Gossner & Simon 2004; Kelbel 1996) or direct sampling and assessment of leaf damage (Ashbourne & Putman 1987). In these studies mostly mobile adult stages were studied and almost no species level data exists on immobile larval stages.

To assess the suitability of DNA barcoding as tool to identify immobile larval stages feeding on native *Quercus robur* and alien *Q. rubra*, caterpillars were sampled from both tree species in a mixed young broad-

leaved tree stand in Southern Bavaria. Geometrid larvae were selected as target group due to the high coverage of this family in BOLD database (95% of the Bavarian geometrid species barcoded).

Material and Methods

Study area

The study was conducted in an afforestated area located near Landau an der Isar in Southern Germany (48°38'N; 12° 45'E) between May and July 2007. The area belongs to the growth region "Tertiäres Hügelland" (district "Östliches Niederbayerisches Tertiärhügelland"). The potential natural vegetation is beech forests (Luzulo-Fagetum, Galio-Fagetum) with Silver fir, *Abies alba*, on periodically wet sites (Walentowski et al. 2006). Altitude of the site is 410 m above sea-level with a mean annual precipitation of 750 mm and a mean annual temperature of 7°C. The stand was afforested by *Q. robur* and *Q. rubra* as dominant tree species, admixed with *Fagus sylvatica*, *Betula pendula* and *Alnus glutinosa* on former agricultural land in 1993.

Caterpillar sampling

In the studied stand, 30 trees of *Q. robur* and *Q. rubra*, each distributed over the whole stand, were selected pairwise for the assessment of Geometrid caterpillars. Sampled oak trees were about 17 years old and 8 to 10m high. Two branches at different heights (breast height and 2-3.5m height), from each tree, were beaten in order to sample caterpillars. A beating tray (diameter 0.5m; area 0.2m²) with a telescopic rod was positioned below each branch and the branch was then vigorously shaken with a crook attached to another telescope bar. The arthropods falling off the branches were sampled with a sampling jar mounted on the collecting funnel. All arthropods were preserved in 100% alcohol, pooled for each of the 60 tree samples. Additionally, branches were cut to control for specimens that did not fall down by beating. All specimens were subsequently sorted to taxonomic orders and Geometridae larvae were analysed by DNA barcoding.

DNA analysis

For DNA analysis, larvae were cut vertically and one central segment (A3 or A4) used for analysis, in order to preserve anterior and posterior parts which are more important for larval morphology. After tissue sampling scissors and pincers were decontaminated by cleaning in 100% alcohol and subsequent exposure to an open flame. Tissues were transferred to a lysis plate under ca 0.5 ml 100% alcohol. Plates were sent to CCDB, Canada, University of Guelph (Paul Hebert) for extraction, PCR and sequencing. The target gene was the mtDNA cytochrome oxidase I gene (COI), 5' barcode fragment (658 bp), using standard high-throughput protocol (Ivanova et al. 2006) and analysed in the Barcode of Life Datasystems (BOLD; Ratnasingham & Hebert 2007). Additional analysis was performed by MEGA4 (Tamura et al. 2007). The terms 'sequence variation' and 'genetic difference' refer to the analysis of the COI 5' barcode fragment (658 bp) by neighbour joining trees based on the Kimura-2-Parameter model. Genetic distances are given in % minimum pairwise distance, intraspecific genetic variation in % maximum pairwise distance. Images, and further details such as voucher hosting institution, GPS coordinates, sequence data and trace files can be obtained online from BOLD-database (2009), public projects FBLGE and FBLGL.

Results

Altogether 58 caterpillars were sampled, 24 Noctuidae, 13 Tortricidae, 13 Geometridae and eight other Microlepidoptera. Of the 13 Geometridae 10 (77%) could be sequenced to full fragment length. All ten of these larvae were successfully identified to species level based on DNA barcoding. Five species were identified: *Cyclophora punctaria* (Linnaeus, 1758), *Chloroclysta siterata* (Hufnagel, 1767), *Operophtera brumata* (Linnaeus, 1758), *Agriopis marginaria* (Fabricius, 1776), and *Ectropis crepuscularia* (Denis & Schiffermüller, 1775).

Intraspecific genetic variation (maximum pairwise distance) was below 1.5% in all available Bavarian data for the five study species (Figure 1). However, *Ectropis crepuscularia* was characterised by a deep 'intraspecific' split possibly referring to cryptic diversity and requiring further taxonomic study which is currently in progress. In this study, *Ectropis crepuscularia* is defined strictly as only the 'common' haplotype, which includes >80% of the Bavarian individuals. Nearest neighbours to the five geometrid species are shown in Table 1. The minimum pairwise distance indicates that identification is unambiguous in all cases.

species	Nearest neighbour	Minimum pairwise distance	remark
Cyclophora punctaria	*C. linearia* (Linnaeus, 1758)	4.3%	
Chloroclysta siterata	*Plemyria rubiginata* (Denis & Schiffermüller, 1775)	7%	*C. miata* not sequenced so far.
Operophtera brumata	*O. fagata* (Scharfenberg, 1805)	7.3%	
Agriopis marginaria	*A. aurantiaria* (Hübner, 1799)	8.5%	
Ectropis crepuscularia s.str.	'*E.* near *crepuscularia*'	5.7%	Undescribed sister species or polymorphic haplotype
Ectropis crepuscularia s.str.	*Paradarisa consonaria* (Hübner, 1799)	10.0%	

Table 1: Nearest neighbours and minimum pairwise distances (COI-gene; Kimura 2 parameter, analysed in BOLD) to the five observed geometrid species.

Five individuals belonging to two species were found on alien *Quercus rubra*, five individuals belonging to three species on native *Quercus robur*. Interestingly, no species was found on both tree species. On *Quercus robur* we found *Cyclophora punctaria* (1 ind.) , *Chloroclysta siterata* (2 ind.) and *Agriopis marginaria* (2 ind.), on *Quercus rubra Operophtera brumata* (4 ind.) and *Ectropis crepuscularia* (1 ind.).

Discussion

This pilot study is one further element to emphasize the great potential of DNA reference profiles for biodiversity assessments. Re-identification was easy through the identification engine of BOLD (Hidden Markov Model; cf Ratnasingham & Hebert 2007) which allows examination of each barcode against the whole database. The re-identification was also un-ambiguous in the 382 Bavarian geometrid moth species (out of a total of 405 species; >800 individuals) so far sequenced. No case was found with two different species sharing the same COI barcode, all species were separated by a genetic distance of at least 3%, with one exception: *Ennomos autumnaria* (Werneburg, 1859) and *Ennomos quercinaria* (Hufnagel, 1767) (2.5%). This shows that potential pitfalls such as introgression or gene transfer (through *Wolbachia*) were irrelevant for our study. In three Bavarian geometrid species substantial intraspecific divergence was found, these do not, however, affect the results of our study. The larvae identified as '*Ectropis crepuscularia*' belonged to the most abundant haplotype, which is present in 80% of the Bavarian populations of that 'species' (traditional species concept). Taxonomical studies are in progress to clear up (1) whether the two observed haplotypes refer to cryptic diversity or to genetic polymorphism and (2) which of both clades have to be attributed nomenclaturally to the name '*crepuscularia*'.

What does this mean for synecological studies? These results clearly indicate that DNA barcoding is a promising method to overcome problems which occur in traditional studies on Lepidoptera caterpillars. These difficulties include problems with determining most caterpillars to species level, high failure rates in rearing adults from larval samples and time and resource consuming procedures necessary for caterpillar identification. Consequently, previous ecological studies primarily focused on adults (mostly sampled by light trapping, e.g. Chao et al. 2009; Hawes et al. 2009), were based on higher taxonomic levels (Gossner & Simon 2004) or analysed leaf damage caused by caterpillars (e.g. Dietz et al. 2004). DNA barcoding might enable the investigation of many ecological questions, for instance studies on herbivorous insects on alien tree species. While trapping of adults is always biased because these species may not actually be feeding on the tree on which they are sampled; larval samples gained by branch beating or insecticide fogging allow more reliable analyses of the herbivores feeding on a particular host. Moreover, DNA barcoding will allow analyses of a high number of replicates within a short time and therefore results in statistically meaningful data. Not all problems will be solved by DNA barcoding, but it will open up the chance to study many ecological questions of high theoretical and advanced relevance in a more sophisticated way.

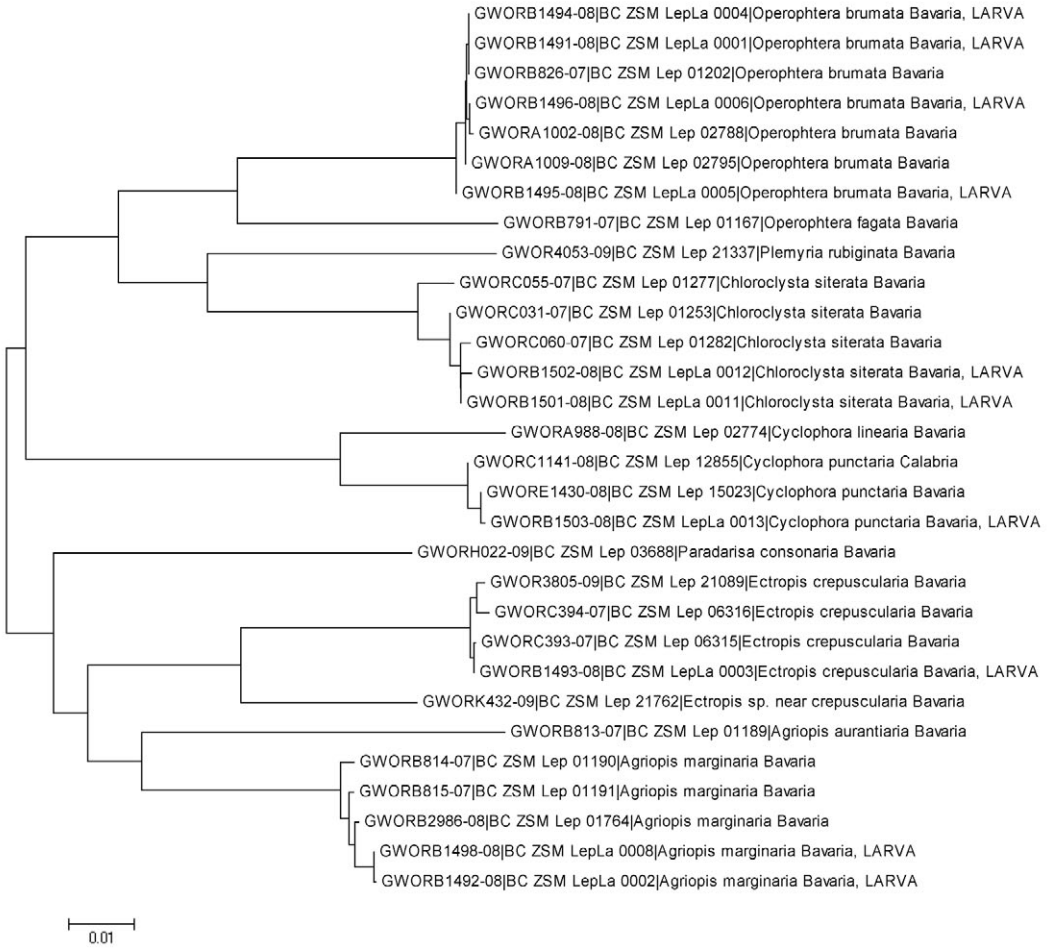

Figure 1: Neighbour joining tree diagram (COI-gene; MEGA4, Kimura 2 parameter, pairwise deletion) of the 10 Bavarian geometrid caterpillars sampled in present study (LARVA) including data from three additional specimens of the 5 species, and one specimen of the nearest neighbour species (data from BOLD, vouchers from Bavarian State Collection of Zoology ZSM).

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Zusammenfassung

Ökologisch arbeitende Entomologen sind stets mit dem Problem der Artidentifikation umfangreicher Ausbeuten konfrontiert, z. B. beim Studium der ökologischen Auswirkungen der Einführung von Neophyten. Dies bereitet vor allem bei Larvalstadien von Insekten oft unüberwindliche Hindernisse, obwohl Schmetterlingsraupen als substratgebundene, herbivore Organismen eine wichtige Schlüsselgruppe bei Biodiversitätserhebungen darstellen könntne. Trotz der zunehmenden Wahrnehmung von DNA barcoding als vielversprechender Methode zur Identifikation von Organismen aller Art, gibt es noch immer wenig Evaluationen ihres Potentials bei der Erfassung herbivorer Larvalstadien. Wir sammelten hierzu Schmetterlingsraupen auf einheimischen sowie eingebürgerten Eichen (*Q. robur* versus *Q. rubra*) in Süddeutschland. Die Raupen aller fünf nachgewiesenen Geometridenarten konnten zweifelsfrei über ihren genetischen Barcode identifiziert werden. Unsere Ergebnisse zeigen, dass DNA barcoding viele der Probleme lösen kann, die derzeit im Zusammenhang mit Erfassung von Larvalstadien bestehen.

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