Characterization and comparison of poorly known moth communities through DNA barcoding in two Afrotropical environments in Gabon

Sylvain Delabye[1,2]*, Rodolphe Rougerie[3]*~, Sandrine Bayendi[4], Myrianne Andeime-Eyene[4], Evgeny V. Zakharov[5], Jeremy R. deWaard[5], Paul D.N. Hebert[5], Roger Kamgang[6~], Philippe Le Gall[6~], Carlos Lopez–Vaamonde[7~], Jacques-François Mavoungou[8,9], Ghislain Moussavou[8~], Nicolas Moulin[10~], Richard Oslisly[11,12~], Nil Rahola[13~], David Sebag[14~], Thibaud Decaëns[15$~]

* Contributed equally to the paper

$ Corresponding author

~ Members of the ECOTROP team. Remaining members of this team, and their affiliations, is given in the Acknowledgements section.

Addresses:

[1] Faculty of Science, University of South Bohemia in České Budějovice, Branišovská 1760, 37005, České Budějovice, Czech Republic

[2] Biology Center, Institute of Entomology, The Czech Academy of Science, Branišovská 31, 37005, České Budějovice, Czech Republic

[3] Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, 57 rue Cuvier, CP 50, 75005 Paris, France.

[4] Institut de Recherches Agronomique et Forestière (IRAF–CENAREST), Libreville, Gabon

[5] Centre for Biodiversity Genomics, Biodiversity Institute of Ontario, University of Guelph, Guelph, ON, Canada N1G 2W1

[6] Laboratoire Evolution, Génomes, Comportement, Ecologie (EGCE UMR 247, IRD–CNRS–Université Paris-Sud), Avenue de la Terrasse, Bâtiment 13, Boite Postale 1, 91198 Gif sur Yvette, France

[7] Unité de Zoologie Forestière (URZF UR 633, INRA-Orléans), 2163 Avenue de la Pomme de Pin, CS 40001 Ardon, 45075 Orléans Cedex 2, France

[8] Institut de Recherches en Ecologie Tropicale (IRET–CENAREST), Libreville, Gabon

[9] Département de Biologie, Faculté des Sciences, Université des Sciences et Techniques de Masuku, B.P 943, Franceville, Gabon

[10] Nicolas Moulin Entomologiste, 82 route de l'Ecole, 76680 Montérolier, France

[11] Agence Nationale des Parcs Nationaux (ANPN), BP 20379, Libreville, Gabon

[12] Laboratoire Patrimoines Locaux et Gouvernance (PALOC) UMR 208, IRD-MNHN, 57 rue Cuvier - Case Postale 26, 75231 Paris cedex 05, France

[13] International Centre for Medical Research (CIRMF), Franceville, Gabon

[14] Normandie Université, UNIROUEN, UNICAEN, CNRS, M2C UMR 6143, Place Emile Blondel - Bâtiment IRESE A, 76821 Mont Saint Aignan Cedex, France

[15] Centre d'Ecologie Fonctionnelle et Evolutive (CEFE UMR 5175, CNRS–Université de Montpellier–Université Paul-Valéry Montpellier–EPHE), 1919 Route de Mende, F-34293 Montpellier, France; thibaud.decaens@cefe.cnrs.fr; +33 (0)4 6761 3243

Abstract

Biodiversity research in tropical ecosystems – popularized as the most biodiverse habitats on Earth – often neglects invertebrates, yet representing the bulk of local species richness. Insect communities in particular remain strongly impeded by both Linnaean and Wallacean shortfalls, and identifying species often remains a formidable challenge inhibiting the use of these organisms as indicators for ecological and conservation studies.

Here we use DNA barcoding as an alternative to traditional taxonomic approach for characterizing and comparing the diversity of moth communities in two different ecosystems in Gabon. Though sampling remains very incomplete, as evidenced by the high proportion (59%) of species represented by singletons, our results reveal an outstanding diversity. With about 3500 specimens sequenced and representing 1385 BINs (Barcode Index Numbers, used as a proxy to species) in 23 families, the diversity of moths in the two sites sampled is higher than the current number of species listed for the entire country, highlighting the huge gap in biodiversity knowledge for this country. Both seasonal and spatial turnovers are strikingly high (18.3% of BINs shared between seasons, and 13.3% between sites) and draw attention to the need to account for these when running regional surveys. Our results also highlight the richness and singularity of savannah environments and emphasize the status of Central African ecosystems as hotspots of biodiversity.

Keywords: Community Ecology, DNA barcodes, Lepidoptera, Tropical Africa, Taxonomic deficit

Introduction

Tropical ecosystems host unrivalled species richness (Kier et al. 2005; Myers 1984; Myers et al. 2000), a fact that has long captivated public attention and raised concerns about the way to conserve this immense biodiversity (Wilson 1988). Understanding of tropical biodiversity has historically been biased toward the largest organisms such as angiosperms and vertebrates (May 2011), leaving considerable gaps in our knowledge of hyperdiverse groups of smaller animals, especially arthropods. These organisms are nevertheless key to ecosystem functioning (Erwin 1983; Zhang 2011) and the shortfalls in our taxonomic, biogeographic and ecological knowledge are strong impediments against the integration of these organisms in conservation and management strategies (Miller and Rogo 2002; Whittaker *et al*. 2005). Because the few studies addressing this topic predict high extinction numbers for insects (Fonseca 2009; Stork and Habel 2013), it is urgent to lift "the curse of ignorance" (Diniz-Filho *et al*. 2010) by developing multi-scale studies on insect diversity that benefit from the technological revolution of the 'genomic era' (Godfray 2006; Wilson 2003) and its recent developments in biodiversity sciences (Hebert *et al*. 2003a).

The Afrotropical region is one of the Major Tropical Wilderness Areas on earth (Myers 1990; Wilson 2002), i.e. a large and highly diverse area that has seen little impact from human activities until recently (i.e. < 5 inhab km^{-2} and > 75% of the original vegetation still present) (Mittermeier *et al*. 1998). However, recent estimates indicate that annual net deforestation of African tropical rainforests, although less dramatic than in Latin America or Southeast Asia, approached 0.3 million ha/year for the 2000-2010 decade (Achard *et al*. 2014), which could have led to dramatic biodiversity loss. As many as 100 000 insect species have been reported from the area, but Miller and Rogo (2002) suggest that species richness could exceed 600 000 species. In Gabon, a central-

African country which is still covered by 80% of tropical rainforests, insect inventories have only considered butterflies (vande Weghe 2010), a few groups with limited number of species such as Mantodea (Roy 1973), Lucanidae (Maes and Pauly 1998) or Apoidae (Pauly 1998), and groups with specific economical and/or agronomical importance such as Pseudococcidea (Hemiptera) and their parasitoids (Boussienguet *et al*. 1991). A few studies have also targeted terrestrial arthropod assemblages along human disturbance gradients (Basset *et al*. 2004, 2008).

Several authors emphasized the potential of using highly diverse groups, such as Lepidoptera, as environmental indicators (Axmacher et al. 2004a, 2004b; Beck et al. 2013; Kitching et al. 2000; Ricketts et al. 2001). They are indeed key herbivores and an important link within foodwebs as prey or as hosts for parasitoids. Variation in the diversity and structure of lepidopteran communities is thus likely to be representative of changes at other trophic levels. For instance, lepidopteran species depend on their host plant species (or a few closely related plants), and in turn play a fundamental role as pollinators; this connects them closely to plant community structure and composition (Ehrlich and Raven 1964; Novotny et al. 2002b). On the other hand, trophic cascades in food webs are likely to link both host plant and primary consumer assemblages to associated higher trophic levels of predators and parasitoids. Surprisingly however, only a few studies have examined this group in the Afrotropics. The taxonomic deficit and the high number of species that occur in those environments are certainly important causes for this deficit, because they impede reliable inventories and the description of community patterns. In a recent study based on a substantial sampling effort in Papua New Guinea (over 30 000 specimens collected over several years), Ashton et al. (2014) found that no asymptote was reached by species accumulation curves. These authors, however, also suggested that more

limited sampling could be efficient in highlighting differences in the diversity and composition of moth communities among distant localities.

In this study, we use DNA barcodes to document and compare communities of moths in two differing ecosystems of Gabon. Several recent studies have demonstrated the effectiveness of DNA barcoding – a tool for species identification based on a short standardized DNA fragment (Hebert et al. 2003b) – in documenting species diversity of lepidopteran communities in regions where species assemblages are very diverse and when many species are undescribed (Janzen et al., 2009; Lamarre et al. 2016; Lees et al. 2014; Zenker et al. 2016). With this approach we aim at evaluating the sampling effort required to produce relevant census of these communities, to document seasonal variation in community composition, and if species-turnover (β-diversity) as revealed from our data is reflecting significant differences in richness and composition that can be linked to the different habitats sampled. Finally, we discuss the contribution of our study to the current knowledge of moth diversity in Gabon and in the Afrotropical region, with special reference to information compiled in the AfroMoths database (De Prins 2016; De Prins and De Prins 2017).

Material and Methods

Study sites

Moths were collected at two locations (named *Lopé 2* and *Ipassa* research station) in the province of Ogooué-Ivindo, in Gabon (Figures 1 and 2):

- *Lopé 2* site is situated in the northern part of Lopé National Park, about 12 km south from Lopé village and the Dr. Alphonse Mackanga Missandzou Training Centre (CEDAMM, Wildlife Conservation Society; coordinates: S0°13’9.699” / E11°35’5.6394”; altitude: 300m). Vegetation

comprises a mosaic of forest and shrub savannah (Figure 2A). Shrub savannah is dominated by Poaceae and Cyperaceae like *Anadelphia arrecta*, *Andropogon pseudapricus*, *Schizachyrium platyphyllum*, *Hyparrhenia diplandra* or *Ctenium newtonii* and by a shrub layer with *Crossopteryx febrifuga* and *Nauclea latifolia* (White and Abernethy 1997). Forest patches are mainly secondary to mature okoumé rainforests, the dominant forest type in western Gabon, dominated by *Aucoumea klaineana ("okoumé")*, *Lophira alata, Desbordesia glaucescens*, *Scyphocephalium ochochoa*, *Dacryodes buttneri*, *Santiria trimera*, *Sindoropsis le-testui* and *Uapaca guineensis* (Ben Yahmed and Pourtier 2004; White and Abernethy 1997).

- The *Ipassa* research station (Institut de Recherches en Ecologie Tropicale) is situated in the northern part of Ivindo National Park, 12 km from the city of Makokou (coordinates: N0°30'38.1456" / E12°48'1.2594"; altitude: 500m). The site is mainly surrounded by mature Guineo-Congolean rainforest showing both Atlantic and continental influences (Doumenge et al. 2004; Nicolas 1977; White 1983), with *Baphia leptobotrys* and *Millettia laurentii* dominating the tree cover, as well as *Scorodophloeus zenkeri*, *Plagiostyles africana*, *Dichostemma glaucescens*, *Santiria trimera*, *Polyalthia suaveolens* and *Poncovia pedicellaris* (Figure 2B).

The two sites are 160 kilometers apart and share a similar seasonal cycle typical of the equatorial transition zone, with short (January-February) and long (June-September) dry seasons. The average monthly temperature is 24°C while mean annual precipitation is 1500 mm at Lopé and 1700 mm at Ipassa.

Moth sampling

Sampling was conducted at both sites in November 2009 and at *Lopé 2* in February-March 2011 during a field class organized in the Lopé National Park (ECOTROP field class -

http://www.ecotrop.com/ECOTROP). We used a standard light trap technique consisting of a 250W UV (mercury vapor) bulb placed 4-5 meters above the ground to attract insects (Figures 2A and 2C). Two low voltage lamps (80W) were positioned on both sides of a vertical white sheet positioned below the UV bulb. Specimens were collected during dark-moon phases from dusk to dawn (6pm to 6am, local time) in order to collect species with varying flight times (Lamarre et al. 2015). Overall, four collecting nights were carried out in each site in 2009 (10th to 14th of November at *Lopé 2* and 14th to 18th of November at *Ipassa*), and three additional nights at the end of the short dry season at *Lopé 2* in 2011 (27th February, 1st and 4th March). Our sampling design was therefore relevant to compare observed communities between sites from the samples collected during the rainy season in 2009, and to investigate seasonal turnover at *Lopé 2*.

Our study focuses on macro-moths, i.e. moths whose wingspan were >1 cm, which includes the nocturnal part of Macrolepidoptera as well as larger representative of non-macrolepidopteran families (so-called "Microlepidoptera"), and exclude the smaller species of other families of Macrolepidoptera (Figure 2D). Each night, we sampled specimens of as many species of macro-moths as could be distinguished morphologically when collecting. Moths were killed using a cyanide jar or by an injection of ammonia into the thorax for larger species, and were placed in glassine envelopes marked with a code unique to each sampling event. Specimens are currently deposited in the Museum national d'Histoire Naturelle in Paris, where they are available for further taxonomic study.

DNA barcoding and taxonomic assignments

The day after collecting, specimens were sorted into morphospecies, i.e. groups of specimens that were readily distinguishable from their external morphology. A maximum of four specimens per morphospecies and per collecting night were then selected for molecular analyses. A small piece

of tissue (generally a complete leg or its tarsus for the largest species) was sampled for each of them (Figure 2D). DNA extraction was carried out at the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph following a standard automated protocol (Ivanova et al. 2006; Hajibabaei et al. 2005). Tissue lysis occurred in 50µl of lysis buffer and proteinase K [0.02 mg/µL] incubated at 56ºC overnight. A 658 bp segment of the 5' region of the COI mitochondrial gene used as a standard DNA barcode was amplified through PCR using the primer pair LepF1/LepR1 (Hebert et al. 2004). Samples failing to amplify after this first PCR pass were re-processed using the primer sets LepF1/MLepR1 and MLepF1/LepR1 that target 307 bp and 407 bp overlapping fragments, respectively (Hajibabaei et al. 2006). A standard PCR reaction protocol was used for all PCR amplifications and products were checked on a 2% E-gel 96 Agarose (Invitrogen). Unpurified PCR amplicons were sequenced in both directions using the same primers as those used for the initial amplification, and following standard CCDB protocols (http://ccdb.ca/resources/) (Hajibabaei et al. 2005). Trimming of primers, sequence editing and contig assembly were carried out at CCDB using CodonCode software (CodonCode Corporation, Centerville, MA, USA). All sequences were aligned and inspected for frame-shifts and stop codons for removal of editing errors and possible pseudogenes, and then uploaded in the Barcode of Life Data systems (BOLD, Ratnasingham and Hebert 2007). All records – including specimen and sequence data – can be accessed publicly in BOLD and GenBank, and were assembled within BOLD dataset DS-LOPELEP1.

Species identification of specimens using either DNA barcodes or morphology could not be achieved for all the specimens, because of the incompleteness of the current DNA barcode library for the region, and because of the lack of taxonomic expertise for many of the moths collected. Also, the use of provisional morphospecies was intractable considering the large number of

specimens and the need for a thorough processing of individuals (spreading of wings, genitalia dissections) for a reliable assessment of observed species diversity (Zenker et al. 2016). As a consequence we used DNA barcodes to delineate molecular taxonomic units (MOTUs) as a proxy for species. More specifically, we used Barcode Index Numbers (BINs) derived from the automated MOTU delineation tool implemented in BOLD (Ratnasingham and Hebert 2013), and which have already been used to consistently approximate species in Lepidoptera (Hausmann et al. 2013; Kekkonen and Hebert 2014). In two families, Saturniidae and Sphingidae, species were carefully identified (by RR and TD) on the basis of morphology and the results were used to test their correspondence with BINs.

A "reverse taxonomy" approach (Markmann and Tautz 2005) using DNA barcode results coupled with the BOLD identification tool as well as the topology of the NJ tree failed to produce species identification for most of our query sequences. However, we were able to provide a family-level identification for the majority of individuals analyzed using either their general morphology or DNA barcode analysis. For this second approach, the richness of the BOLD DNA barcode library, with records for more than 100 000 species of Lepidoptera – proved very useful using a simple query for best close matches in the database. Instead of applying a threshold to generate family (or occasionally subfamily and genus) assignment, we verified the proposed assignments by comparing images and, where relevant, by examining the specimens and confirming the proposed taxon on the basis of its morphology.

Community data analyses

The α-diversity at each site was assessed by plotting rarefaction curves and their extrapolations for both species richness and sample coverage (i.e. a 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), using specimen numbers as a measure of sampling intensity. These analyses were carried out using the *iNEXT* package (Hsieh et al. 2014) for R 3.0.2 (R Development Core Team 2004). We then used the *Vegan* package (Oksanen et al. 2013) to calculate several diversity indices: observed richness (defined as the total number of observed BINs at a given sampling site or on a given date), Chao1, ACE and second order jacknife diversity estimators, and Fisher α-diversity index. We also used *iNEXT* to calculate the number of species observed given a constant level of sampling coverage, and *Vegan* for the estimation of species richness rarefied to a constant level of sampling intensity (i.e. a constant number of specimens collected). We finally used *fisherfit*, *prestonfit* and *prestondistr* functions of *Vegan* to plot rank-abundance diagrams and fit Fisher's logseries, Preston's lognormal and truncated lognormal models to abundance data for each sampling site.

To assess β-diversity among sampling sites (for samples collected in 2009) and seasons (in *Lopé 2* site only), we calculated an average Sørensen's index of dissimilarity using the package *Vegan* (Oksanen et al. 2013):

$$\beta_{BC} = (b+c)/(2\,a + b + c)$$

where *a* is the number of species (here BINs) shared between two sites B and C, and *b* and *c* are the numbers of unique BINs for sites B and C.

We used the *betapart* package to decompose β-diversity into two components (Baselga 2010): nestedness (i.e. when the composition of communities with a smaller species number is a subset of a richer community) which reflects non-random processes of species loss, and spatial turnover which results from species replacement as a consequence of environmental sorting or spatial and historical constraints (Qian et al. 2005; Ulrich et al. 2009; Wright and Reeves 1992). Analyses of

β-diversity were carried out with and without singletons (i.e. BINs represented by a single specimen in the dataset), as their inclusion can lead to overestimation of β-diversity.

Results

Species richness at the regional scale

We obtained 3494 (97.7%) sequences from the 3576 specimens selected for DNA barcoding. These sequences included representatives of 1385 BINs representing 23 families of Lepidoptera (Table 1) and only 6 specimens (6 BINs) could not be identified to family level. Noctuidae, Erebidae and Geometridae represented about one third of the BINs and sampled individuals, whereas 10 other families were each represented by less than 10 specimens. More than half of the BINs (796 in total, 59%) were represented by a single individual in our data set (i.e. singleton).

Morphological examination of specimens in the families Saturniidae (177) and Sphingidae (267), led to the distinction of 42 and 63 species, respectively, of which only two (in family Saturniidae) could not be identified to species and were given a provisional name (*Orthogonioptilum* mgab_RR01 and *Dogoia* mgab_RR01). The correspondence between morphologically assigned species and BINs was nearly perfect: 42 species versus 43 BINs in Saturniidae (98%) and 63 versus 66 in Sphingidae (95%) (see DNA barcode NJ trees in gen-2018-0063.R1Suppla and gen-2018-0063.R1Supplb). In other families, 112 species (representing 121 BINs) were formally identified by taxonomic experts (see acknowledgments) or through DNA barcode matches in BOLD. Overall, with Saturniidae and Sphingidae included, these species represent about 16% of all BINs (230/1385).

Comparison between the number of BINs observed in our study and the list of recognized species and subspecies for Gabon, as derived from the AfroMoths online database (De Prins 2016; De

Prins and De Prins 2017), revealed the strong taxonomic deficit and the lack of exploration (i.e. biodiversity surveys, collecting activities) that characterizes moth diversity in the Afrotropics. Afromoths is based on the survey of 7355 published sources for the whole Afro-tropical region (as of August 8th, 2017) and the authors' own studies. It lists 1301 moth species and subspecies for Gabon, belonging to 36 families. Our survey (Figure 4), limited to macro-moths collected during only 11 nights at two sites, revealed 1385 BINs in just 25 families. Three families (Bombycidae, Brahmaeidae, and Lecithoceridae) detected in our study lack published records for Gabon in the AfroMoths database. For 10 of the 22 other families, the number of BINs recorded in our study exceeded the number of known species (Table 1). Large differences were observed for Cossidae (1 species in AfroMoths versus 11 BINs), Crambidae (9 vs. 52), Erebidae (309 vs. 369), Geometridae (184 vs. 220), Lasiocampidae (68 vs. 101), Noctuidae (71 vs. 224), and Pyralidae (6 vs. 70), which may represent the most understudied families or those yet incompletely surveyed in the AfroMoths database.

In the few families that are well-studied for this region, we collected approximately half the known number of species (48.2%, sd=6.9, N=4 – including Saturniidae (43 BINS vs. 110 species listed in AfroMoths, 39%), Eupterotidae (15 vs. 32, 47%), Sphingidae (66 vs. 124, 53%) and Lasiocampidae (101 vs. 188 as listed by P. Basquin, personal communication, 54%).

Species richness and diversity patterns between sampling sites

Our survey revealed a total of 823 BINs (1604 specimens analyzed) and 782 BINs (1890 specimens analyzed) in the *Ipassa* and *Lopé 2* sites, respectively (Table 1). Sampling resulted in a high proportion of singletons at both sites (64% and 59% of BINs in *Ipassa* and *Lopé 2*, respectively; 57% of all BINs when combining the two sites), and the distributions of BIN

abundance are a strong fit to a log-series model (gen-2018-0063.R1Supplc). While observed richness was similar between the sites, we collected fewer BINs in *Lopé 2*, despite collecting three additional nights in this site during the dry season.

For comparison of the two sites, we only considered specimens collected during the wet season when sampling efforts were identical. The four collecting nights at each site resulted in the capture of 1604 and 1110 specimens, which belonged to 823 and 481 BINs for *Ipassa* and *Lopé 2*, respectively (Table 2). Richness estimators indicate that species richness ranged between 1250 and 1850 species at *Ipassa* and between 700 and 1200 species at *Lopé 2*. Rarefaction curves clearly show a higher richness in *Ipassa* (Figure 5a), while sampling coverage rate was slightly higher at *Lopé 2* (73% versus 68% at *Ipassa*) (Table 2, Figures 4b and 4c). Overall, the moth communities at both sites showed a similar relative abundance of the different families, both in terms of specimen numbers and BINs, although observed richness in the most diverse families was consistently higher in *Ipassa*, with the exception of Crambidae and Pyralidae, which had more BINs at *Lopé 2* (Table 1).

Comparison of BINs collected during the wet season at *Lopé 2* and *Ipassa* revealed only 158 BINs shared by the two sites, 13.8% of the total number analyzed. Sørensen's index of β-diversity calculated between the two sites was 0.76 for the whole dataset and 0.42 after singletons were removed (Table 3). In both cases, β-diversity was mainly explained by spatial turnover (71.0% and 67.6%, respectively) and to a lesser extent by nestedness (29.0% and 32.4%).

Seasonal changes in moth assemblages at Lopé 2

We generated DNA barcodes for 1110 and 780 specimens from *Lopé 2* during the rainy and the dry seasons, respectively. Observed richness during the wet season was slightly higher (478 BINs

versus 441 BINs during the dry season), but this trend was reversed after rarefying richness to a constant sampling effort or a constant sampling coverage. Rarefaction curves and diversity estimators were also quite similar, the latter ranging between 650 and 1100 for both seasons (Figure 5).

During the dry season, we collected moths belonging to 17 families versus 21 families during the wet season. Seven families were not shared between the two sampling seasons, but all were represented by few BINs (maximum 2) and individuals (maximum 2), excepting one BIN in the family Thyrididae for which 18 specimens were collected in the wet season. Overall, the diversity for each family was similar for the two sampling periods (Table 1) with a few exceptions: the Crambidae (31 vs. 16 BINs), Pyralidae (37 vs. 18), and Saturniidae (31 vs. 9), which were all more diverse during the wet season, and the Sphingidae (40 vs. 28) that was more diverse during the dry season. Out of a total of 782 BINs, 144 (i.e. 18.5%) were found during both the rainy and the dry seasons. Sørensen's index of dissimilarity between seasons was 0.69, largely explained by temporal turnover (95.4%), but it dropped to 0.23 and was evenly explained by turnover and nestedness after removing singletons from the data set (Table 3).

Discussion

DNA barcodes for the study of moth diversity in the tropics

Overall, we documented in our study a number of molecular units (BINs) that was higher than the total number of species listed for the country in AfroMoths database, including three families not documented so far (De Prins and De Prins 2017). Considering the relatively shallow geographical range and temporal extent of our study, this result highlights the weakness of the current knowledge of moth diversity in the Afrotropics, despite the remarkable efforts by De Prins and

De Prins (2017) to synthesize and centralize this knowledge in the AfroMoths database. Our results clearly highlight the value of DNA barcoding for producing a rapid and accurate census of moth diversity in a poorly studied tropical region. Because this approach facilitates comparisons between sampling campaigns through barcode matches (as exemplified here between sites, but it can also be applied between countries as currently in progress with a similar campaign in Central African Republic), its systematic implementation would represent a powerful mean to address both the Linnean and Wallacean shortfalls (Lomolino 2004), i.e. the inadequacies in taxonomic and distributional knowledge that characterise most invertebrate taxa in poorly studied regions such as the Congo basin (Whittaker *et al*. 2005).

In our study, the large number of BINs without taxonomic assignation at species level (1155 out of 1385) corresponds both to already known species not yet documented in the BOLD libraries and to species that are new to science. The number and proportion of the latter remains unclear and further study by expert taxonomists of the specimens collected is needed, as well as continued efforts to populate DNA barcode reference libraries. In addition, the inflation of species numbers in many families may reflect an incomplete census of Gabonese records in past studies, a considerable task initiated in the Afromoths database, but certainly suffering from the absence of recent dedicated efforts to synthesize lepidopteran diversity data for this country. The bombycid *Amusaron kolga* (Druce, 1887) and brahmaeid *Dactyloceras lucina* (Drury, 1782) for instance represent new records for their respective families in Gabon, but are species known to occur in neighbouring countries of the Congo basin (De Prins and De Prins 2017). In Lasiocampidae the number of species listed in AfroMoths (68) is identical to the number of species reported from an independent literature survey by a specialist of this family on the African continent (P. Basquin, personal communication). Furthermore, this same taxonomic

authority (unpublished results) has recorded approximately 188 Gabonese lasiocampid species in natural history collections worldwide, which clearly demonstrates how insufficient the published data are for this family at the regional scale and is consistent with the number of BINs (101) reported in our study.

The very close fit we found between species names and BINS in the Saturniidae and Sphingidae families supports previous assessments of BINs as good proxies for species in Lepidoptera where empirical studies (e.g. Hausmann *et al*. 2013) revealed only few occurrences of discrepancies between morphologically identified and molecular species (for instance one species divided in two or more BINS, or multiple species merged within a single BIN; see Ratnasingham and Hebert, 2013). Within the two families thoroughly investigated here, mismatches between BINs and species are cases where supposedly well-defined morphological species appeared to be split into two or three distinct BINs. These require further study using an integrative approach and may represent cases of cryptic species, i.e. species that cannot be distinguished from morphological characters, or that present subtle morphological and/or ecological traits previously ascribed to intra-specific variation or thought to be insignificant for species-level recognition (Janzen et al. 2009; Janzen et al. 2013; Rougerie et al. 2014). Alternatively, these BIN "splits" may also cause an overestimation of species diversity if they represent cases of pseudogene amplification (although this is considered unlikely here because of the absence of indels, stop codons or unusual amino acid substitutions), insufficient sampling of genetic variation biasing BIN assignments, or *Wolbachia* infections isolating lineages within species (Smith *et al*. 2012). Geographical structure of populations in poorly mobile species could also cause strong genetic structure that may inflate the number of BINs per species, but we consider this unlikely in the present study because of the small geographical distance between sampled sites, the absence of

geographical barriers, and the generally high vagility of moth species. DNA barcoding has revealed many cases of cryptic diversity in Lepidoptera since its broad integration in the taxonomic toolbox of lepidopteran taxonomists (e.g. Vaglia *et al*. 2008; Rougerie et al. 2012, 2014) and we consider it is very likely that speciose and less studied families such as Erebidae, Geometridae and Noctuidae will also reveal many such cases, leading to an increase of species numbers in these groups compared to available checklists only based on morphologically recognized species.

Moth diversity at Ivindo and Lopé National Parks

Among the 1385 BINs found in our samples, 796 (i.e. 58% of the total) were represented by a single specimen, which is a high singleton proportion compared to the average of 32% found by Coddington et al. (2009) in a review of tropical arthropod studies. There are little or no biological explanations for the high proportion of rare species usually found in tropical insect surveys (Novotný and Basset 2000). Rather, this pattern can be attributed to undersampling of highly diverse communities (Coddington et al. 2009), suggesting that caution should be taken when interpreting the observed patterns of community composition and structure. It also suggests that the estimates of species richness derived from our results probably represent a low estimation of the actual diversity of these ecosystems. Both rarefaction curves and sampling coverage indices (Figure 5, Table 2) support this idea, suggesting that at least twice the number of collected species may occur in the study area.

We found only a few studies that assessed moth local richness in tropical rainforest or savannah ecosystems and that can be readily compared with our own results. Ashton et al. (2014) sampled 791 to 2795 species and produced Chao1 estimates ranging from 1478 to 3666 among three rainforest locations in Malaysia. In Costa Rica, Janzen et al. (2009) published a census of 2349

species using a DNA barcode-based assessment of macro-moth assemblages in the Area de Conservación Guanacaste (see also Janzen and Hallwachs, 2016). On the other hand, Hawes et al. (2009) reported 98 species of Arctiinae (Erebidae), 43 of Saturniidae and 5 of Sphingidae in a primary forest area of Brazilian Amazonia, which is well below our findings in the present study. Variations in the number of species observed among studies are however difficult to interpret, because they can both reflect real differences in species diversity, but can also be biased by differences in sampling efforts and/or sampling performed in different seasons. In fact, the moth sampling by Ashton et al. (2014) and Hawes et al. (2009) was done through 264 and 30 collecting nights per study site, respectively, while the survey of Janzen et al. (2009) was conducted over decades and involved additional sampling methods (in particular, the mass rearing of caterpillars). Comparing the results obtained in different studies and with different sampling intensity requires standardization through rarefaction procedure (Gotelli and Colwell 2001). Applying this approach to the data from Ashton et al. (2014) produces a result different from what can be directly deduced from observed richness (Nakamura A., Ashton L.A., Kitching. R.L., personal communication). For instance, species numbers in their Malaysian sites ranged from 290 to 475 after standardization to a constant sampling effort of 1000 individuals, and between 100 to 270 at a constant sampling coverage of 50%, which was lower to what we found in our two study sites (Table 2). This suggests that Central African rainforests may represent an important hotspot for moth diversity.

Variation in moth diversity and composition among study sites

Our analyses of moth assemblages during the rainy season in the rainforest of *Ipassa* and the savannah/forest landscape of *Lopé 2* unveiled significant differences in both species diversity and composition. As expected from differences in vegetation coverage, the observed and estimated

richness were both higher in *Ipassa*. Plant diversity is indeed higher in the rainforest landscape of *Ipassa* than in the shrubland savannahs and peaty marshes that dominate the landscapes of the northern part of Lopé National Park (White and Abernethy 1997). In addition, despite presenting a comparable structure, forests at *Ipassa* are more humid and present higher tree diversity when compared with the gallery forests of *Lopé 2*. These features presumably offer a broader diversity of ecological niches in terms of trophic resources and microhabitats, in particular via the important diversity of epiphytes and lianas (Ben Yahmed and Pourtier 2004).

Difference in species assemblage composition among sites was high, with only 13.3% of BINs found in both. This high β-diversity was mainly attributed to spatial turnover, meaning that undersampling may only weakly account for this variation. This is in contrast with other studies that reported relatively low β-diversity of insect herbivores in comparable tropical rainforest habitats (Basset et al. 2012; Novotny et al. 2007). This also concords with other studies having reported high species turnovers among sites as long as these comprised enough variability in vegetation types (Beck and Chey 2007; Ødegaard 2006). In fact, contrasted composition of dominant forest tree species among our study sites may have selected for different assemblages of herbivorous species, as leaf-chewing insects are usually specialized on a single genus of host plants (Novotny et al. 2002a, 2002b). Similarly, the presence of herbaceous ecosystems and secondary forests at *Lopé 2* may have also driven the presence of specific species assemblages associated with these open habitats. The high diversity of Crambidae and Pyralidae observed at this site compared to *Ipassa* could for instance be linked to species preferences within these groups for herbaceous host-plants (Kitching et al. 2000).

Even if additional sampling is necessary to confirm this finding, these preliminary results suggest that landscapes dominated by a savannah-forest patchwork may host substantial levels of

herbivore insect diversity with a high compositional specificity at species level compared to typical tropical rainforests. This argues in favor of a better consideration of savannah ecosystems in both global estimates and conservation strategies of insect biodiversity.

Seasonal variation of moth assemblages

At *Lopé 2* we found little difference in species richness of moth assemblages collected during the rainy and the dry seasons. In contrast, BINs compositions clearly differed from one season to the other, with only 18.3% of the BINs collected being observed in both seasons, and this temporal β-diversity being clearly explained by seasonal turnover rather than by nestedness (Table 3). Composition may simply be influenced by the level of vegetation development during the seasonal cycle, which is well known to influence the phenology of lepidopteran species, or by different climatic preferences linked to the feeding and/or reproductive activity of the moths.

From a methodological point of view, these results highlight the importance of standardizing the period of sampling to provide fully comparable results among different localities. They also suggest the need of sampling different seasons to obtain a reliable inventory of species at a given study site, as the assemblages observed at the rainy season (the usually preferred period for moth collecting) clearly do not provide a representative overview of the actual species composition of the focal community.

Conclusion

Our study highlights the usefulness of utilizing DNA barcodes for performing rapid analyses of taxonomic diversity and composition of moth assemblages in poorly studied areas. It also stresses the need to accelerate biodiversity inventories in those areas that have been insufficiently explored regarding moths and other poorly studied invertebrates. Central Africa clearly is one of

those areas and our results represent the first robust assessment of moth diversity in Gabonese forests and savannahs, highlighting a strongly understudied fauna. The material collected and the DNA barcode library released with this study are thus important contributions and we expect that they will serve the development of knowledge on the diversity and distribution of African moths. In general, studies combining molecular data and traditional taxonomic expertise are critically needed to better document invertebrate communities in tropical areas, especially in the regions where anthropogenic pressures are high and where species extinctions remain unaccounted for because species simply remain undocumented.

Acknowledgements

Fieldwork was supported by grants from the University of Rouen, SCALE Research Federation, French Embassy at Libreville ("Service de Coopération et d'Action Culturelle") and IRD ("Action Thématique Structurante" call). Logistical support was provided by ANPN, the National Agency for National Parks (Libreville, Gabon), WCS (Libreville, Gabon) and CEDAMM (National Park, Lopé, Gabon), Research Station on Gorilla and Chimpanzee (SEGC, Gabon), as well as "Agence des Universités Francophones" and the numerical campus of Libreville. Sequencing costs were covered by grants to PDNH from Genome Canada and from the Ontario Ministry of Research, Innovation and Science. Patrick Basquin (for Lasiocampidae) and Ugo Dall Asta (for Lymantriinae) provided taxonomic assistance. We also thank the institutions that made this project possible: CENAREST for research authorizations, IRET and IRAF (Libreville, Gabon), Science University of Masuku (Franceville, Gabon (Libreville, Gabon), CIRMF (Franceville, Gabon), University O. Bongo (Libreville, Gabon), and University of Douala (Cameroun).

The ECOTROP team is a multidisciplinary and international consortium of university teachers and researchers working together at organizing field classes in the domain of environmental and ecological research in the tropics. From 2011 to 2014, they coordinated four successive editions of a training course in biodiversity assessment in the North of the Lope National Park in Gabon, producing some of the results included in this paper. The following table gives the list of the members not already listed as authors on the manuscript:

Names	**Contact**
Emilie Arlette APINDA LEGNOUO	CENAREST, IRET, Libreville, Gabon. Email: ea.apindalegnouo@yahoo.fr
Diego AYALA	MIVEGEC, IRD, CRMF, Franceville, Gabon. Email: diego.ayala@ird.fr
Marlucia BONIFACIO MARTINS	Museu Paraense Emilio Goeldi, Belem, Para, Brazil. Email: marlucia@museu-goeldi.br
Laurent BREMOND	ISEM, Université de Montpellier, EPHE, IRD, CNRS, Montpellier, France. Email: laurent.bremond@umontpellier.fr
Barabara EVRARD	Normandie Univ, UNIROUEN, UNICAEN, CETAPS 76000 Rouen, France. Email: barbara.evrard@univ-rouen.fr
Damien FEMENIAS	Normandie Univ, UNIROUEN, UNICAEN, CETAPS 76000 Rouen, France. Email: Damien.Femenias@univ-rouen.fr
Flore KOUMBA PAMBO	CENAREST, IRAF, Libreville, Gabon. Email: csar_cenarest@yahoo.fr
Boris MAKANGA	CENAREST, IRET, Libreville, Gabon. Email: makanga.boris@gmail.com
Mike MAKAYA MVOUBOU	Université des Sciences et Techniques de Masuku, Franceville, Gabon. Email: makayamvoubou@yahoo.fr
Elise MAZEYRAC	WCS-Gabon, La Lopé, Gabon. Email: elise.mazeyrac@ntymail.com
Judicaël OBAME NKOGHE	CIRMF, Franceville, Gabon. Email: judicael.obame@live.fr
Marjolaine OKANGA-GUAY	LAGRAC, Université Omar Bongo, Libreville, Gabon. Email: m_okanga_guay@yahoo.fr

Didier OREVOUNO	Ecole Nationale des Eaux et Forêts, Libreville, Gabon. Email: orevouno2001@yahoo.fr
Johan OSZWALD	LETG, CNRS, Université Rennes 2, Rennes, France. Email: johan.oszwald@univ-rennes2.fr
Christophe PAUPY	MIVEGEC, IRD, CNRS, Université de Montpellier, Montpellier, France. Email: christophe.paupy@ird.fr
Olivia SCHOLTZ	WCS-Gabon, Libreville, Gabon. Email: Olivia_scholtz@hotmail.com
Maurice TINDO	Université de Douala, Cameroun. Email: tindodouala@yahoo.com
Michel VEUILLE	ISYEB, CNRS, MNHN, UPMC, EPHE, Paris, France. Email: veuille@mnhn.fr

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Figure Captions

Figure 1. Location of the study sites; Dark grey areas on the upper right map represent National Parks in Gabon. The *Ipassa* site is located near Ivindo.

Figure 2. Photos of the two study sites and sampling methods: A) View of the savannah-forest patchwork in Lopé National Park, showing the position of the light trap (*Lopé 2*, November 2009); B) Rainforests at *Ipassa* research station at the edge of the Ivindo river (November 2009); C) Light trapping at *Lopé 2* in March 2011; D) Tissue sampling for DNA barcoding during the ECOTROP field class in March 2011.

Figure 3. Diversity and composition of the macro-moth sample at the two locations (*Lopé 2* and *Ipassa*): the circular phylogram represents the results of a Neighbor Joining analysis in BOLD of 3,494 COI sequences clustering into 1,385 BINs; barcodes obtained for specimens from *Ipassa* are in green while those from *Lopé 2* are in grey. The pie chart represents the relative contribution (ordered) of the different families and sub-families (for Erebidae) of moths collected in the two sites; numbers within brackets indicate the number of BINs and number of specimens sampled, respectively.

Figure 4. Comparisons between the numbers of BINs observed in this study for 28 families and sub-families of macro-moths (dashed bars) and the numbers reported from Gabon in the AfroMoths online database (grey bars; De Prins and De Prins 2017).

Figure 5. Individual-, sample-, and coverage-based rarefaction and extrapolation curves for the two study sites and for two seasons at *Lopé 2* (DS: dry season, WS: wet season): A) Size-based rarefaction/extrapolation curves; B) Sample coverage plotted against the number of individuals; C) Coverage-based rarefaction/extrapolation (rarefaction curves are represented in solid lines,

extrapolation curves in dashed lines; shaded areas represent a 95% confidence intervals based on a bootstrap method with 200 replications).

720 **Tables**

721 **Table 1.** Number of individuals and number of BINs collected for different families/sub-families
722 of macro-moths at the two study sites and for two seasons at *Lopé 2*, and number of species listed
723 in the AfroMoths online database (grey bars; De Prins and De Prins 2017) for the same
724 families/sub-families (WS= wet season; DS= dry season).

	Ipassa (WS)		Lopé (WS)		Lopé (DS)		Lopé		Total	
	# ind	# BINs	# ind	# BINs	# ind	# BINs	# ind	# BINs	# ind	# BINs
Bombycidae	2	2							2	2
Brahmaeidae	2	1	2	1			2	1	4	1
Cossidae	6	5	5	4	8	4	13	8	19	11
Crambidae	17	14	73	31	19	16	92	43	109	52
Drepanidae	6	4			1	1	1	1	7	5
Erebidae (Arctiinae)	198	71	131	41	75	37	206	65	404	113
Erebidae (Erebinae)	75	38	71	24	47	34	118	46	193	72
Erebidae (Lymantriinae)	220	103	47	32	79	54	126	73	346	164
Other Erebidae	61	33	102	18	22	18	124	32	185	60
Eriocottidae			2	2			2	2	2	2
Eupterotidae	13	10	22	3	5	3	27	5	40	15
Euteliidae			1	1	4	2	5	2	5	2
Geometridae	293	153	130	63	117	62	247	107	540	220
Lasiocampidae	88	55	80	42	74	36	154	61	242	101
Lecithoceridae			1	1	2	1	3	2	3	2
Limacodidae	31	15	20	14	8	7	28	18	59	30
Noctuidae	199	125	103	66	95	69	198	124	397	224
Nolidae			2	2			2	2	2	2
Notodontidae	155	77	72	31	45	27	117	49	272	104
Psychidae	1	1	2	2	6	4	8	4	9	5

Pyralidae	65	29	82	37	25	18	107	49	172	70
Saturniidae	62	32	79	31	36	9	115	33	177	43
Sphingidae	98	44	58	28	111	40	169	47	267	66
Thyrididae	4	4	18	1			18	1	22	5
Tineidae			2	1			2	1	2	1
Tortricidae	5	4			1	1	1	1	6	5
Uraniidae			1	1			1	1	1	1
Zygaenidae	1	1					0		1	1
Not identified	2	2	4	4			4	4	6	6
Total	***1604***	***823***	***1110***	***481***	***780***	***443***	***1890***	***782***	***3494***	***1385***

727 **Table 2.** Summary of macro-moth data sets collected at the two study sites and for two seasons in
728 *Lopé 2* (numbers in parentheses represent the 95% confidence intervals based on a bootstrap
729 method with 200 replications).

	Ipassa (WS)	**Lopé (WS)**	**Lopé (DS)**	**Lopé**
Number of indiduals collected	1604	1110	780	1890
Observed richness	823	481	443	782
Proportion of singletons (%)	63.85	63.61	64.93	59.31
Sampling coverage (%)	67.32 (± 2.95)	72.44 (± 2.27)	63.14 (± 3.02)	75.54 (± 1.77)
Richness at constant sampling coverage of 50%	469.2 (± 13.8)	197.6 (± 7.0)	313.7 (± 12.9)	330.4 (± 7.6)
Richness at constant sampling intensity of 1000 indiv.	599.1 (± 8.9)	449.9 (± 4.4)	511.4 (± 25.6)	521.5 (± 9.5)
Chao1 estimated richness	1837.0 (± 130.6)	1011.6 (± 107.9)	869.5 (± 70.9)	1513.4 (± 96.5)
ACE estimated richness	1849.4 (± 27.6)	1120.6 (± 20.7)	1054.4 (± 22.9)	1629.5 (± 26.2)
First order jackniffe estimated richness	1269.2 (± 286.2)	728.5 (± 169.7)	663.7 (± 166.9)	1211.4 (± 197.0)
Fisher alpha	678.3 (± 36.5)	318.2 (± 21.0)	419.3 (± 32.3)	491.5 (± 25.0)

730

Table 3. Comparison of macro-moth species assemblages between the two study sites and for two seasons in *Lopé 2* showing the Sørensen index of dissimilitude (with singletons removed or not from the dataset) and its partitioning into geographical/seasonal turnover and nestedness.

	Sørensen	**Turnover (%)**	**Nestedness (%)**
Ipassa vs Lopé 2	0.75	70.97	29.03
Same without singletons	0.40	67.57	32.43
Wet vs dry season (Lopé 2)	0.69	95.39	4.61
Same without singletons	0.23	54.76	45.24

Captions for Supplementary materials

gen-2018-0063.R1Suppla. Neighbour Joining (NJ) tree based on K2P distances for the Sphingidae and Saturniidae moths collected in *Ipassa* (terminals labelled as 'makokou' in the tree) and *Lopé 2* (labelled as 'La Lope'). The tree was produced with records in BOLD dataset DS-LOPELEP1 using BOLD-alignment and default settings.

gen-2018-0063.R1Supplb. Images of specimens in NJ tree of gen-2018-0063.R1Suppla; numbers of images correspond to numbers of terminals in gen-2018-0063.R1Suppla tree.

gen-2018-0063.R1Supplc. Rank-abundance diagrams for *Ipassa* (left panels) and *Lopé 2* (right panels): (A) and (C) represent Fisher's logseries functions fitted on abundance data; (B) and (D) represent Preston's lognormal (red lines) and truncated lognormal (blue lines) models.