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ARTICLE

DNA barcodes reveal deeply neglected diversity and numerous invasions of micromoths in Madagascar[1]

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Abstract: Madagascar is a prime evolutionary hotspot globally, but its unique biodiversity is under threat, essentially from anthropogenic disturbance. There is a race against time to describe and protect the Madagascan endangered biota. Here we present a first molecular characterization of the micromoth fauna of Madagascar. We collected 1572 micromoths mainly using light traps in both natural and anthropogenically disturbed habitats in 24 localities across eastern and northwest Madagascar. We also collected 1384 specimens using a Malaise trap in a primary rain forest at Andasibe, eastern Madagascar. In total, we DNA barcoded 2956 specimens belonging to 1537 Barcode Index Numbers (BINs), 88.4% of which are new to BOLD. Only 1.7% of new BINs were assigned to species. Of 47 different families found, Dryadaulidae, Bucculatricidae, Bedelliidae, Batrachedridae, and Blastobasidae are newly reported for Madagascar and the recently recognized Tonzidae is confirmed. For test faunas of Canada and Australia, 98.9%–99.4% of Macroheterocera BINs exhibited the molecular synapomorphy of a phenylalanine in the 177th complete DNA barcode codon. Non-macroheteroceran BINs could thus be sifted out efficiently in the Malaise sample. The Madagascar micromoth fauna shows highest affinity with the Afrotropics (146 BINs also occur in the African continent). We found 22 recognised pests or invasive species, mostly occurring in disturbed habitats. Malaise trap samples show high temporal turnover and alpha diversity with as many as 507 BINs collected; of these, astonishingly, 499 (98.4%) were novel to BOLD and 292 (57.6%) were singletons. Our results provide a baseline for future surveys across the island.

Key words: Africa, invasive alien species, Lepidoptera, Malaise trap, plant pests.

Résumé : Madagascar est un haut lieu de l'évolution au niveau mondial, mais sa biodiversité unique est menacée, essentiellement en raison de perturbations anthropogéniques. Une course contre la montre est engagée pour décrire et protéger le biote malgache menacé. Dans ce travail, les auteurs présentent une première caractérisation moléculaire des « microlépidoptères » de Madagascar. Les auteurs ont récolté 1572 spécimens en utilisant principalement des pièges lumineux (dans des milieux tantôt naturels, tantôt perturbés par l'Homme) au sein de 24 localités dispersées à travers l'est et le nord-ouest de Madagascar. Ils ont aussi collecté 1384 spécimens à l'aide d'un piège Malaise dans une forêt primaire humide à Andasibe. Au total, les codes-barres ADN ont été séquencés pour 2956 spécimens correspondant à 1537 BIN, dont 88,4 % sont inédits dans BOLD. Seuls 1,7 % des nouveaux BIN ont été assignés à une espèce connue. Parmi les 47 familles trouvées, les Dryadaulidae, Bucculatricidae, Bedelliidae, Batrachedridae et Blastobasidae sont signalés pour la première fois de Madagascar. La faune étudiée présente la plus grande affinité avec l'écozone afrotropicale (dans laquelle se retrouvent 146 BIN). Les auteurs ont trouvé 22 ravageurs connus ou espèces envahissantes, principalement dans les habitats perturbés. Les spécimens provenant des pièges Malaise diffèrent au fil du temps et présentent une grande diversité alpha, leur total pouvant atteindre 507 BIN. Parmi ceux-ci, étonnamment, 499 (98,4 %) sont inédits dans BOLD et 292 (57,6 %) ont été vus une seule fois. Ces résultats fournissent un référentiel pour de futures études dans d'autres régions de l'île.

Mots-clés : Afrique, espèces envahissantes, Lepidoptera, piège Malaise, ravageurs de plantes.

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Introduction

Madagascar is one of the top priority global hotspots for biodiversity conservation with high endemicity and under large anthropogenic pressure (Vences et al. 2009). There is an urgent need to describe what remains of the unique biota of Madagascar so as to locate hotspots of biodiversity and endemism and to protect them. Conservation efforts in Madagascar are mainly focused on vertebrates (Herrera 2017; Jenkins et al. 2014) and plants (Royal Botanic Gardens Kew 2017). Arthropods are rarely taken into account in conservation in Madagascar, despite the fact that many species are micro-endemics at greatest risk of extinction (Danielczak et al. 2017; Wesener and Rudolf 2017; Wesener et al. 2014).

With up to 4900 described species currently listed from Madagascar (Viette 1990; Krüger 2007; Lees and Minet 2003; Libert 2014; Lees 2016; De Prins and De Prins 2018), the order Lepidoptera (moths and butterflies) is a significant component of the arthropod biota. As lepidopterans have been widely used as bioindicators of habitat disturbance (Kremen 1994; Enkhtur et al. 2017, Hawes et al. 2009), they could provide a strong signal for conservation efforts and priorities. Unfortunately, Madagascan Lepidoptera are relatively poorly known—particularly the "micromoths", a polyphyletic group excluding Macroheterocera and butterflies (Lees et al. 2003) of about 1600 described species (Viette 1990; De Prins and De Prins 2018)—with many species yet to be described (Lees and Minet 2003). Biodiversity assessment studies rarely take into account micromoths because of the difficulty in identifying them, the general lack of taxonomic expertise, and the need for specialised technical skills for specimen mounting and dissecting. The use of DNA barcoding, however, has proved an efficient and affordable method to alleviate this taxonomic impediment. Operational taxonomic units derived from DNA barcodes can accurately and objectively represent species diversity and then be used to survey micromoth diversity in poorly known and hyperdiverse areas of the World (Lees et al. 2013; Miller et al. 2016).

The Barcode of Life Datasystem (BOLD; www.boldsystems.org; Ratnasingham and Hebert 2007) now contains nearly six million DNA barcodes and represents a huge resource to accelerate identification and quantify biodiversity. However, the coverage for the Madagascan lepidopteran fauna is very sparse. Nevertheless, the use of Barcode Index Numbers (BINs) (Ratnasingham and Hebert 2013) as proxies for species allows the assessment of hyperdiverse groups that are taxonomically poorly known, such as micromoths (Schmidt et al. 2017; Aagaard et al. 2017; Lees et al. 2013; Lopez-Vaamonde et al. 2012). As of 29 June 2018 (including the current study) there were 2852 DNA barcode BINs for Madagascar out of a total of 113 161 lepidopteran BINS, according to a search of the BIN database in the public portal of BOLD. Van Nieukerken et al. (2011) estimated 157 424 described species of Lepidoptera, and the upper bounds for true richness may be as much as half a million species (Solis and Pogue 1999). Very few of all these BINs representing Madagascan Lepidoptera are yet publicly identified on BOLD to described species. As of 30 March 2018 there were only 287 publicly released species names according to the BIN portal of BOLD, of which only 277 had correctly composed names; 173 represented Macroheterocera, 77 represented butterflies, and only 27 represented micromoths, 24 of which were Tortricoidea and Pyraloidea. Furthermore, only 201 of these species had BIN numbers allocated. Progress in DNA barcoding the described fauna of Madagascan Lepidoptera lags far behind most countries. The first implementation of the Global Malaise Program in Madagascar (Bio-Inventory and Collections Unit, Biodiversity Institute of Ontario 2015) provided a local instance, where identification of Lepidoptera samples below Order level was problematic by external morphology (Lepidoptera wings being poorly preserved) or was very time consuming by individual sequence queries. We tested whether a previously observed simple molecular synapomorphy in the DNA barcode (Lees et al. 2011) was reliable enough to filter out the clade Macroheterocera from such samples.

From a biogeographic point of view, Madagascar has a very unbalanced or disharmonic fauna, with some taxa overrepresented and some underrepresented relative to the mainland source area (Briggs 1987). Indeed, the Madagascan fauna is characterised by a significant number of large endemic radiations such as lemurs and tenrecs now extinct on mainlands (Poux et al. 2005) and a large number of major continental lineages that appear not to have established at all on the island (e.g., the lack of poritiine lycaenids, which are highly diverse in Africa, is evident; Lees et al. 2003). The lepidopteran fauna of Madagascar is, in particular, quite dissimilar to that of southern Africa, much more so than the relatively more harmonic fauna of the neighbouring island fauna of La Réunion (Krüger 2007). Southern Africa has twice as many described lepidopteran species described as Madagascar, while Noctuoidea is overrepresented in Madagascar. By contrast, "primitive" Lepidoptera (defined as consisting of the non-ditrysian grade of micromoths that includes groups from Micropterigoidea to Tischerioidea; Krüger 2007), as well as Tineoidea and Gelechioidea, are, in particular, underrepresented in Madagascar. However, these general faunistic patterns are based on current checklists, which are particularly incomplete for the Madagascan lepidopteran fauna and also biased towards the best-studied families (e.g., Viette specialized on the noctuid fauna of both Madagascar and La Réunion; Viette 1963, 1965, 1967).

Finally, many microlepidopteran species are highly invasive and serious pests of agricultural and ornamental plants (Lopez-Vaamonde et al. 2010). Despite their potential economic and ecological impact, there is limited information available on invasive insects in Madagascar (Paulian and Viette 1955; Fisher et al. 1998; Kull et al. 2014; Irwin et al. 2010).

The main aims of the study were (*i*) to carry out a survey of micromoth diversity using DNA barcodes across several sites in Madagascar from disturbed to primary rainforests using DNA barcodes; (*ii*) to identify any molecular synapomorphy(ies) within the DNA barcode fragment that would allow us to more accurately identify samples and to better evaluate sequence queries where external morphology was problematic; (*iii*) to characterize as far as possible the biogeographic origins of the Madagascan microlepidopteran fauna (based purely on proximity, a predominantly African mainland affinity would be expected); and (*iv*) to identify the presence of any cosmopolitan, invasive, agricultural, and forestry pest species, which should be more prevalent in disturbed habitats than in well preserved ones.

Material and methods

Specimen collection

Micromoths were collected in non-protected areas (by C.W.) between October–November 2013 and March 2015 primarily using light traps. The light traps consisted of two to three light towers with 15 W ultraviolet fluorescence actinic tubes (www.bioform.de) operated by lithium batteries (Li-Ion Akku HELLPOWER 12V/10.5Ah 116.60Wh). Micromoths were sampled from nine collecting sites in disturbed habitats around the Nosy Be area (northwestern Madagascar) (Table 1). All these specimens were deposited at the Natural History Museum of Carinthia (Austria).

Specimens were also collected, largely within protected areas, across eastern Madagascar primarily using light traps consisting of 160 W blended tungsten/mercury-vapour lamps or 15 W actinic lights (Bioquip) powered by a generator (Honda EX350) (lights suspended on a white sheet with a protective transparent tarpaulin). These specimens were collected in November–December 2011, January–February 2014, and November 2014. All these specimens were deposited at the Natural History Museum in London.

One Townes-style Malaise trap (standard for the Global Malaise Trap Program, Geiger et al. 2016) was set up in two sites within

Table 1. Collection localities for light trapping or day netting and the two seasons of Malaise trapping in this study.

Locality	Region	Habitat	Latitude and Longitude (decimal degrees)	Elevation (m)
Ambilobe	Mainland	Scrubland	–13.108 to –13.163, 49.097	25–40
Mont Passot	Nosy Be	Degraded forest	–13.282, 48.259	25
Ambaro	Nosy Be	Degraded forest	–13.31, 48.187	20
Dzamandzar	Nosy Be	Open fields	–13.333, 48.196	25
Fascene	Nosy Be	Open field surrounded by degraded forest	–13.344, 48.299	75
Hell-Ville	Nosy Be	Degraded forest	–13.367, 48.283	15
Lac Ampobilava	Nosy Be	Degraded forest	–13.395, 48.241	40
Lac Djabala	Nosy Be	Degraded forest	–13.386, 48.244	40
Ambanoro	Nosy Be	Degraded forest	–13.389, 48.3	75
Ambondro	Nosy Be	Gardens	–13.382, 48.197	10
Ambanja	Mainland	Scrubland	–13.701, 48.464	40
Manongarivo Réserve Spéciale, Antsatrotro Mt	Mainland	Protected forest	–14.082, 48.366	1235
Marojejy National Park	Mainland	Protected forest	–14.433, 49.761	700
			–14.44, 49.74	1540
Anjanaharibe Sud Réserve Spéciale, below Anjividibe summit	Mainland	Protected forest above dry stream bed	–14.739, 49.462	1540
Anjanaharibe Sud Réserve Spéciale, Indri Camp	Mainland	Protected forest	–14.741, 49.497	960
Anjanaharibe Sud Réserve Spéciale	Mainland	Protected forest	–14.743, 49.464	1450
Anjozorobe Mananara Lodge	Mainland	Degraded forest	–18.436, 47.942	1300
Feo-ny-ala, Andasibe	Mainland	Hotel near protected forest	–18.947, 48.419	945
Mantadia National Park, Belakato trail	Mainland	Protected forest	–18.82, 48.436	1000
Andasibe, Malaise trap M1	Mainland	Protected forest	–18.9484, 48.4256	1000
Andasibe, Malaise trap M2	Mainland	Protected forest	–18.9438, 48.4316	1050
Ankazomivady	Mainland	Degraded high plateau forest	–20.778, 47.178	1710
			–20.7948, 47.1773	1830
Andringitra National Park, camp	Mainland	Protected forest	–22.147, 46.946	1570
Andringitra National Park	Mainland	Protected forest	–22.1504, 46.9487	1625
Sahavondronina, 7 km W Vohiparara, Community forest at Ranomafana National Park	Mainland	Open field surrounded by protected forest	–21.278, 47.331	1230

Andasibe-Mantadia National Park, specifically the forest originally designated as the Réserve Spéciale d'Analamazaotra (for short, we refer hereafter to this reserve as its current popular name Andasibe; it was also popularly known as Perinet). Andasibe is a ~810 ha fragment of the once far larger Analamazaotra rainforest (Table 1). One site was sampled for 65 days at the end of the wet to beginning of the dry season (from 1 April 2014 to 28 May 2014) (M1) at 1000 m elevation; a second site, 0.8 km away from the first site and at 1050 m, was sampled for 67 days at the end of the dry to beginning of the wet season (from 1 September to 6 November 2014) (M2) (elevations adjusted for coordinates in Table 1 using Google Earth). Each sample was collected in a 500 mL plastic Nalgene bottle that was filled with 375 mL of 95% ethanol and then attached to the trap head. The catch was harvested weekly and brought to the University of Antananarivo where the bulk ethanol was replaced with fresh 95% ethanol before storage at –4 °C. Samples were then drained and sent to the Centre for Biodiversity Genomics in Canada (CBG; www.biodiversitygenomics.net).

DNA barcoding

In most cases only one specimen was DNA barcoded per morphospecies for light-trapped and day-netted specimens. Morphospecies were defined using external morphology, mainly wing pattern. DNA was extracted using the hind legs of pinned specimens or entire body extracts in the case of smaller Malaise-trapped Lepidoptera. DNA barcodes (658 bp of the COI mitochondrial gene) were generated using traditional Sanger sequencing at CBG using standard high-throughput protocols (Ivanova et al. 2006).

Malaise trap samples were also processed at CBG as part of the Global Malaise Program (http://biodiversitygenomics.net/projects/gmp/) following the protocol described in deWaard et al. (2017), which involves unidirectional sequencing—so those sequences are usually shorter than 658 bp. Larger moths were pinned and smaller ones were kept in their original wells. A randomly selected example of each BIN was imaged at CBG, as the order Lepidoptera captured via this method tend not to be in good condition for external morphological analysis.

DNA sequences, along with the voucher data, images, and trace files, are deposited in the Barcode of Life Data Systems (BOLD v4) (Ratnasingham and Hebert 2007, www.barcodinglife.org), and the sequences were deposited in GenBank. All data are available in BOLD through the public dataset DS-MICROMA (dx.doi.org/10.5883/DS-MICROMA).

To aggregate barcodes of the polyphyletic group micromoths (which includes some larger moths such as thyridids) from the Malaise trap data set, we tested whether a previously noticed molecular synapomorphy for the clade Macroheterocera (Lees et al. 2011) was reliable enough to partition out all non-macroheterocerans. To do this, we used a test dataset of two well-identified lepidopteran faunas, namely that of Australia (n = 13 163 BINs analyzed) and Canada (4684 BINs analyzed).

In the case of the Malaise trap sample, which had been predetermined to Lepidoptera before sequencing, we first filtered out all Papilionoidea (butterflies), which could be verified by batch queries on BOLD because all genera and most species had already been DNA barcoded.

To determine the number of BINs novel for this study for BOLD, we derived the number of uniques and non-uniques from our dataset front page (Column marked "Data Summary"), by clicking on the BIN total. However, we subtracted 36 BINs from the total of 44 "non-unique" BINs pertaining to the Malaise trapping. In fact, these 36 BINs were novel to BOLD as a result of the Global Malaise-Madagascar 2014 (GMTAD) project, but included sequences in CBG which were private data with respect to DS-MICROMA.

Data analyses

Diversity analyses were carried out on both Malaise and light trap/day-netted samples. Community analyses were performed only on

Malaise samples from Andasibe as it was the only site for which abundance data were collected.

Data analyses was performed in R ver.3.4.3 (R Development Core Team 2004) using different packages for community and species richness analyses.

iNEXT (Chao et al. 2014; Hsieh et al. 2016) was used to calculate α-diversity and to generate accumulation curves using 50 resampling replicates with replacement (Chao et al. 2014). BINs were used as species proxies (Ratnasingham and Hebert 2013) and plotted against both the sampling coverage (i.e., measure of sample completeness that estimates the proportion of the total number of individuals in a community that belong to the species represented in the sample) and the total number of individuals taken as a measure of sampling intensity. Analyses were performed for late wet to early dry (M1) and late dry to early wet (M2) seasons, both covered by the sampling at Andasibe.

Abundance Coverage Estimator (ACE) (Gotelli and Colwell 2010) and Chao1 (Chao et al. 2009) were calculated with the package Vegan ver. 2.4-6 (Oksanen et al. 2016) to estimate the potential species richness in accordance with the sampling intensity.

Distributional data analysis was performed by extracting from BOLD a list of all countries for which each BIN has been barcoded. Each appearance of a BIN per country was assigned to a biogeographical region (Afrotropical, Australasian, Nearctic, Neotropical, Oriental, and Palearctic) by looking at the corresponding countries associated to the records in BOLD. Each BIN was counted only once per region, but one BIN might be spread over multiple regions.

Specimen identification

Specimens were identified by both external morphology (without dissection) and by using DNA barcode queries using all data present in BOLD. For each sequence queried, we used the "Current Database" and the "Search Database" query option "All Barcode Records" on the Identification Engine of BOLD, and then we built a NJ tree in BOLD to find the nearest neighbour. Then we searched for the minimum corresponding p-distance(s) in the list of 99 top hits. We looked, in particular, for interspecific query tails among the hit list that seemed informative, i.e., with the hit(s) showing potential signal standing proud of the noise of background hits (often evident as the sequence similarity value directly preceding the sharpest inflexion in the similarity score graph before it starts to asymptote), or otherwise stated 'Non-informative' under Taxonomy Notes. Particular note was taken when nearest hits were derived from apparent local radiations. Amino acid information was also considered, in particular, ignoring similarity values for irrelevant hits inside or outside of the Macroheterocera (see below), and also looking qualitatively at unusual codon changes shared between taxa as shown in Conservation plot mode against a reference sequence in Bioedit v7. In most cases, the sequence divergence(s), to the nearest identified BINs on BOLD, expressed as 100-Similarity, are noted under "Taxonomy notes" (see DS-MICROMA, within the specimen pages in the taxonomy section), particularly for records from the BOLD projects MADAM and MIMAD. In that field, we were often able to specify closely related BIN numbers by building a corresponding Image database for the Tree Based Identification query. We assigned species-level identifications for sequence queries to known species on BOLD, usually only in the case of low pairwise divergence hits (0–2% divergence, i.e., 100–0.98 similarity). We also identified some query sequences as higher taxonomic ranks (e.g., genus, family), in some cases where a tail of subsequent hits also showed clear taxonomic consistency at that higher taxonomic level in a grouping in the NJ tree. As application of strict thresholds may generally be misleading, particularly for supraspecific ranks, and as no support levels are specified on BOLD NJ trees, we also used independent ML analyses in PHYML 3.0 (Guindon et al. 2010) to identify barcoded specimens by examining their phylogenetic position within a clade containing identified individuals—some of which were downloaded from BOLD—at the best justifiable taxonomic rank. In Phyml 3.0, we used default options, except for GTR (or automatic model selection), all parameters estimated, and SPR. In general, we looked for ABayes support levels >0.94 to assume nestedness within a clade. Identifications from the light-trapped and day-netted samples run alongside the malaise samples in an ML analysis helped the identifications of Malaise samples. We specified the identification method(s) or combination thereof (e.g., External morphology, COI-5P (NJ), COI-5P (ML), COI-5P (codons), i.e., amino-acid based identification) under the field Identification Method (see also DS-MICROMA, taxonomy section).

We compared the 1572 light-trapped and day-netted moths with specimens, including where possible, accessible types, deposited in the two most important reference collections of Madagascan Lepidoptera, namely the Muséum national d'Histoire naturelle (MNHN, Paris) and the Natural History Museum (NHMUK, London), and to illustrations in reference works. We have not attempted an exhaustive type comparison with our specimens and anticipate that more matches will come to light as the collections are digitised and (or) as DNA sequencing of the types is attempted.

Results

DNA barcodes and identification rates

We successfully barcoded 2956 micromoth specimens (1572 light-trapped and day-netted micromoths and 1384 micromoths collected by Malaise trap) belonging to 1537 BINs (6 of 2956 samples do not qualify as full barcodes and so lack BIN numbers). Those 1537 BINs belonged to 44 families as currently classified in BOLD (see Table 2, where 47 family-level groupings are specified; these include families currently lumped on BOLD). Further, 32.7% of BINs (503 out of 1537 BINs) were identified to genus level and 6.2% of BINs (95 out of 1537 BINs) were identified to species level. Many of those identified BINs correspond to well-known cosmopolitan species more likely to have been DNA barcoded elsewhere (Table 3).

In total, 88.4% of BINs (1358 out of 1537 BINs) obtained are new to BOLD and only 179 BINs (13.2%) were already in the BOLD database.

By analysing barcoded lepidoptera faunas from Canada and Australia, we found that almost all Macroheterocera show a phenylalanine rather than leucine or other character state in the 177th complete codon (5′–>3′) of the (up to) 658 bp nucleotide sequence.

For the Australian fauna, 99.4% of 4093 BINs exhibiting a phenylalanine in the 177th position pertain to sequences identified as macroheteroceran families, while 11 exceptions belong to the genus *Aristeis* (Oecophoridae) and one to another Oecophoridae genus. Five others belong to Crambidae: Acentropinae, one to Crambinae, two to Lecithoceridae, two to Gelechiidae, two to Tineidae: Harmacloninae, and one to Heliozelidae. Exceptions to the reliability of this synapomorphy (total $n = 26$, discounting an apparently contaminated Lycaenidae) are not only rare in general, but phylogenetically they are also very narrowly represented. Also, true conversely for this dataset, 99.5% of 9070 BINs exhibiting another state than a phenylalanine in that position (usually leucine) are identified as belonging to non-macroheteroceran families, including those of butterflies. Of the exceptions ($n = 48$), 21 BINs belonging to Oenosandridae and three belonging to Nolidae, as well as three of seven belonging to Geometridae, three of eight belonging to Erebidae, and three of seven belonging to Noctuidae, seem correctly identified (the rest are micromoths from images), and one imaged Saturniidae also represents a micromoth. For the Canadian Lepidoptera fauna (4684 BINs analysed), the presence of a phenylalanine in this position is 98.9% reliable as a surrogate for Macroheterocera (99.75% reliable when excluding Crambidae: Acentropinae and Tineidae: Meessiinae), whereas the presence of another character state is 99.8% reliable for

Table 2. Number of BINs per family and method of collection (Malaise, light trapping, and netting by day).

Family	Records	BINs	BINs Malaise	BINs light trap + day-netted	Shared BINs	BINs identified to species	No. of described species
Micropterigidae	2	2	0	2	0	0	0
Opostegidae	6	5	0	5	0	0	0
Nepticulidae	50	28	21	7	0	0	1
Heliozelidae	0	0	0	0	0	0	1
Adelidae	7	7	0	7	0	0	1
Tischeriidae	0	0	0	0	0	0	1
Psychidae	41	23	15	8	0	0	19
Eriocottidae	45	12	2	10	0	0	0
Dryadaulidae*	6	3	3	0	0	0	0
Tineidae	450	159	95	65	1	1	40
Lyonetiidae s.auct. (Cemiostomidae)	2	2	1	1	0	0	2
Bucculatricidae	2	2	1	1	0	0	0
Gracillariidae	73	55	17	40	2	2	22
Bedelliidae	8	4	1	3	0	0	0
Praydidae	2	2	0	2	0	2	2
Lyonetiidae s.str.	0	0	0	0	0	0	1
Argyresthiidae	29	5	0	5	0	0	9
Yponomeutidae	5	5	2	3	0	0	6
Ypsolophidae	0	0	0	0	0	0	1
Plutellidae‡	0	0	0	1	0	0	1
Tonzidae	1	1	0	1	0	0	0
Glyphipterigidae‡	29	20	11	10	1	0	7
Alucitidae	3	3	0	3	0	0	4
Pterophoridae	31	19	1	19	1	4	65
Copromorphidae	9	5	0	5	0	0	1
Carposinidae	8	3	0	3	0	0	2
Epermeniidae	30	12	0	12	0	0	7
Immidae	34	21	17	5	1	0	1
Choreutidae	4	3	0	3	0	1	1
Galacticidae	3	1	0	1	0	0	1
Tortricidae	260	150	19	132	1	19	342
Brachodidae	1	1	0	1	0	1	8
Cossidae	1	1	0	1	0	0	26
Dudgeonidae	0	0	0	0	0	0	1
Metarbelidae	0	0	0	0	0	0	2
Sesiidae	0	0	0	0	0	0	32
Epipyropidae	2	2	1	1	0	0	3
Lacturidae	2	1	0	1	0	0	9
Limacodidae (incl. Chrysopolominae)	11	10	1	9	0	7	70
Somabrachyidae	0	0	0	0	0	0	1
Zygaenidae	0	0	0	0	0	0	5
Gelechioidea i.s., includes (*Orygocera*, *Prothamnodes*, *Trichocirca decaryanum*)	87	53	27	26	0	0	27
Depressariidae s.l. (Stenomatidae: *Herbulotiana*, *Amontes*)	6	4	2	3	1	0	18
Depressariidae s.l. (Peleopodidae: Oditinae)	230	131	54	81	4	0	61
Depressariidae s.l. (Ethmiidae)	1	1	0	1	0	0	19
Depressariidae s.s. (Depressariidae: Depressariinae, Cryptolechiinae)	2	2	0	2	0	0	6
Oecophoridae	62	28	9	20	1	1	23
Lecithoceridae	219	79	51	31	3	0	28
Xyloryctidae§	0	0	0	0	0	0	1
Autostichidae	0	0	0	0	0	0	2
Elachistidae s.s.	85	8	8	0	0	0	1
Momphidae	0	0	0	0	0	0	1
Batrachedridae	4	4	0	4	0	0	0
Coleophoridae	0	0	0	0	0	0	1
Blastobasidae	13	7	1	6	0	1	0
Scythrididae	21	14	7	7	0	1	5
Stathmopodidae	27	17	0	17	0	0	4
Cosmopterigidae	105	59	13	47	1	2	14
Gelechiidae	313	178	36	145	3	6	32
Whalleyanidae	0	0	0	0	0	0	2

Table 2 (*concluded*).

Family	Records	BINs	BINs Malaise	BINs light trap + day-netted	Shared BINs	BINs identified to species	No. of described species
Thyrididae	4	3	0	3	0	2	32
Hyblaeidae	0	0	0	0	0	0	4
Callidulidae	0	0	0	0	0	0	4
Pyralidae	171	107	14	93	0	10	271
Crambidae	253	162	25	139	2	34	346
Unknown/i.s. Lepidoptera	190	113	52	62	1	0	1
TOTAL	2950†	1537	507	1053	23	94	1598

Note: Families are ordered systematically and those recorded for the first time for Madagascar are in bold. All micromoth families are shown for completeness of the total microlepidopteran count (species included in Viette 1990 that are not synonyms of other species, although not listed for Madagascar in Afromoths, have been included in the count). Some other amendments have been made such as there is only one Elachistidae described from Madagascar (Parenti 2006; Koster and van Nieukerken 2017). See text regarding Depressariidae s.l. Families largely ordered following van Nieukerken et al. (2011), Regier et al. (2014) for Tineoidea, and Sohn et al. (2013, 2015) for Yponomeutoidea and Gelechioidea.

*Placement in this family based entirely on COI data, specimens in poor condition.

†An additional six barcodes are too short to be allocated BINs so a total of 2950 barcodes have BINS and are subject to analysis.

‡"*Iridostoma*" *catatella* Viette, 1956 is misplaced in *Iridostoma* Meyrick, 1909; this species is here transferred from Plutellidae to the Glyphipterigidae: Acrolepiinae (provisionally as *Acrolepia catatella* comb. nov.).

§Currently included in Lecithoceridae is an *Epichostis*-like species, *Lecithocera ojejyella* Viette, 1958. In Gelechioidea i.s. we include 18 sequences representing nine BINs of *Epichostis*-like moths as indicated in the field Extra info. There are currently no *Epichostis* barcodes on BOLD, and we are currently uncertain if *L. ojejyella* together with these BINs might represent true Xyloryctidae.

non-macroheteroceran Lepidoptera (exceptions include one geometrid, one nolid, and two noctuids). We did not detect any cases of such parallelisms in our Madagascan dataset, suggesting that the synapomorphy was fully reliable for this fauna, but in the case of filtering of the malaise sample, Macroheterocera were only represented by 170 BINs (whereas Papilionoidea were represented by 20 BINs).

Within the 507 BINs collected with the Malaise trap, 50 BINs (9.9%) have been identified to genus and only three BINs (0.59%) have been identified to species level (*Angustalius malacellus*, *Bradina admixtalis*, and *Lobesia aeolopa*). The only other five BINs already on BOLD were a cosmopterigid *Stilbosis* sp. (BOLD:ABY7721, Kenya), a spilomeline (BOLD:ACT8113, South Africa), two tortricids, and another spilomeline from Ranomafana (*Pandemis* sp. BOLD:ACO0519; *Olethreutes* sp. BOLD:ACS0054; and *Herpetogramma* sp. BOLD:ACT6691). The remaining 499 BINs, 270 of which are singletons and 109 doubletons, are at present only known as endemic to Andasibe. Two BINs (BOLD:ACS0229, an *Elachista* and BOLD:ACS1392, Tineidae: Hieroxestinae) have more than 70 individuals ($n = 75$ and 91, respectively), but even these abundant taxa have as yet no species name.

A total of 113 BINs were not identified to family level. According to NJ building on BOLD and (or) external morphology of pinned specimens, these 113 BINs were overwhelmingly dominated by possible or probable Gelechioidea (>77%) that could not be reliably assigned at present to family. Around 16% may represent tineoids, and superfamily was unassigned even tentatively for 6%. Maximum divergences among all of those unidentified BINs to any other BIN was no smaller than 14.3%. Over 40% of those unknown BINs were more closely related to one or more unidentified Madagascan BINs than to BINs outside Madagascar.

Only 23 BINs were shared between the samples collected with Malaise (507 BINs) and those collected with light trapping and day netting (1053 BINs) (Table 2). Of the non Malaise-trapped material, approximately 92% were light-trapped and the remainder day-netted, so there was a strong bias towards nocturnal activity. The low number of shared BINs is particularly striking for tineids, considering the high diversity of this family (95 BINs collected with Malaise and 65 BINs collected by other methods), with only one BIN shared (Table 2).

Some groups were much more strongly represented in Malaise samples such as Nepticulidae, Tineidae, Immidae, Lecithoceridae, and Elachistidae. Other families were much better represented in the mainly light-trapped samples than in the Malaise, notably Gracillariidae, Tortricidae, Cosmopterigidae, Gelechiidae, Pyralidae, and Crambidae (Table 2).

Taxonomic composition and biogeographical distribution

Figure 1 shows the difference in distribution of BINs per family between light trap and Malaise trap samples. The three families with the highest number of BINs within the 507 BINs collected with Malaise traps are Tineidae (95 BINs, 18.7%), Depressariidae s.l. ('Peleopodidae': Oditinae) (54 BINs, 10.7%), and Lecithoceridae (51 BINs, 10.1%). Within the 1053 BINs collected by light trapping and day netting, the three most representative families are Gelechiidae (145 BINs, 13.8%), Crambidae (139 BINs, 13.2%), and Tortricidae (132 BINs, 12.5%).

Of the up to 47 different micromoth families found, Dryadaulidae, Bucculatricidae, Bedelliidae, Batrachedridae, and Blastobasidae are newly reported for Madagascar (Table 2). Other families, namely Micropterigidae, Opostegidae, Tonzidae, and Eriocottidae, have been previously reported from Madagascar, but they have no described species (Krüger 2007; Lees and Minet 2003; Davis and Stonis 2007; Gibbs 2016; Kobayashi et al. 2018).

Analysis revealed that 55 BINs show a widespread distribution over more than one biogeographical region (Table 3). Out of the 162 BINs shared between Madagascar and other biogeographical regions, 146 BINs (90.1%) occur in Africa and 105 are found only in the Afrotropical region. More surprisingly, 49 BINs (30.3%) detected in Madagascar also occur in Australasia, 29 BINs (17.9%) occur in the Oriental region, 27 BINs (16.7%) occur in the Palearctic, 18 BINs (11.1%) occur in the Neotropics, and 17 BINs (10.5%) occur in the Nearctic (Fig. 2).

Invasive and pest species

Of the above 55 BINs that show a widespread distribution occurring outside the Afrotropical region, at least 40% (22 out of 55) are known to be pests and (or) invasive somewhere in their distribution range, and at least an additional five species occasionally feed on crops or may be minor pests. At least 50.9% (28 out of 55) are recorded for the first time in Madagascar (Table 3).

All widespread BINs, except nine, are identified to species level. These exceptions include a tineid (BOLD:ACS7592), a glyphipterigid (BOLD:AAY2216) previously barcoded from Australia but 1.7% divergent, a tortricid unidentified to genus (BOLD:ACS7628), one cosmopterigid of the genus *Gisilia* (BOLD:ACS6187), one *Ascalenia* (BOLD:AAG0134), two crambids of the genus *Herpetogramma* (BOLD:ACD5135 and BOLD:AAB6841), a gracillariid of the genus *Stomphastis*

Table 3. Species detected during this study in Madagascar that are known to occur outside the Afrotropical region.

Family: subfamily	Species	BIN	Distribution	New record for Madagascar	Pest	Reference
Tineidae: Erechthiinae	*Erechthias minutalis*	BOLD:ABW6327	Cosmopolitan	Yes	No	—
Tineidae: Hieroxestinae	*Opogona* sp.	BOLD:ACS7592	Madagascar, Oriental	Yes	No	—
Gracillariidae: Acrocercopinae	*Dialectica scalariella*	BOLD:AAL3278	Palearctic, Afrotropics, and Australia	Yes	No	—
Gracillariidae: Ornixolinae	*Stomphastis* sp.	BOLD:AAM6667	Madagascar, Australia	Yes	No	—
Praydidae	*Prays nephelomima*	BOLD:AAM9790	Madagascar, Australia	Yes	Yes	Jamieson et al. 2008
Praydidae	*Prays citri*	BOLD:AAW5122	Palearctic, Afrotropics, and Australia	?see text	Yes	Lopez-Vaamonde et al. 2010
Glyphipterigidae	*Glyphipterix* sp.	BOLD:AAY2216	Australia, Madagascar	Yes	No	—
Pterophoridae: Platyptiliinae	*Hepalastis pumilio*	BOLD:AAD4253	Cosmopolitan	No	No	—
Pterophoridae: Platyptiliinae	*Stenoptilia* sp.	BOLD:AAD0716	Cosmopolitan	Yes	No	—
Pterophoridae: Platyptiliinae	*Sphenarches anisodactylus*	BOLD:AAD0725	Cosmopolitan	No	No	—
Tortricidae: Olethreutinae	Genus sp.	BOLD:ACS7628	Palearctic, Afrotropics, Oriental	Yes	No	—
Tortricidae: Olethreutinae	*Bactra venosana*	BOLD:ABZ1079	Afrotropics, Oriental, Australia	Yes	No	—
Tortricidae: Olethreutinae	*Crocidosema lantana*	BOLD:AAH5763	Nearctic, Neotropics, Afrotropics, and Australia	Yes	No	—
Tortricidae: Olethreutinae	*Cydia choleropa*	BOLD:ABW2540	Afrotropics, Oriental	Yes	No	—
Tortricidae: Olethreutinae	*Dudua aprobola*	BOLD:AAT9574	Cosmopolitan	Yes	**No**	—
Tortricidae: Olethreutinae	*Lobesia aeolopa*	BOLD:AAJ2244	Afrotropics, Oriental	No	Yes	Evans 1970
Tortricidae: Olethreutinae	*Lobesia vanillana*	BOLD:ABV8007	Réunion, Madagascar	No	Yes	Brown et al. 2014
Tortricidae: Olethreutinae	*Thaumatotibia leucotreta*	BOLD:AAE7729	Afrotropics, intercepted in Palearctic and Nearctic	No	Yes	Baker et al. 2013
Cosmopterigidae: Cosmopteriginae	*Anatrachyntis simplex*	BOLD:ABX3349	Cosmopolitan	Yes	Yes	Heckford 2004
Cosmopterigidae: Cosmopteriginae	*Cosmopterix athesiae*	BOLD:AAE4001	Palearctic, Afrotropics	Yes	No	—
Cosmopterigidae: Cosmopteriginae	*Cosmopterix* sp. cf. *attenuatella*	BOLD:AAC1744	Cosmopolitan	Yes	No	—
Cosmopterigidae: Chrysopeliinae	*Ascalenia* sp.	BOLD:AAG0134	Cosmopolitan	Yes	No	—
Cosmopterigidae: Chrysopeliinae	*Gisilia* sp.	BOLD:ACS6187	Bangladesh, Madagascar	Yes	No	—
Gelechiidae: Anacampsinae	*Aproaerema simplexella*	BOLD:ACK6985	Cosmopolitan, invasive in Afrotropics	Yes	Yes	Buthelezi et al. 2013
Gelechiidae: Dichomeridinae	*Dichomeris acuminatus*	BOLD:AAB6409	Cosmopolitan	Yes	No	—
Choreutidae	*Tebenna micalis*	BOLD:AAH9855	Cosmopolitan	Yes	No	—
Pyralidae: Galleriinae	*Achroia grisella*	BOLD:ACO9701	Cosmopolitan	No	No	—
Pyralidae: Galleriinae	*Galleria mellonella*	BOLD:AAA0965	Cosmopolitan	No	Yes	Kwadha et al. 2017
Pyralidae: Pyralinae	*Hypsopygia nostralis*	BOLD:AAI3521	Nearctic, Neotropics, and Afrotropics	Yes	No	—
Pyralidae: Phycitinae	*Cadra cautella*	BOLD:AAB9605	Cosmopolitan	Yes	Yes	Paulian and Viette 1955
Pyralidae: Phycitinae	*Cryptoblabes gnidiella*	BOLD:AAW5129	Cosmopolitan	Yes	Yes	Silva and Mexia 1999
Pyralidae: Phycitinae	*Ectomyelois ceratoniae*	BOLD:AAU4812	Cosmopolitan	No	Yes	Morland, 2015
Pyralidae: Phycitinae	*Etiella zinckenella*	BOLD:AAB7420	Cosmopolitan	No	Yes	Van Den Berg et al. 1998
Pyralidae: Phycitinae	*Thylacoptila paurosema*	BOLD:AAV8326	Afrotropical, Oriental, and Australia	No	**No**	—
Crambidae: Acentropinae	*Parapoynx fluctuosalis*	BOLD:AAA0473	Cosmopolitan	No	Yes	Yen 2014
Crambidae: Crambinae	*Angustalius malacellus*	BOLD:AAV9127	Palearctic, Afrotropics	No	**No**	—
Crambidae: Pyraustinae	*Isocentris filalis* (=*Hyalobathra retinalis*)	BOLD:AAL8896	Afrotropical, Oriental	No	No	—
Crambidae: Spilomelinae	*Bocchoris inspersalis*	BOLD:AAC5466	Cosmopolitan	No	No	—
Crambidae: Spilomelinae	*Cnaphalocrocis trapezalis*	BOLD:AAC0297	Cosmopolitan	No	Yes	Shankara Murthy and Nagaraj 2014
Crambidae: Spilomelinae	*Cnaphalocrocis exigua*	BOLD:AAO9362	Afrotropics, Oriental, Oceania	Yes	Yes	Barrion et al. 1991
Crambidae: Spilomelinae	*Diasemiopsis ramburialis*	BOLD:AAD0296	Old World (Africa, Oriental, Australia)	No	**No**	—
Crambidae: Spilomelinae	*Diaphania indica*	BOLD:AAB1719	Cosmopolitan	No	Yes	Paulian and Viette 1955; Shimizu 2000
Crambidae: Spilomelinae	*Eurrhyparodes bracteolalis*	BOLD:AAD1173	Cosmopolitan	No	**No**	—
Crambidae: Spilomelinae	*Herpetogramma licarsisalis*	BOLD:AAA3965	Palaeotropics, Australasia, Hawaii, Canaries	No	Yes	Lopez-Vaamonde et al. 2010
Crambidae: Spilomelinae	*Herpetogramma* sp.	BOLD:AAB6841	Afrotropics, Oriental	No	No	—

Table 3 (*concluded*).

Family: subfamily	Species	BIN	Distribution	New record for Madagascar	Pest	Reference
Crambidae: Spilomelinae	*Herpetogramma* sp.	BOLD:ACD5135	Oriental	Yes	No	—
Crambidae: Spilomelinae	*Hymenoptychis sordida*	BOLD:AAF8520	Old World (Africa, Oriental, Australia)	No	No	—
Crambidae: Spilomelinae	*Hyalobathra olesialis*	BOLD:ACN7820	Afrotropical, India, Australia	Yes	No	—
Crambidae: Spilomelinae	*Maruca fuscalis*	BOLD:AAD9057	Australia	Yes	No	—
Crambidae: Spilomelinae	*Maruca vitrata*	BOLD:AAB2756	Pantropical	No	Yes	Sharma et al. 1999
Crambidae: Spilomelinae	*Omiodes indicata*	BOLD:AAB5389	Cosmopolitan	No	Yes	Favetti et al. 2018
Crambidae: Spilomelinae	*Palpita vitrealis*	BOLD:AAC1043	Cosmopolitan	No	Yes	Hayden and Buss 2013
Crambidae: Spilomelinae	*Pyrausta phoenicealis*	BOLD:AAF5760	Cosmopolitan	No	Yes	Yamada 1979
Crambidae: Spilomelinae	*Salbia haemorrhoidalis*	BOLD:AAD3428	Cosmopolitan	Yes	No	—
Crambidae: Spilomelinae	*Spoladea recurvalis*	BOLD:AAA3666	Cosmopolitan	No	Yes	Paulian and Viette 1955

Note: We also indicate those species that are known to be pests and (or) invasive. Those known to feed on crops but not widely acknowledged pests are in bold.

(BOLD:AAM6667) with barcodes from Australia (also currently without associated species name), and a pterophorid of the genus *Stenoptilia* (BOLD:AAD0716) with barcodes from Africa, Asia, and Australia (Table 3).

In total, 38.2% (21 out of 55 BINs) of widespread BINs belong to the family Crambidae, a family known for many highly dispersive species (Lopez-Vaamonde et al. 2010).

Species richness and turnover

The analysis of 1384 microlepidopterans (three of which are without BIN allocations) collected over 16 weeks of Malaise trapping revealed a total of 507 BINs. Astonishingly, nearly all (499 BINs or 98.4%) were novel to BOLD given an also surprisingly small overlap (4.5% of the Malaise sample, 2.2% of others, and 1.5% of the total sample) with the principally light-trapped samples in this study. Moreover, 57.6% (292 out of the 507 Malaise BINs) are represented by singletons (i.e., by single individuals) in our data set (Fig. 3). The high number of singletons demonstrates that even 16 weeks and two seasons are entirely inadequate to sample with Malaise traps most of the species that must be present in the studied area.

The Malaise trap automatically collected a total of 335 micromoths (representing 165 BINs) at the end of the wet to beginning of the dry season (M1), whereas 1046 individuals (representing 404 BINs) were collected at the end of the dry to beginning of the wet season (M2).

The BINs shared between Malaise trap samples collected at M1 and M2 are only 12.2% (62 BINs out of 507 BINs), but with many individuals (528 specimens out of 1384 individuals collected, 38.1%). Therefore many of the species collected during the two periods belong to relatively common species.

Figure 4 shows a clear temporal turnover with a strong relationship between temporal distance of samples and amount of species overlap. For each site (M1 and M2) taken separately and compared, a clear decline in sample overlap with temporal distance is evident. The intercept for M2 is higher than M1, but slopes, p-values, and R-squared values are very similar.

Rarefaction curves show that both species diversity and sampling coverage indices were perhaps surprisingly higher at the end of the wet towards early dry season (M1) than at the end of the dry towards early wet season (M2) (Figs. 5A, 5B). They also show that 16 weeks of Malaise sampling is not nearly enough to capture all the Lepidoptera diversity in the studied area (Fig. 5C). We collected with one Malaise trap 507 BINs at Andasibe, whereas both non parametric indices, Chao 1 index and ACE, suggest that at least twice as many species could occur in the studied area (Fig. 6).

Discussion

Massive 'Linnean shortfall' of micromoths in Madagascar

The majority of BINs (1358 of 1537) found in our study are new to BOLD and most of them remain unidentified to species level, as we only recovered 1.7% (23 out of 1358 BINs) of corresponding taxonomic assignation (several of these were assigned using external morphology and are new to BOLD). Remarkably, despite the impressive lepidopteran coverage on BOLD, we do not know the names of some of the most abundant species in Madagascan ecosystems, and without comprehensive barcoding of museum collections, it is difficult to be sure what names may already be available for them.

In total, 115 of 507 BINs (22.7%) in the Malaise trap were identified to five families within Tineoidea, one of which is newly reported for Madagascar (Fig. 1). Among the non-tineoid Ditrysia, exceptional diversity was found among Gelechioidea identified to families, which with 181 BINs (208 including Gelechioidea *incertae sedis*) form 35.7% (41%) of the entire Malaise sample. Of these, Lecithoceridae (with 51 BINs by Malaise) was the richest family, whereas elsewhere in the Depressariidae assemblage (Depressarii-

Fig. 1. Distribution of Barcode Index Numbers (BINs) over 47 different families collected with light traps or hand netting by day at 24 sites across Madagascar (light grey) and with the Malaise trap at Andasibe (black). Unknown refers to individuals that could not be identified at family level. See text regarding Depressariidae s.l./s.s. (Gelechioidea i.s. are only those currently under Depressariidae in BOLD that do not fit in the expanded categories).

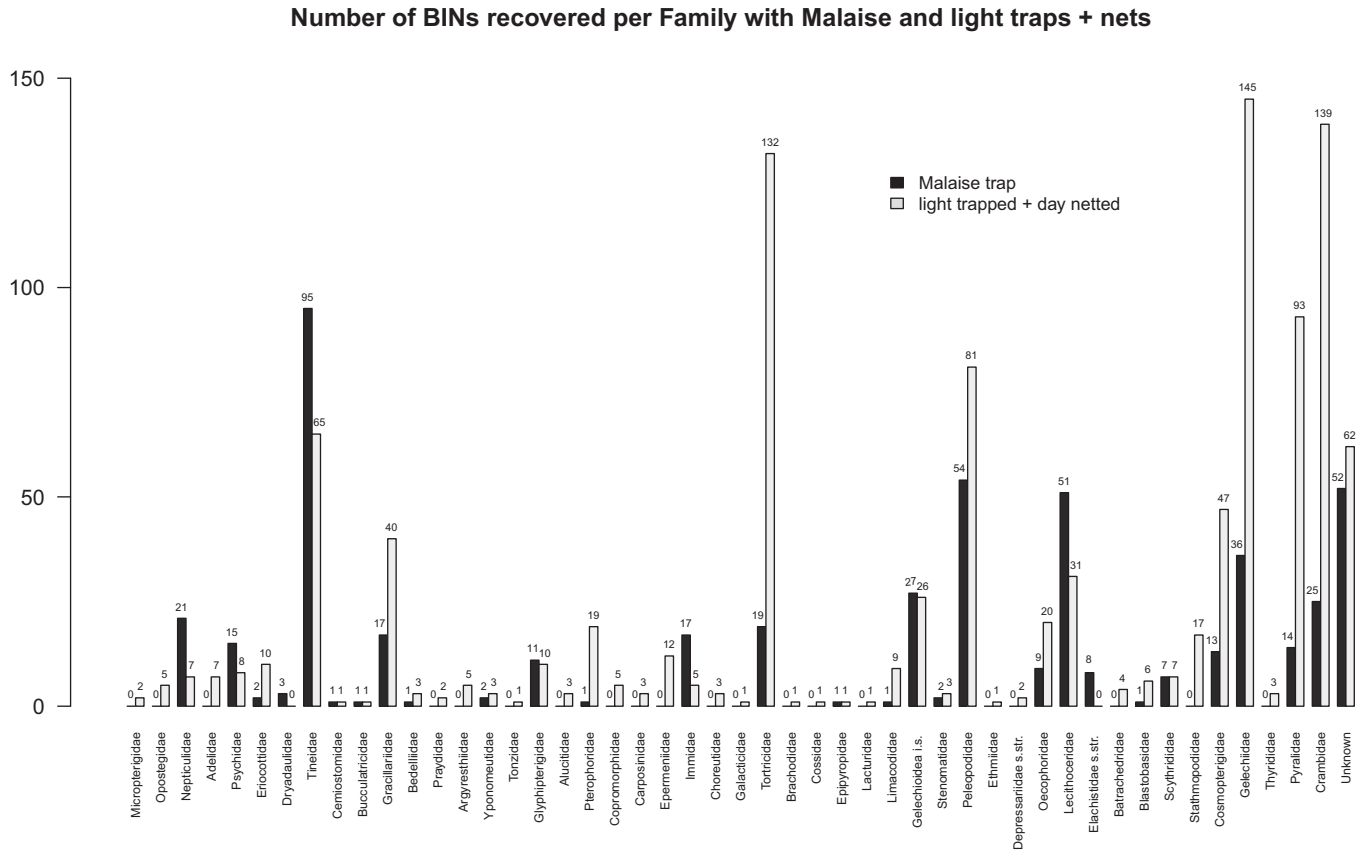
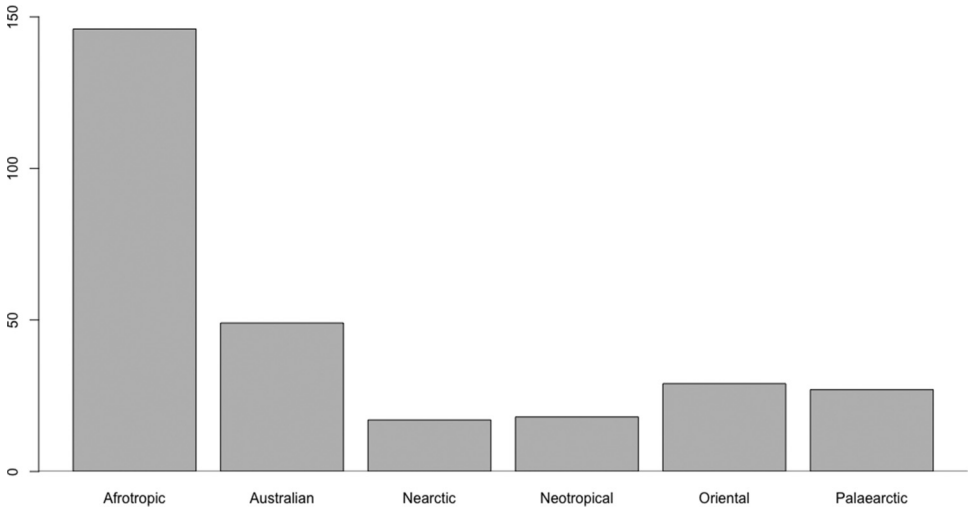


Fig. 2. Distribution of Barcode Index Numbers (BINs) over biogeographic regions. Notice one BIN can appear in several biogeographic regions.



nae s.l. on BOLD), the Malaise trap sampled a large diversity of the local radiation of Oditinae (54 BINs) and Oecophoridae only had nine BINs, most of these in *Metachanda*. This depressariid assemblage (Sohn et al. 2015), not yet adequately sorted at family level but probably including numerous Peleopodidae, comprise a high proportion of leaf litter detritivores (this provisional classification, including Gelechioidea *incertae sedis*, as well as Stenomatidae and Lecithoceridae, is included in Table 2). In the Malaise trap, we also found eight BINs of Elachistinae (Elachistidae), a leaf mining group reported by Lees and Minet (2003) but with only one reported (Parenti 2006) and one undescribed (Lees and Minet 2003: 751) Madagascan species (De Prins and De Prins 2018 duplicate *Pauroptila* in Parametrioninae, but it is here placed in Cosmopterigidae; see also Koster and van Nieukerken 2017). In the Malaise-trapped Gelechiidae, Dichomeridinae with 27 BINs clearly form another significant local radiation. The Malaise trap evidently cap-

Fig. 3. Abundance data for the 507 Barcode Index Numbers (BINs) detected in the Malaise trap samples. Notice that 57.6% (292 out of 507) of the BINs are singletons. Two BINs (BOLD:ACS0229, *Elachista* and BOLD:ACS1392, Tineidae: Hieroxestinae, exemplars illustrated left to right) have more than 70 individuals but no species name.

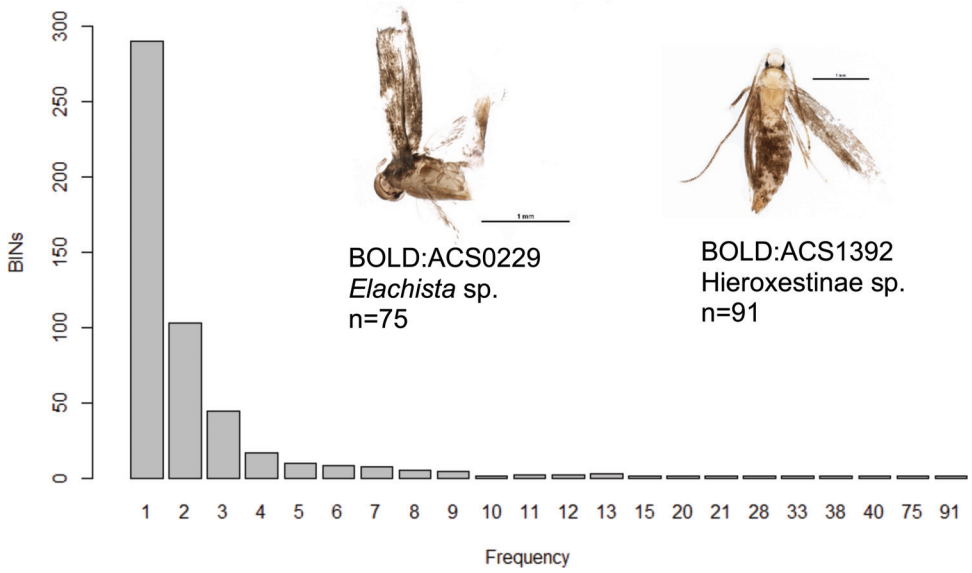
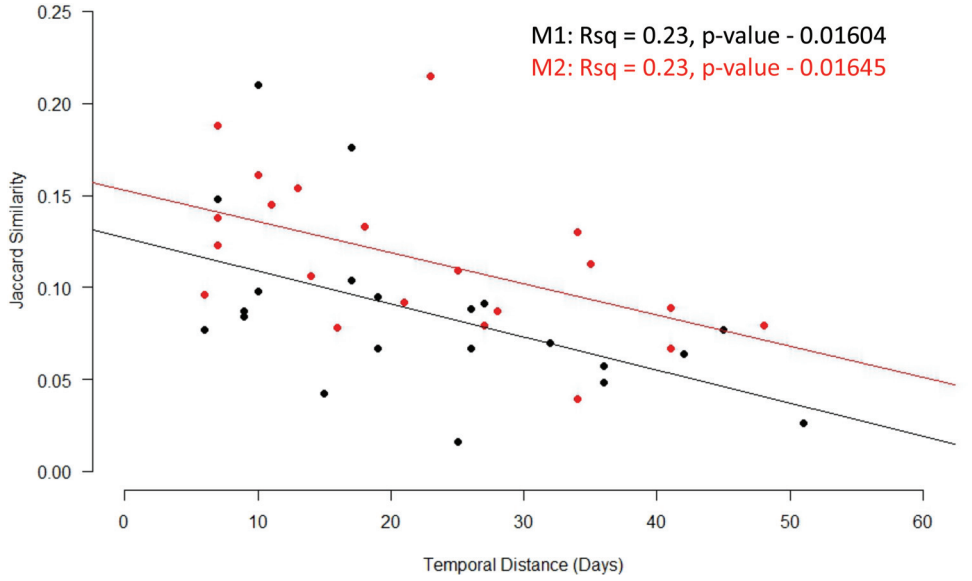


Fig. 4. Shared species decay plot for Malaise trap samples collected at two sites in Andasibe: Malaise trap 1 (M1: sampled from 1 April 2014 to 28 May 2014) in black; Malaise trap 2 (M2: sampled from 1 September 2014 to 6 November 2014) in red.



tures diurnal and nocturnal species, and the use of such a passive and stationary sampling method allowed us to recover three families not detected by light trapping and a much better diversity of some local radiations. The Malaise sampling, however, was clearly limited in finding 27 rather than 45 families (using the expanded Depressariidae classification in Table 2), but the light trapping and day netting encompassed a greater geographic range and number of sampling sites.

The large differences in taxonomic composition between the two main collecting methods could be explained by differing geography, sampling times, and human versus malaise collecting bias. Indeed, each method, of course, has its own inherent strong taxonomic biases, but the Malaise trap was largely free of human bias and its capture proportions reflect its passively sampled abundances. Indeed, Malaise trapping is likely to be the most unbiased method, as we did not control for all the possible biases (location, time of day, weather conditions, local flora, wind, etc.) that may have affected our acquired sample composition, in particular for light trapping.

The low number of "primitive" Lepidoptera in Madagascar reported by Krüger (2007) is probably an artefact of insufficient sampling. The only primitive non-ditrysians found in the Malaise trap samples were Nepticulidae (21 BINs with an additional seven BINs, all at light), a family with only one described species (*Fomoria scobleella* (Minet, 1990)) in Madagascar. Other non-ditrysians found by other methods (such as adelids) were hand netted by day (apart from one *Nemophora* sampled at light). The micropterigids (two BINs), a group also known to enter Malaise traps, were also hand netted by day, but it is likely that the Malaise trap did not intersect with their particularly narrow flight phenology. It may be that such lineages as adeloids actually need special sampling techniques and habitat surveys for their detection. Interestingly, we

Fig. 5. Accumulation curves for Malaise trap samples over the two periods corresponding to the end of the wet to beginning of the dry season (M1) and the end of the dry to beginning of the wet season (M2). (A) Species diversity (BINs) per number of individuals; (B) Sample coverage per number of individuals; (C) Species diversity (BINs) per sample coverage.

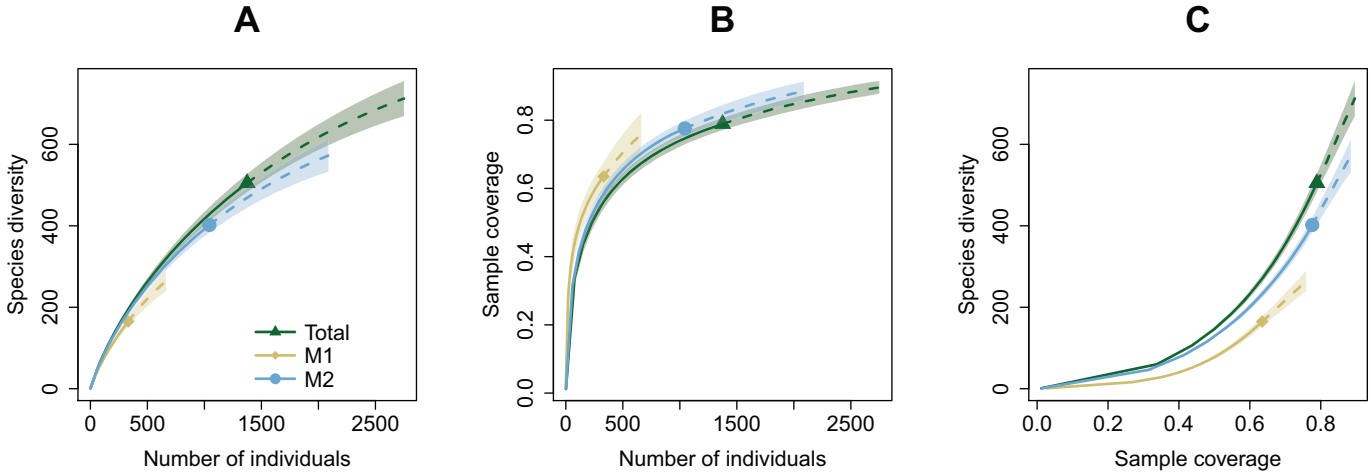
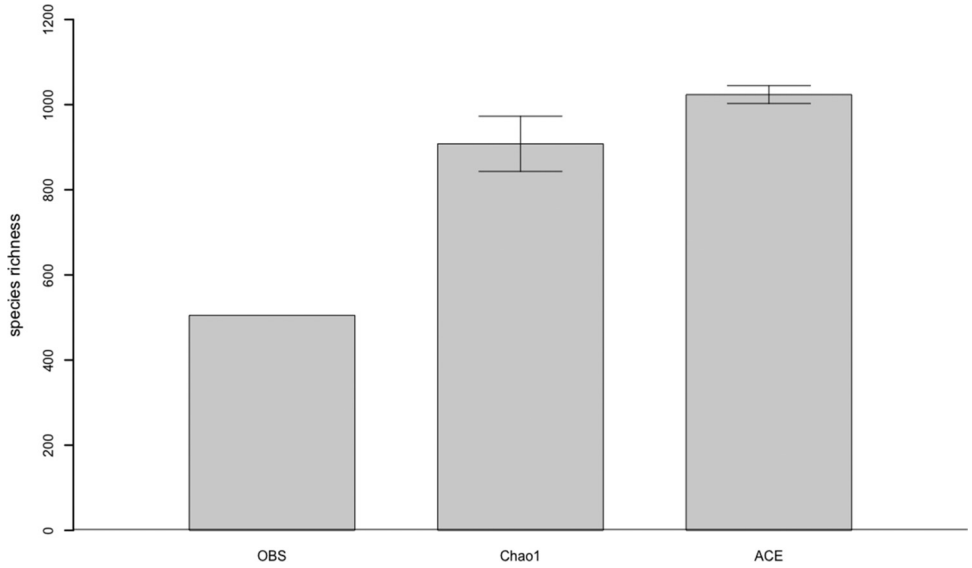


Fig. 6. Species richness observed (OBS) and estimated, based on Chao1 and ACE analyses, for the sites sampled with a Malaise trap at Andasibe.



also noticed the absence of heliozelids, which does not fit field observations of their leaf-mines in Madagascar, and the fact that one species, *Antispila merinaella* Viette, 1956, has been already described. The diverse primitive moth group Exoporia, which includes the Hepialidae, has not yet been detected in Madagascar, but they are exceptionally depauperate in tropical Africa (in fact unknown there in tropical rainforests) and so might be really absent in Madagascar or present in poorly sampled habitats. Targeted samplings, such as plant internal feeder rearings, leaf mine and gall collections, soil and periphyton layer analysis, and vigorous netting during peak emergence synchronized to rains may increase the probability of discovering such primitive groups on the island.

These observations, reinforced by the apparent high seasonal turnover found in our study, demonstrate the paucity of knowledge regarding Madagascan lepidopteran diversity, notably for micromoths, and its true taxonomic makeup. They also highlight the lack of progress in sequencing identified and unidentified museum collections, as well as the extreme shortfall in documentation of true diversity of the fauna resulting from what remains to be found in the wild and then described. The actual number of public BINs on BOLD for Madagascan Lepidoptera (2852 BINs) relative to all described Madagascan species (~4900 species) is about 58.2%. This may seem like substantial progress, but a high percentage of those BINs are unlikely to correspond to checklists of Madagascan Lepidoptera. Most micromoths in Madagascar must be undescribed: the ratio of micromoths to Macroheterocera in the French checklist, for example, is about 2.1 and if such a ratio were to hold for Madagascar too, where ~3000 described Macroheteroceran species are currently known, one would expect to find at least 6300 micromoths. The paucity of well identified moth DNA barcode clusters highlights the gap in reference barcode libraries for Madagascar and the urgency of this task in regard to conservation of this biodiversity hotspot. Indeed, a comprehensive DNA barcoding library of Madagascan moths is needed, and that could be achieved by rapid digitization and sequencing of specimens deposited at the two main collections of MNHN and NHMUK. A good example of how that could be achieved is the DNA barcoding of the Australian National Insect Collection (Hebert et al. 2013).

For micromoths, the numbers of BINs from our study (1537 BINs) equals approximately the number of non-macroheterocerans in

the current list as updated in Table 2 (based on a resolution of Viette 1990 and De Prins and De Prins 2018). There are around 1510 described species for the micromoth families detected here (1539 including Gelechioidea *incertae sedis*) out of 1598 species (Table 2). Ultimately, we would anticipate a very low overlap between the current checklist of described species and the list of BINs in our samples, in the hypothetical case that the types in museums were successfully DNA barcoded. We were able to identify relatively few (162) specimens down to species level (94 species representing 95 BINs), either using DNA barcode searches on BOLD (for the Malaise trap, just three species) or using pinned material in the MNHN. Comparative analysis of the external morphology of our pinned material with museum reference collections suggests that a large percentage of our barcoded material are likely to represent undescribed species. Moreover, some higher taxa have so few described species that we can be almost certain (given a likely very high species endemism rate in these groups) that the undescribed rate is also very high in those groups (e.g., only one nepticulid and eight hieroxestine tineids are described). For the 27 families that show more BINs than described species (notable among which are Gelechiidae and Tineidae), the number of BINs (889) exceeds the number of described species (270) by 619; only 15 of these BINs are identified to species. For the remaining 34 families (with 1298 described species), a minor proportion of their 482 BINs are likely to intersect greatly with their described species, considering only 79 of those BINs could be identified, 70 of which represent just four families (Crambidae, Pyralidae, Tortricidae, and Limacodidae). These figures alone allow a range of 45%–93% undescribed species, with a tendency towards the upper figure, among the 1371 BINs identified to family (or family-level grouping). An additional 166 BINs were not even identified to family. It is of paramount importance to DNA barcode the reference collections deposited in both MNHN and NHMUK, using new barcoding technologies (e.g., Zuccon et al. 2012) and with a particular focus on types (Hausmann et al. 2016) to more precisely estimate the Linnean shortfall (Cardoso et al. 2011) in Madagascan moths. Micromoths will be more challenging in this respect because of the need to minimise tissue removal on holotypes, but as an alternative, morphologically linkable non-primary type material is frequently available. This need for reference libraries from collection types also echoes the call for the barcoding effort to be extended to local metabarcoding studies, that all need to be linked into the BOLD system to improve database comprehensiveness (Porter and Hajibabaei 2017).

Molecular synapomorphy to identify Macroheterocera

We found that the presence of a phenylalanine in the 177th position of the barcode fragment is shared by almost all Macroheterocera analysed from the two test data sets. We have determined that phenylalanine is a shared character state of the clade Macroheterocera that is very seldom reversed or paralleled within the Lepidoptera. Its precise reliability is hard to gauge owing to the possibility of false positive and false negative identifications, but appears to be of the order of 99.5%. This molecular synapomorphy allowed us to filter out reliably non-macroheteroceran barcodes from data sets where external morphology of voucher specimens is poorly preserved (e.g., Malaise trap samples), although butterflies, which share a leucine with most micromoths in the homologous position, need to be independently removed. Use of this character should be very useful to barcoders of Lepidoptera and also provides a means when using the identification engine to evaluate nearest neighbours (e.g., from irrelevant families) that are spuriously close to the sequence being queried.

Higher taxonomic diversity of micromoths in Madagascar

Our survey detected 38 micromoth families previously recognized for Madagascar (including confirmation of the newly recognized Tonzidae) and added five new families (Dryadaulidae, Bucculatricidae, Bedellidae, Batrachedridae, and Blastobasidae). It also added four higher taxa that may be valid at family level but are currently included on BOLD as subfamilies, respectively, of Lyonetiidae (s.l.) (Cemiostomidae) and Depressariidae (s.l.) (Ethmiidae, Peleopodidae, Stenomatidae; *Orygocera* is *incertae sedis* and needs to be excluded from the latter). Seventeen micromoth families previously listed for Madagascar (Viette 1990; Lees and Minet 2003; Sohn 2015; De Prins and De Prins 2018) that we did not detect in this survey were Heliozelidae, Tischeriidae, Lyonetiidae (s. str.), Ypsolophidae, Plutellidae, Dudgeoneidae, Metarbelidae, Sesiidae, Zygaenidae, Somabrachyidae, Xyloryctidae, Autostichidae, Momphidae, Coleophoridae, Hyblaeidae, Callidulidae, and Whalleyanidae. Whalleyanidae is included in Thyrididae and *Boisduvalodes tamatavana* in Limacodinae in De Prins and De Prins 2018, but here we follow Lees and Minet 2003 (see p. 758, note 22) in treating the former as a valid family and the latter as a representative of Somabrachyidae. That brings the Madagascan micromoth fauna to as much as 64 families. It is not at all surprising that we did not find the other undetected families as they are essentially diurnal (Heliozelidae, Ethmiidae, Sesiidae, Zygaenidae, Hyblaeidae, and Callidulidae), are rare and represented by only one or two described species (Tischeriidae, Lyonetiidae s.s., Ypsolophidae, Dudgeoneidae, Metarbelidae, Somabrachyidae, Autostichidae, and Momphidae), or occur outside the sampled region (Whalleyanidae). The main surprise is the absence of Sesiidae and Zygaenidae, known to occur in Malaise traps, and Heliozelidae, mentioned earlier.

It is always an exciting possibility that one or more new families could be represented in our dataset, considering that the 113 unknowns to family level include a number of local still unidentified radiations and exhibit sometimes striking divergences to any other BIN from Madagascar or outside it.

Invasive and pest species

We found 55 species with a widespread distribution range mostly outside the Afrotropical region. Of those, 28 species appear to be new to Madagascar as they have not been recorded previously by others (Viette 1990, checklist; Martiré and Rochat 2008; and the T@RTS database; Gilligan et al. 2014). Therefore, it is unknown whether these newly recorded species are established on the island or represent interceptions of new arrivals.

Most of the widespread species and the 22 pests recorded in Table 3 were detected in disturbed habitats of the Nosy Be area. However, we found some pest species in primary habitats. For example, in primary forest in Andringitra we found both *Prays citri* and *Diasemiopsis ramburialis* for the first time, although the former is not clearly distinguishable from the sympatric *P. oleaeoides* Gibeaux, 1985 (B. Heckford, pers. comm.), raising the possibility *P. citri* has been present there for decades.

These results show the impoverishment and homogenization of the micromoth fauna in disturbed areas and the importance of preserving intact primary forests (Watson et al. 2018). In addition, they highlight the importance of DNA barcoding as a biosurveillance tool to facilitate the identification and detection of plant pests (Frewin et al. 2013).

Biodiversity assessments

DNA barcoding has facilitated the use of hyperdiverse groups such as micromoths in biodiversity studies (Miller et al. 2016). Traditionally, especially in the tropics, micromoths have been largely ignored in biodiversity assessment. This study adds much motivation to this type of effort, considering also that they are so straightforward to distinguish with DNA barcodes (e.g., to separate from very small erebids, or within types of sampling like Malaise trapping, where wing pattern identification is rendered for the most part impractical). Identifying micromoths to family level or below, however, still requires a large effort and integrated morphological and molecular analysis. Here, building a comprehensive DNA barcode reference library on BOLD with as complete

taxonomic information as possible alongside lists of BINs will prove indispensable for assisting future identification, surveys, and comparisons of poorly known faunas such as that of Madagascar. Hopefully such efforts will stimulate a new wave of species description while time is left to highlight disappearing forest regions, with slash and burn for hill rice cultivation now exacerbated by downwards spiralling poverty and the rosewood logging crisis. They will also assist agriculturalists and horticulturalists to identify threats to plants via documentation of plant pests and invasive species.

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