Insect science

Insect Science (2011) 18, 349–362, DOI 10.1111/j.1744-7917.2010.01366.x

ORIGINAL ARTICLE

DNA barcoding-based species delimitation increases species count of *Eois* (Geometridae) moths in a well-studied tropical mountain forest by up to 50%

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Abstract The genus *Eois* comprises an important part of megadiverse assemblages of geometrid moths in mountain rainforests of southern Ecuador. In this study we report: (i) on the construction of a DNA barcode library of *Eois* for identification purposes; and (ii) the exploration of species diversity through species delimitation by pair-wise distance thresholds. COI barcode sequences were generated from 408 individuals (at least 105 species) collected on a narrow geographic scale (~40 km^2) in the Reserva Biológica San Francisco. Analyses of barcode sequence divergence showed that species delimitations based solely on external morphology result in broad overlap of intra- and interspecific distances. Species delimitation at a 2% pair-wise distance threshold reveals a clear barcoding gap. Fifty-two previously unrecognized species were identified, 31 of which could only be distinguished by an integrative taxonomy approach. Twelve additional putative species could only be recognized by threshold-based delimitation. Most splits resulted in two or three newly perceived cryptic taxa. The present study increased the number of *Eois* species recorded from that small area of Andean mountain forest from 102 to 154 (morphology- plus integrative taxonomy-based) or even 166 (sequence-based), leaving the species accumulation curve still far from reaching an asymptote. Notably, in no case did two or more previously distinguished morphospecies have to be lumped. This barcode inventory can be used to match larvae to known adult samples without rearing, and will therefore be of vital help to extend the currently limited knowledge about food plant relationships and host specialization.

Key words cryptic species, Ecuador, integrative taxonomy, Larentiinae, larvae

Introduction

In recent years DNA barcoding has developed into a quick and increasingly inexpensive tool for species-level identification of all Metazoa. A 658 bp fragment from the 5′ part of the mitochondrial cytochrome c oxidase subunit I (COI) gene, as amplified by the primers LCO1490/HCO2198 described by Folmer *et al.* (1994), has been proposed as a universal marker for animals (Hebert *et al.*, 2003a, 2003b). Two major applications of DNA barcoding are: (i) the exploration of species boundaries in insufficiently known taxa; and (ii) the identification of already known species, especially in cases where morphological differentiation is minimal or remains unexplored, as is the case in the early life cycle stages of many arthropod species.

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The conceptual foundation for DNA barcode-aided species discrimination is the assumption that interspecific genetic distances exceed intraspecific distances by such a margin that a distinct gap exists. The presence of this so-called "barcoding gap" allows one to set a threshold for species discrimination. Sequence pairs with distances below the threshold are thought to be conspecific, whereas pairs with distances above the threshold are accepted as belonging to separate species. Proponents of DNA barcoding claim that this assumption is virtually always true and deviations are either caused by a negligible number of cases of incomplete lineage sorting or can be attributed to shortcomings in traditional taxonomy (i.e. failure to recognize cryptic species) of the taxa in question (Barrett & Hebert, 2005; Hajibabaei *et al.*, 2006; Hebert *et al.*, 2003a, 2003b, 2004a). The universal presence of a barcoding gap would then allow for instant species delimitation even in previously unknown taxa. However, a number of case studies where the power of DNA barcoding, to distinguish morphologically or otherwise well-defined species, has been subject to scrutiny show that this does not always apply. Recent examples that demonstrate problems with the assumption of a barcoding gap come from diverse groups such as lycaenid butterflies (Wiemers & Fiedler, 2007), ithomiine butterflies (Elias *et al.*, 2007), orthopterans (Trewick, 2008), harvestmen (Boyer *et al.*, 2007), or land snails (Davidson *et al.*, 2009). However, the limited performance of DNA barcoding in these case studies could be attributed to shortcomings in taxonomy of the respective target group (i.e. classical taxonomy failed to recognize cryptic species) or to occurrences of very young species splits.

One major objective of DNA barcoding is identification of species without the need for taxonomic expertise. It is quite obvious that the success of identification through barcoding is crucially dependent on comprehensive taxon sampling (Elias *et al.*, 2007; Wiemers & Fiedler, 2007). The short barcode sequences contain only very limited phylogenetic information and correct assignments of focal samples do require quite closely related sequences from a template library with which to compare. DNA barcodes are usually not suitable for taxonomic assignments above the species level. Furthermore, barcoding is bound to fail when applied to non-monophyletic, recently diverged species and in cases of hybridization. Funk and Omland (2003) found that in a sample of 2 319 animal species, 23% were not monophyletic. Many publications tried to highlight the conceptual shortcomings of DNA barcoding (e.g., DeSalle *et al.*, 2005; Meyer & Paulay, 2005).

The first objective of this study is to test the utility of DNA barcoding to explore species richness of one diverse, taxonomically understudied, tropical moth genus and the applicability of standard pair-wise distance thresholds within this context.

Eois Hübner (Lepidoptera: Geometridae, Larentiinae) is a speciose genus of rather small-sized moths and comprises an important part of a megadiverse assemblage of geometrid moths in the mountain rainforests of southern Ecuador (Brehm *et al.*, 2005). Over the past 10 years, the geometrid fauna of one particular locality, the Estación Científica San Francisco (ECSF), situated in the Reserva Biológica San Francisco (RBSF), has served as a paradigm to investigate patterns in species diversity and community structure of tropical moths (e.g. Brehm *et al.*, 2003a, 2003b, 2005; Brehm & Fiedler, 2003, 2005; Hilt *et al.*, 2006; Fiedler *et al.*, 2008). The moth fauna of that area is arguably the best known of all Andean mountain forests. In these ecosystems, representatives of *Eois* account for 8.1% of the morphospecies and 10.2% of all individuals of geometrid ensembles (Brehm *et al.*, 2005). The genus *Eois* occurs in the Americas, ranging from Mexico to Argentina, as well as in South-east Asia, Australia and Africa (Scoble, 1999). Scoble (1999) recognized 250 species, the majority of which (207) occur in the Neotropical region. Brehm *et al.* (2005) found 102 *Eois* species, exclusively delimited by wing patterns, to occur in the RBSF and adjacent areas between 1 000–2 700 m elevation.

Host plant associations of Neotropical *Eois* are incompletely known, although a number of host plant records for *Eois* have accumulated in recent years as part of massive campaigns to elucidate tropical food webs. The predominant host plant family is the Piperaceae with *Piper* being by far the most commonly used genus. A smaller number of host records exist from Chloranthaceae, Monimiaceae and Gesneriaceae (Bodner *et al.*, 2010; Connahs *et al.*, 2009; Dyer *et al.*, 2009; Dyer & Gentry, 2009; Janzen & Hallwachs, 2009). In order to firmly establish host plant records in the absence of reliable identification literature for life cycle stages, most tropical Lepidopteran larvae must be collected and then reared to adulthood for identification. This approach is often troubled by high mortality of caterpillars through parasitoids and pathogens as well as by the massive need for manpower. In this situation identification of larvae through DNA barcodes, without the need of rearing individuals to adulthood, provides an elegant solution. The second objective of this study is therefore to generate a barcode library that allows larvae to be matched to sequences obtained from identified adult moth vouchers. The use of DNA barcode sequences for the matching of larvae to adult stages can be considered a well-established method and has been applied in a number of studies (e.g., Miller *et al.*, 2005;

Webb *et al.*, 2006; Ahrens *et al.*, 2007; Pfenninger *et al.*, 2007).

Material and methods

Collection and DNA sequencing

Most moths (327 individuals, 80%) used in this study were collected at light traps in a small area of mountain ravine (quebrada) forests (approximately 1 km^2 in extension) in the surroundings of the Estación Científica San Francisco (southern Ecuador, 3°58′S, 79°04′W, elevational range 1 850–2 000 m a.s.l.). Light-trapping in the ravine forest occurred at nine sites (Zimmermann, 2005; Günter *et al.*, 2008, Table S1). All geometrid moths arriving at a weak light source (2 × 15 W tubes, placed in a gauze cylinder at ground level) were manually collected in the time interval between 19:00–22:00 h during 30 nights in February to May 2005. Moths were stored in a freezer and subsequently representatives of *Eois* were sorted out. In all, *Eois* specimens accounted for approximately 12% of the geometrids sampled. Most *Eois* specimens were sequenced except for a few abundant species (> 20 individuals) where 70%–80% of the specimens were sequenced. All other samples were taken in November 2008 from nearby lower and higher sites using the same methodology. Sixty-eight samples were obtained from lower elevations (Parque Nacional Podocarpus, Bombuscaro entrance, 4°06′S, 78°58′W, ca. 1 000–1 025 m a.s.l.), and 13 samples from higher elevations (12 from 3°59.7′S, 79°04.1′W, 2 670 m a.s.l., one from 4°06.7′S, 79°10.5′W, 2 985 m). Collection sites for all specimens are indicated in Table S1; for details about the area see Günter *et al.* (2008). Moths were killed with cyanide and stored at −20°C until DNA extraction. Heads and/or legs of moths were homogenized with ceramic beads using a Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France) set to 5 000/min for 2 × 20 s. When extracting DNA from larvae the homogenization step was skipped and larvae were instead cut into small pieces. The remaining protocol was identical for larvae and adults stages. DNA extraction was performed with the DNEasy Tissue Kit (Qiagen, Hilden, Germany) or the Peqgold Tissue DNA mini Kit (Peqlab, Erlangen, Germany) according to the protocol supplied with the respective kit. The target fragments were amplified using the Fermentas polymerase chain reaction (PCR) system (Fermentas, Burlington,ON, Canada). PCR reactions were set up with 2.5 μL of 10 × $(NH_4)_2SO_4$ PCR buffer, 2 μL 25 mmol/L $MgCl_2$, 0.1 μL 10 mmol/L dNTPs, 1 μL of each primer, 1 μL genomic DNA, 1 μL *Taq* polymerase and filled to 25 μL with PCR-grade H_2O. A PCR cycler program modified from Hebert *et al.* (2003a) was used. PCR reactions were purified by digestion with shrimp alkaline phosphatase and exonuclease for 15 min at 37°C followed by 15 min at 80°C for enzyme deactivation. Sequencing reactions were set up with 1 μL ABI BigDye 3.1 (Applied Biosystems, Carlsbad, CA, USA), 1 μL primer, 1 μL template DNA and filled to 10 μL with PCR grade H_2O and sequenced on an ABI capillary sequencer. PCR products were sequenced in both directions. PCR and sequencing was performed with primer pairs LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′)/HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′), LCO1490/Nancy (5′-CCC GGT AAA ATT AAA ATA-3′) or LepF (5′-ATT CAA CCA ATC ATA AAG ATA TTG G-3′)/LepR (5′-TAA ACT TCT GGA TGT CCA AAA AAT CA-3′). The thermal cycler was set to 25 cycles of 20 s at 94°C, 20 s at 48°C and 4 min at 60°C.

Morphological examination of moths and species coding

Moths were either spread or the wings of the right body side were cut off and mounted on a piece of cardboard. Photographs in dorsal and ventral views were taken, resized and visually examined using Adobe Photoshop versions 8 and 9. Moths were provisionally identified by comparison of wing patterns with type material or photographs of type material (98% of all described Neotropical species available, Brehm *et al.* submitted). Examination of type specimens revealed that ∼87% of species from Ecuador recognized by morphological sorting are still formally undescribed (G. Brehm, F. Bodner, P. Strutzenberger, F. Hünefeld, F. K. Fiedler, submitted). All *Eois* morphotypes that could not be matched to type material or to a morphospecies already found by Brehm *et al.* (2005) were subsequently treated as separate novel entities.

In order to unequivocally label the entities encountered, three 'types' of species names were assigned in the present study (Table 1, second column). (1) When a specimen in our sample could be conclusively matched to a described species, it was designated as being identical to that species (e.g. *Eois borrata* 396). Being matched to a described species implies that the species was already known from morphological sorting prior to this study. Numeric identifiers after the species epithet are unique species code numbers used in all ecological studies of the RBSF Lepidopteran fauna thus far. (2) Specimens that could not be matched to a described species with sufficient certainty but have a wing pattern that is highly similar to a described species were assigned as 'near' the described species (e.g. *Eois* spnr *azafranata*). If species epithets in such cases are

Table 1 List of all 118 species covered in this study. The number of individuals per species is indicated for all three means of species delimitation. Species newly recognized for the RBSF area are printed in bold; species recognized by integrative taxonomy are marked with grey shading; species that were only recognized by threshold delimitation are underlined.

Species	Name	No. individuals morpho	No. individuals 3% threshold	No. individuals 2% threshold
Sp001	*E.* spnr *azafranata* 397	9†	9†	4
Sp002	***E.* spnr *azafranata* 397**	Included in Sp001	Included in Sp001	**4**
Sp003	*E.* sp. 696	7‡	6	6
Sp004	***E.* spnr *cobardata***	**2**	**2**	**2**
Sp005	*E. escamata* 390	2	2	2
Sp006	*E.* sp. 977	2	2	2
Sp007	*E.* spnr *adimaria* 399	3	3	3
Sp008	***E.* spnr *adimaria***	**2**	**2**	**2**
Sp009	*E.* sp. 385	7	7	7
Sp010	***E.* spnr *heza***	**2**	**2**	**2**
Sp011	*E.* sp. 400	4	4	4
Sp012	*E.* spnr *golosata* 374	12	7	7
Sp013	***E.* spnr *golosata* 374**	Included in Sp012	**4**	**4**
Sp014	*E.* spnr *margarita* 398	21	5	**5**
Sp015	***E.* spnr *margarita* 398**	Included in Sp014	**16**	**16**
Sp016	***E.* spnr *borrata***	**3**	**3**	**3**
Sp017	***E.* spnr *borrata***	**6**	**6**	**6**
Sp018	***E.* sp.**	**4**	**4**	**4**
Sp019	*E. planetaria* 383	7	7	7
Sp020	*E.* spnr *restrictata* 837	3	3	3
Sp021	*E. chrysocraspedata* 1029	24	24	24
Sp022	*E.* sp. 425	12	12	12
Sp023	*E.* spnr *paraviolascens* 423	22†	11	11
Sp024	***E.* sp.**	**2**	**2**	**2**
Sp025	***E.* spnr *paraviolascens* 423**	Included in Sp023	**11**	**11**
Sp026	*E.* sp. 411	8	8	8
Sp027	*E.* spnr *trillista* 414	3	3	3
Sp028	***E.* sp.**	**2**	**2**	**2**
Sp029	*E.* sp. 427	3	3	3
Sp030	*E.* spnr *odatis* 419	7	7	7
Sp031	*E.* sp. 836	4	4	4
Sp032	*E. angulata* 376	2	2	2
Sp033	*E.* spnr *nigrosticta* 388	3	3	3
Sp034	*E.* sp. 405	2	2	2
Sp035	*E. biradiata* 410	6	6	4
Sp036	***E. biradiata* 410**	Included in Sp035	Included in Sp035	**2**
Sp037	***E.* spnr *lunifera***	**2**	**2**	**2**
Sp038	*E.* spnr *lunifera* 395	2	2	2
Sp039	*E.* sp. 394	7	7	7
Sp040	***E.* spnr *nigrinotata***	**3**	**3**	**2**
Sp041	***E.* sp.**	**5**	**5**	**5**
Sp042	***E.* sp.**	**2**	**2**	**2**

to be continued.

Table 1 Continued.

Species	Name	No. individuals morpho	No. individuals 3% threshold	No. individuals 2% threshold
Sp043	***E.* spnr *guapa***	**4**	**4**	**4**
Sp044	***E.* spnr *violada***	**7**	**7**	**7**
Sp045	*E.* spnr *violada* 403	19	19	19
Sp046	*E. chasca* 392	16	16	16
Sp047	***E.* spnr *lilacea telegraphica***	**4**	**4**	**4**
Sp048	*E.* spnr *inflammata* 515	3	3	3
Sp049	*E.* spnr *encina* 412	2	2	2
Sp050	***E.* spnr *pallidicosta***	**2**	**2**	**2**
Sp051	*E.* spnr *pallidicosta* 1023	5	5	5
Sp052	***E.* spnr *goodmanii***	**8**	**8**	**8**
Sp053	***E.* spnr *goodmanii***	**4**	**4**	**4**
Sp054	***E.* spnr *goodmanii***	**4**	**4**	**4**
Sp055	***E.* spnr *goodmanii***	**5**	**4**	**4**
Sp056	*E.* spnr *muscosa* 803	6	6	6
Sp057	***E.* spnr *olivacea***	**2**	**2**	**2**
Sp058	***E.* spnr *olivacea***	**2**	**2**	**2**
Sp059	*E.* spnr *olivacea* 416	23	9	9
Sp060	***E.* spnr *olivacea* 416**	Included in Sp059	**14**	**14**
Sp061	***E.* spnr *serrilineata***	**3**†	**2**	**2**
Sp062	*E.* spnr *catana* 426	10	10	10
Sp063	*E.* sp. 409	3	3	3
Sp065	*E.* spnr *camptographata* 739	3	3	3
Sp066	***E.* sp.**	**2**	**2**	**2**
Sp067	*E.* sp. 820	6	6	6
Sp068	*E.* spnr *lucivittata* 384	3	3	3
Sp069	*E.* sp. 386	1	1	1
Sp070	***E.* spnr *serrilineata***	Included in Sp061	**1**	**1**
Sp071	*E. antiopata* 799	1	1	1
Sp072	***E.* spnr *paraviolascens***	**1**	**1**	**1**
Sp073	*E.* spnr *inflammata* 402	1	1	1
Sp074	***E.* sp.**	**1**	**1**	**1**
Sp075	*E.* spnr. *olivacea* 408	1	1	1
Sp076	*E.* sp. 961	1	1	1
Sp077	*E.* sp. 1070	1	1	1
Sp078	***E.* spnr *chasca***	**1**	**1**	**1**
Sp079	*E.* sp. 961	1	1	1
Sp080	*E.* spnr *ignefumata* 389	1	1	1
Sp081	***E.* spnr *goodmanii***	**1**	**1**	**1**
Sp082	*E.* spnr *sagittaria* 377	1	1	1
Sp083	***E.* spnr *goodmanii***	Included in Sp055	**1**	**1**
Sp084	***E.* spnr *borrata***	**1**	**1**	**1**
Sp085	*E.* sp. 420	1	1	1
Sp086	***E.* spnr *concatenata***	**1**	**1**	**1**
Sp087	***E.* spnr *nigrinotata***	Included in Sp040	Included in Sp040	**1**
Sp088	***E.* spnr *golosata* 374**	Included in Sp012	**1**	**1**

to be continued.

Table 1 Continued.

Species	Name	No. individuals morpho	No. individuals 3% threshold	No. individuals 2% threshold
Sp089	***E.* spnr *concatenata***	**1**	**1**	**1**
Sp090	***E.* spnr *pallidicosta***	**1**	**1**	**1**
Sp091	***E.* spnr *nigrinotata***	**1**	**1**	**1**
Sp092	*E.* sp. 382	1	1	1
Sp093	*E.* sp. 2128	1	1	1
Sp094	*E.* spnr 2129	1	1	1
Sp095	***E.* sp.**	**1**	**1**	**1**
Sp096	***E.* sp. 696**	Included in Sp003	1	1
Sp097	***E.* spnr *violada***	**1**	**1**	**1**
Sp100	***E.* spnr *pararussearia***	**1**	**1**	**1**
Sp101	***E.* spnr *paraviolascens***	**1**	**1**	**1**
Sp102	*E.* sp. 1041	1	1	1
Sp103	***E.* spnr *olivacea***	**1**	**1**	**1**
Sp104	***E.* sp.**	**1**	**1**	**1**
Sp105	***E.* spnr *olivacea***	**1**	**1**	**1**
Sp106	***E.* spnr *nigrinotata***	**1**	**1**	**1**
Sp107	***E.* spnr *goodmanii***	**1**	**1**	**1**
Sp108	***E.* spnr *deleta***	**1**	**1**	**1**
Sp109	***E.* spnr *delicatula***	**1**	**1**	**1**
Sp110	*E.* spnr *ignefumata* 30	1	1	1
Sp111	***E.* spnr *fucosa***	**1**	**1**	**1**
Sp112	*E. borrata* 396	1	1	1
Sp113	***E.* spnr *camptographata***	**1**	**1**	**1**
Sp114	***E.* spnr *azafranata* 397**	Included in Sp001	Included in Sp001	**1**
Sp115	***E.* spnr *goodmanii***	**1**	**1**	**1**
Sp116	***E.* sp.**	**1**	**1**	**1**
Sp117	***E.* spnr *azafranata***	**1**	**1**	**1**
Sp118	***E.* spnr *azafranata***	**1**	**1**	**1**
Sp119	***E.* sp.**	**1**	**1**	**1**
Sp120	***E.* spnr *trillista***	**1**	**1**	**1**
Sp121	*E.* spnr *lavendula* 851	1	1	1

†Monophyly of the species is not supported in maximum likelihood trees.

‡Strong evidence against monophyly of the species is present in maximum likelihood trees; species: newly adopted numerical code of species as recognized in this study.

Name: species names and numerical identifiers according to Brehm *et al.* (2005).

followed by a numeric identifier, these species had again already been known from previous investigations of the local fauna (e.g. *Eois* spnr *azafranata* 397). In contrast, species assigned as 'spnr' but lacking a numeric identifier in Table 1 were newly recognized in the course of this study (e.g. *Eois* spnr borrata). (3) Specimens where no match could be made to any named species in reference collections were designated as unknown species without a species epithet (i.e. *Eois* sp.). Again, species with a numeric identifier were already known prior to this barcoding study (e.g. *Eois* sp. 1070), whereas species without such a numeric identifier are newly recognized ones (e.g. *Eois* sp.). In view of this complexity the identity of species is hereafter exclusively defined by a novel 3-digit numeric code (e.g. Sp067) (see Table 1, first column) and not by the assigned name (i.e. identical species epithets alone do not necessarily imply conspecificity).

Sequence data processing

Proofreading of sequences and contig assembly was done with ChromasLite Version 2.01, ChromasPro Ver

1.34 (Technelysium Pty Ltd, Tewantin, Queensland, Australia, http://www.technelysium.com.au/) and DNAStar Lasergene SeqMan Pro Ver. 7.1 or Ver. 8 ('DNASTAR Inc., Madison, WI, USA, http://www.dnastar.com/). Sequences were either 658 or 676 bp in length, longer sequences were cropped to 676 bp in length. All sequences were aligned manually using Bioedit Ver 7.0.4.1 (Hall, 1999). Sequence data was prepared for analysis using the programs FORCON version 1.0 and MEGA version 4 (Tamura *et al.*, 2007). Sequences were screened for unusual nucleotide composition and the presence of stop codons to control for possible nuclear mitochondrial pseudogene (NUMT) amplification; see Song *et al.* (2008) for a review on potential problems associated with NUMTs in DNA barcoding.

Sequence analyses

Pair-wise Kimura-2-parameter distances (Kimura, 1980) were calculated with PAUP* (Swofford, 1999). This particular measure of genetic distance has been chosen to facilitate comparability with other DNA barcoding studies where it has been used extensively. Distances were analyzed with Microsoft Excel for Mac version 12.1.0. Maximum likelihood trees were calculated with RAxML (Stamatakis, 2006) performing a search for the best known likelihood tree with 500 replicates and bootstrapping using the rapid hill climbing algorithm with 1 000 replicates. Maximum likelihood trees and neighbor-joining diagrams were midpoint rooted. Maximum likelihood trees were used to assess the monophyly of threshold-defined species in a phylogenetic context and therefore their status under the phylogenetic species concept. In the analysis of barcode sequences a comparison has been made between species delimited by a sequence divergence threshold of 3% and 2%, respectively, and species delimited on morphospecies level. Morphospecies were initially defined after morphological examination (see above), and through the application of an integrative taxonomy approach (Schlick-Steiner *et al.*, 2010) in cases where the amount of sequence divergence between seemingly conspecific individuals made the recognition of morphological differences possible in hindsight. Threshold-based species delimitation was assisted by neighbor-joining diagrams. Every monophyletic clade with at least one internal sequence pair with a distance of less than the threshold value was considered one species. Species represented by only a single individual (45 species) were excluded from analyses of intra- versus interspecific genetic distances.

Species accumulation curves

To visualize the progress in *Eois* species coverage at the RBSF area we produced species accumulation curves (with 50 randomizations) using the software EstimateS 8.20 (Colwell, 2009). They were calculated separately: (i) for the data set collected between 1999 and 2003 (species only sorted by wing patterns; this corresponds to the species list published by Brehm *et al.*, 2005); (ii) for the data on newly recognized species gathered from 2005 to 2008 (as presented in this study, using DNA barcodes); and (iii) for the entire sample.

Results

We obtained COI barcode sequences from 408 individuals of *Eois*, ranging in length from 635 to 676 bp; 94.7% of sequences were at least 658 bp in length and the average length was 671 bp. All sequenced specimens are listed in Table S1 along with their assignment to species according to all three modes of species delimitations. Sequences were deposited in Genbank and accession numbers are indicated in Table S1 for each specimen. Specimen vouchers are stored in the research collections of P. Strutzenberger (333 specimens), G. Brehm (72 specimens) and F. Bodner (3 specimens). Upon completion of research all specimens will be transferred to the Phyletisches Museum Jena (Germany). Sequence alignment was straightforward and without gaps; the alignment had a length of 676 bp. No cases of NUMT amplification could be detected. Examination of the moths by wing pattern morphology revealed that from a total of 106 *Eois* morphospecies that could be recognized in the sample, 52 (49%) were previously unknown, that is they could not be matched to any of the 102 morphotypes from the RBSF area distinguished thus far. Only eight species in our sample (∼8%) could be assigned to formally described species with certainty. From the 52 'new' morphospecies 31 are closely related, and therefore morphologically similar, to previously known morphospecies and would probably have gone unnoticed in a purely morphological sorting as performed prior to this study. The recognition of those 31 morphospecies was only possible after examination of the maximum likelihood tree (Fig. S1) and neighbor-joining diagrams (not shown) followed by a thorough reexamination of wing patterns. Due to the application of barcodes, 13 morphospecies as delimited by Brehm *et al.* (2005) could be split in an integrative taxonomy approach. Most frequently, such splits resulted in the segregation of earlier defined morphotypes into two or three species (Fig. 1). In only

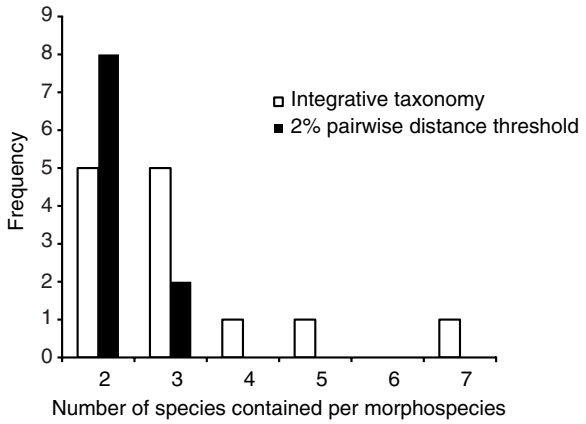

Fig. 1 Frequency distribution of splits of *Eois* morphotypes into 'cryptic' species. White bars: species recognized by integrative taxonomy; black bars: species recognized exclusively by delimitation with a 2% pair-wise distance threshold.

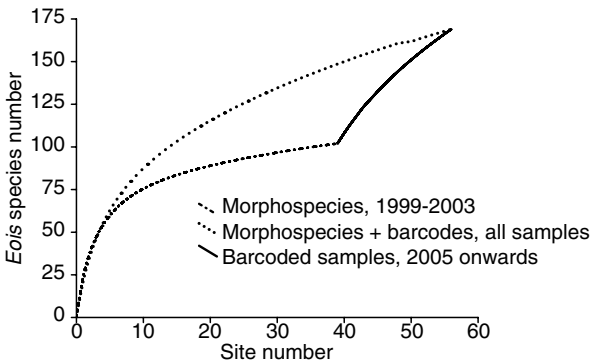

Fig. 2 Randomized species accumulation curves (Mao's Tau as a function of sampling sites: Colwell, 2009) of *Eois* moths in the Reserva Biológica San Francisco (southern Ecuador). Dashed line – morphotype delimitations of samples taken in the years 1999–2003. Solid line – additional species recorded from new samples (2005–2008) which were subjected to DNA barcoding. Dotted line – accumulation across combined data set.

three cases (i.e. *E.* spnr *borrata*, *E.* spnr *olivacea* and *E.* spnr *goodmanii*) did the integrative taxonomy approach demand that morphospecies be split into four, five or even seven species, respectively. We screened our sample for additional, cryptic species by application of a sequence divergence threshold. Delimitation with a 3% divergence threshold produced eight putative additional species. When applying a 2% threshold value, four more 'new' species could be distinguished. All of these cases are missed by the 3% threshold only by a small margin. Note that in case of *E.* spnr *azafranata* Sp042 and *E*. spnr *biradiata* Sp008 the maximum interspecific distance to the respective sister clade is above the 3% threshold. However, the 3% threshold did not split these species, as at least one pair-wise distance was less than 3% (see also Table 1). All species accepted from the sample analyzed in this study are listed in Table 1 with their new numeric codes and highlighting newly recognized species. Splits based on pair-wise distance thresholds always resulted in the species being split into two or three putative species (Fig. 1).

Species accumulation curves are presented in Figure 2. Earlier sampling at 39 sites (> 3 600 specimens) between 1999 and 2003 revealed 102 morphospecies, with the total estimated being 122.5 ± 4.4 (Jacknife1 estimator ± 1 SD, as recommended by Brose and Martinez (2004)). This corresponds to a coverage of 83.3% and would suggest a good sampling for this part of a highly diverse tropical insect fauna. However, for all samples combined, that is including species recognized by integrative taxonomy and species recognized by delimitation at a 2% pair-wise distance threshold, the recorded species number steeply increased to 166. At this lower level of coverage the Jacknife2 estimator should be used (Brose & Martinez, 2004), yielding a species total of 269.8 ± 23.4 (coverage 62.6%). Hence, by additional sampling in ravine forest, as well as at low and high elevation sites, in combination with barcode-based species delimitation, the species accumulation pattern changed distinctly from a fairly good coverage to a still very incomplete one. Incorporating recent samples indicates that about 100 additional *Eois* species might occur in the small area around RBSF in southern Ecuador.

Average intra- and interspecific distances are summarized in Table 2 for morphological species delimitation as well as for delimitation with a 3% and 2% threshold. A plot of intraspecific and interspecific distances (Fig. 3a) reveals that in the case of purely morphology-based delimitation there is substantial overlap of intraspecific and interspecific divergences. Analysis of cumulative error rates with morphology-based delimitation (Fig. 4a) shows that error is minimized with 15% at a 3% barcode sequence divergence (all false positives). False positives are completely eliminated at a threshold of 7.4%, producing 84% false negatives. When a 3% distance threshold for species delimitation is applied, overlap between intra- and interspecific distances is much reduced, but not completely eliminated (Fig. 3b). Error is minimized at a threshold of 3% pair-wise distance, all being false positives (Fig. 4b). Two of the three instances causing overlap between intra- and interspecific divergence are cases where some

Table 2 General characteristics of the dataset used for analysis of intra- versus interspecific distances, relative to the three modes of species delimitation. Note that the number of taxa and species varies because singleton species were excluded from analyses of intra- versus interspecific distances.

	2% threshold	3% threshold	Morphology
Mean interspecific distance	9.31%	9.31%	9.34%
Standard error	0.01%	0.01%	0.01%
Range of interspecific distances	2.88%–15.6%	3.2%–15.6%	3.2%–15.6%
No. of interspecific sequence pairs	63 188	63 875	64 590
Mean intraspecific distance	0.33%	0.40%	1.38%
Standard error	0.01%	0.02%	0.05%
Range of intraspecific distances	0–1.55%	0–4.6%	0–7.3%
No. of intraspecific sequence pairs	1 432	1 466	1 840
No. of taxa	360	362	365
No. of species	68	66	62
Mean number of individuals per species	5.3	5.5	5.6
Range of individuals per species	2–24	2–24	2–24

interspecific comparisons between the two clades in question give distances below the threshold, while others are above the threshold. False positives are completely eliminated at a threshold of 4.8%, producing 34.8% false negatives. Intraspecific distances show a pronounced bimodal distribution when morphological or 3% threshold delimitation is applied. Delimitation at 2% results in a distinct gap between 1.6% and 2.8% sequence divergence (Figs. 3c and 4c).

Discussion

With the discovery of 52 novel morphospecies, the species count in one small area of tropical forest increased from 102 to 154 in the genus *Eois* alone. The number rises further to 162 or even 166 when accepting the species recovered only by threshold delimitation at 3% and 2%, respectively. This overall growth of the species list is quite remarkable, since earlier inventories were based on > 3 600 individuals of the genus *Eois* sampled during massive light-trapping campaigns at 39 sites spread over several years (Brehm *et al.*, 2005; Hilt *et al.*, 2006). However, much of the material available for sequence analysis came from ravine forests that support a distinct flora, including a high number of *Piper* species (Günter *et al.*, 2008; Homeier *et al.*, 2010). It is likely that at least a part of those species serve as host plants for *Eois* and ravine forests are therefore able to support a high number of *Eois* species. Preliminary results suggest that ravine forests do indeed harbor a higher number of *Eois* species than non-ravine forests. In comparison, in the moth family Arctiidae, rather few species were added to the local species list by sampling in ravine forests (Zimmermann, 2005). Twenty-six of the 52 newly discovered *Eois* morphospecies were found in the most densely sampled elevational zone at RBSF (1 800–2 000 m). Seventy-eight *Eois* morphospecies were known from this elevational zone. Hence our findings increase that number by 33% to 104 morphospecies even in this core area of ecological investigations (Beck *et al.*, 2008).

The discovery of 8–12 additional 'cryptic' new species by means of barcode analyses was not unexpected when compared to other recent studies that employ barcodes in tropical insect faunas (e.g. Condon *et al.*, 2008; Smith *et al.*, 2008). The amount of interspecific COI sequence divergence of the eight additional species recognized by the 3% threshold is well within the range of divergence found between other, morphologically well defined, species within *Eois*. Future studies on male and female genitalia morphology will reveal how many of those putative species, currently defined only by pair-wise distances, can be distinguished by morphological characters. However, even if neither in wing patterns nor in genitalia anatomy morphological differences were to be found, the observed high levels of sequence divergence in sympatric populations would remain a strong indicator for reproductive isolation. Yet, to conclusively support the species status of the newly recognized sequence types, it will be necessary to supplement the evidence for these putative species with data on life histories, resource use or microdistribution. Most splits of morphotype

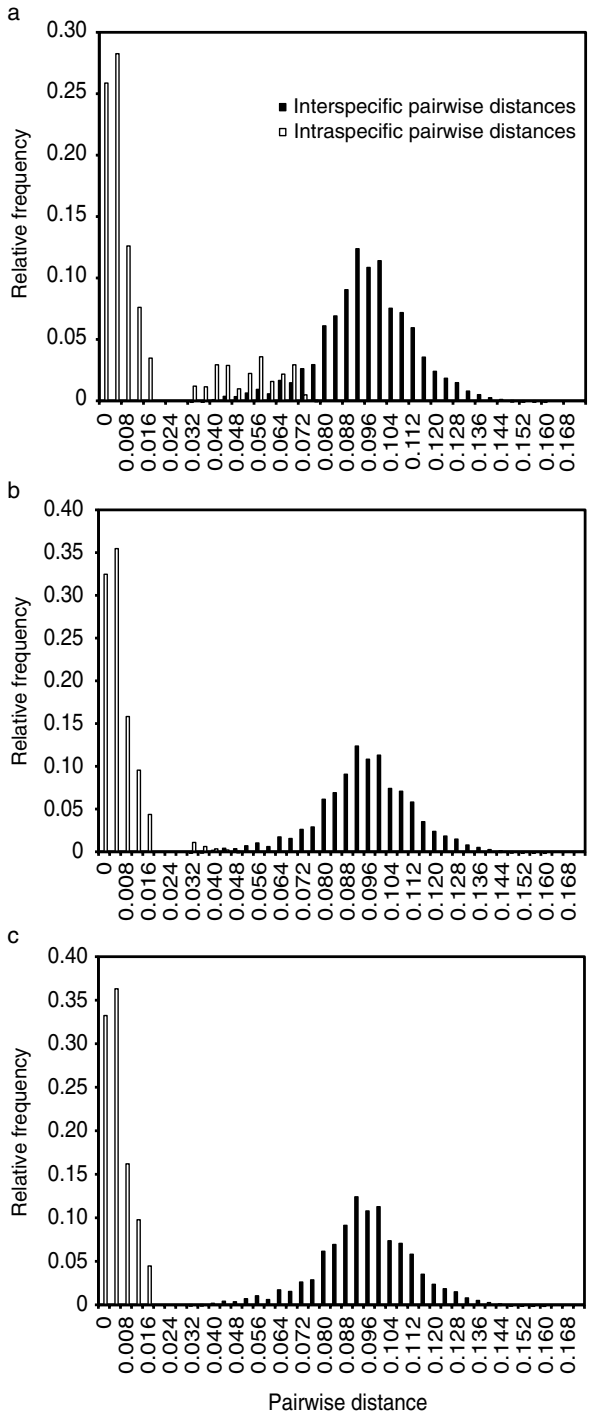

Fig. 3 Relative frequencies of intra- and interspecific COI barcode sequence distances within *Eois* moths from southern Ecuador for morphological species delimitation (a), for 3% threshold delimitation (b) and 2% threshold delimitation (c).

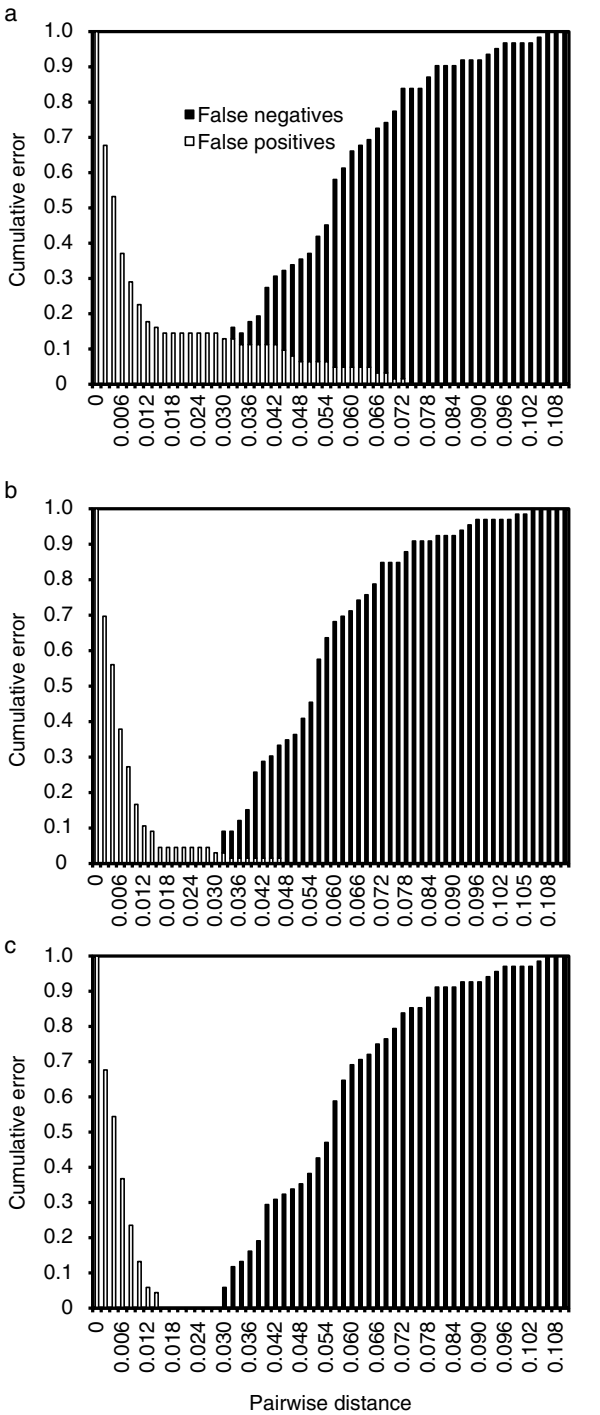

Fig. 4 Plots of cumulative error rates for given threshold values for morphological species delimitation (a), for 3% threshold delimitation (b) and 2% threshold delimitation (c).

delimitations that became necessary lead to the recognition of two or three cryptic species, with only one case of splitting into seven species by integrative taxonomy. Thus, occasions where 10 species are hidden in one (Hebert *et al.*, 2004b; but see Brower, 2006) are likely to be exceptional, even in under-explored tropical insect faunas.

The use of DNA barcodes may substantially increase estimates of local insect species richness (Hajibabaei *et al.*, 2006; Condon *et al.*, 2008), especially in tropical regions where taxonomic coverage and biodiversity inventories are still very incomplete (Foottit & Adler, 2009). Our case study on *Eois* clearly illustrates this. Species accumulation based on morphospecies sorting as done in the years 1999–2003 suggested that species numbers were already approaching saturation. However, combining morphological examinations with the application of DNA barcoding not only increased the number of species but also showed that recorded species numbers are far from approaching local saturation. We now must consider that, in addition to the approximately 154–166 *Eois* species (depending on delimitation) recorded from just a small area in southern Ecuador, about 100 additional species may await discovery. Hence, local diversity of this moth genus in and around the RBSF area in southern Ecuador may well exceed the number of recognized described *Eois* species worldwide.

DNA barcoding performed badly when tested within a framework of purely morphological species delimitation. This is not surprising in a group still lacking proper taxonomic treatment and suggests that the resolution of earlier morphospecies sorting yielded too conservative richness estimations. When using morphology-based species delimitations, error is minimized at 3% sequence divergence, and species delimitation at 2.8% would already result in a clear barcoding gap. This is in good agreement with early claims by proponents of DNA barcoding. A threshold value of 3% for the minimum sequence divergence between congeneric species enabled Hebert *et al.* (2003a) to correctly distinguish 98% of morphologically defined lepidopteran test species. This value has also been confirmed by Barrett and Hebert (2005) for arachnids, and Hebert *et al.* (2004a) found that a 2.7% threshold value for birds identifies 90% of the examined species. Setting the threshold at ten times the mean intraspecific divergence as proposed by Hebert *et al.* (2004a) for identification of potentially new species with minimal false positives would in the case of morphology-based species delimitations within *Eois* result in the threshold being set to 13.8%. This limit would fail to correctly identify any of the included species. When the species delimitation at a threshold of 3% sequence divergence is used as reference, the threshold would be set to 4% corresponding to a total error rate of 25.8%, including one false positive. When using species delimitation at a 2% divergence the threshold would be 3.3% which produces an error rate of 13%, all being false negatives. Hence, a threshold of ten times the mean interspecific divergence does well in minimizing false positives but generates up to 100% false negatives, as in the case of morphology-based species. This is the most obvious scenario when screening for potential new species. Thus, in agreement with Meyer and Paulay (2005) and Davidson *et al.* (2009) we were unable to confirm the applicability of a general standard threshold defined in this way. In the present study a threshold of 2% proved to be the most useful to screen for novel taxa. Yet, the applicability of barcoding in *Eois* from Ecuador was likely to be greatly facilitated by the very limited geographic range of sampling. No intraspecific geographic variation can be expected to occur when all samples come from the same few square-kilometers. Inclusion of samples from more distant populations of the same species might be more challenging for the barcoding approach.

Non-monophyly of species was not important in our data set. With morphological delimitation this occurred in only four cases, and strong support for non-monophyly was only present in the case of Sp003. In the other three cases it could not be determined if the species is really poly- or paraphyletic or if the true relationships could just not be recovered due to insufficient phylogenetic signal. Species defined by a 2% pair-wise distance threshold were all monophyletic with strong bootstrap support. The same was true for 3% threshold delimitation with one exception (Sp001 *Eois* spnr *azafranata*). This provides additional support for the validity of the threshold-based approach. Accuracy of tree-based species identification is expected to be high in all cases, as in morphological species delimitation there are only four cases of non-monophyletic species and only one case with the 3% threshold-based delimitation.

Having established the DNA barcode library for 106 *Eois* species (including 45 species with only one sequence available) from the RBSF area in southern Ecuador we were able to use this information to identify larval samples. Thus far, 87 caterpillars that could not be successfully reared could be matched to adult moths. Thereby we were able to add host plant records for a further 17 species of *Eois*, information that would have been lost without the application of DNA barcoding. Therefore, our case study on the performance of DNA barcoding in a highly species-rich tropical insect genus with unresolved taxonomy highlighted the usefulness of this approach in detecting cryptic species, even in a region where massive sampling campaigns had been performed. Results of

barcode analyses fostered the successful search for additional, albeit subtle morphological characters. This exemplifies how synergistic or reciprocal use of 'classical' and molecular techniques can improve our understanding of biodiversity in the sense of integrative taxonomy (Smith *et al.*, 2008; Schlick-Steiner *et al.*, 2010). The new insights gained into the local species richness of *Eois* now await their application in answering ecological questions pertaining to co-evolution, host plant specificity and niche partitioning.

Acknowledgments

We thank Manuela Zimmermann for providing the majority of moths used in this study and Florian Bodner for providing additional specimens, assistance in species determination and helpful comments on earlier drafts of the manuscript. Furthermore we thank Brigitte Gottsberger, Christine Truxa, Christian Schulze and Martin Wiemers for their various contributions. This study was financially supported by grants from the Deutsche Forschungsgemeinschaft (FOR 402, Fi 547/6-3; FOR 816, Fi 547/10-1). The foundation Nature and Culture International (Loja/Ecuador, Del Mar/USA) provided access to their property for field work. The Ministerio del Ambiente (Ecuador) kindly issued the necessary research permit (002-PNP-DBAP-RLZCH/MA).

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Accepted May 16, 2010

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Best known maximum likelihood tree calculated with RAxML. Values next to nodes are bootstrap support values.

Table S1 All included taxa are listed along with their assignment to species for all three methods of species delimitation as well as length of sequence (bp), collection site (code and elevation), Genbank accession number and the assigned species name. SpeciesM: Species assignment under morphological delimitation; Species2%: Species assignment under 2% threshold delimitation; Species3%: Species assignment under 3% threshold delimitation. Table is sorted after species assignment under morphological delimitation. Codes: Q and 4a, 4b: ravine forest sites; 1a, 1b and BC: sites at Bombuscaro; 11a and KP1: high elevation sites.

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