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Diversity of Diptera in Bohol Island, Philippines, using DNA barcodes

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Abstract

Studying island systems offers a great advantage to look into diversity of organisms because this provides opportunity for both dispersal, isolation mechanisms and key to conservation. The study was conducted to determine the diversity of true flies in Bohol, Island, Philippines. Malaise traps were used to collect samples from different habitats. A total of 94 Molecular Operational Taxonomic Units (MOTUs) clustering at 3% threshold were identified. The result showed a diversity index of H'=3.70. Observed diversity may be influenced by availability of heterogeneous habitats present between sampling sites and longer period of sampling. Sixty-nine (69) presumptive Diptera species belonging to eight (8) families were identified in Bohol. In addition, family Mycetophilidae showed the highest number of species in Bohol at 49.28% of the total species. Moreover, DNA barcodes showed presumptive species belonging to eight families of Diptera were found only in Magsaysay Park, Bohol. These include: Culicidae, Dolichopodidae, Empididae, Mycetophilidae, Sepsidae, Stratiomyidae, Tabanidae, and Tephritidae. This is possibly due to the unique characteristic of habitat in Magsaysay Park, Bohol, hence, a key site for conservation.

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Introduction

Island systems offer advantages for evolutionary and diversity studies owing to unique properties which cater adaptive radiations and high level of endemism in the area (Ricklefs and Bermingham, 2007; Sanchez-Gonzalez *et al.,* 2015). The Philippines as an archipelago provides a good simplified natural model to look into natural populations and test for diversity and genetic differentiation in continuous habitats within islands. As an archipelago, it is of oceanic in origin and was believed to have originated as a set of *de novo* oceanic islands in the early Oligocene. The archipelago provided opportunities for both dispersal and isolation of populations since, it has been subjected to climatic and sea-level changes in the Pleistocene that somehow produced cycles of isolation and aggregation of islands into larger landmasses (Hall, 1998; Esselstyn *et al.*, 2010). One of the prominent islands in the Philippines is the island of Bohol which is considered as the tenth largest island in the Philippines and the main land is surrounded about by 70 smaller islands. The terrain composition is basically rolly and hilly and about half the island is covered in limestone. Its famous chocolate hills are made of limestone left over from coral reefs during the Ice Age period when the island was submerged. The outer areas of the island are low mountain ranges and the interior is a large plateau with irregular landforms. Numerous waterfalls and caves are scattered across the island. Creeks, springs and rivers are present as well (Villegas, 2003).

Herewith, this offers a great advantage to look into diversity of organisms because this provides opportunity for both dispersal and isolation mechanisms. In this respect, the order Diptera is considered as one of the most ecologically diverse groups of insects containing 157,000 described extant species (Thompson, 2008; Inclan and Stireman, 2011). Estimates of the total diversity of Diptera suggest that this order contains a minimum of 1,000,000 to 1,700,000 species (Stork, 1997), indicating that 90% of Diptera species remain undescribed taxonomically (Inclan and Stireman, 2011). To unravel such diversity, it has come to attention the so called 'messy species groups' and 'cryptic' species which are difficult to differentiate based on morphology alone (Bickford *et. al.*, 2007; Ang *et al.*, 2008). As such, the multi-pronged approach of integrative taxonomy is recommended because it has been demonstrated in various studies that 'messy' or cryptic species could be discovered once DNA sequence data become available (Ang *et al.*, 2008) hence, the importance of this study.

Moreover, DNA barcoding has been widely used in species identification and biodiversity research because it has been shown that in many groups, including insects, interspecific variation in DNA sequences of some genes is much higher than intraspecific variation and this provided an opportunity to use DNA sequences for species identification.

The gene region that has been proposed as the standard barcode for identifying flies is a 313bp subsection of the original 648 base-pair region of the mitochondrial cytochrome c oxidase 1 gene ("COI") that is designated as the barcode. COI is proving highly effective in identifying flies and many other animal groups. Once a database of DNA sequences of all species has been developed, specimens can be identified by sequencing their COI sequences and matching them to the database (Hebert *et al.*, 2003). Apart from identification, DNA barcodes can also be used as a tool to pre-sort specimens into alleged species (Lee, 2016). COI barcodes are also important for discovering cryptic, closely related and morphologically similar species. In these cases, DNA sequences are particularly useful for clarifying species boundaries because the sequences are not affected by the environmental variables (Tan, 2010) and barcode is required for morphological research (Scotland *et al.*, 2003; Vogler and Monaghan, 2007). Noteworthy, is that DNA barcode sequences can be used to group specimens into Molecular Operational Taxonomic Units (MOTUs), which function as species proxies, and are especially useful for many downstream applications like investigating site diversity or distribution (Ryberg, 2015). While many species

delimitation techniques based on DNA barcoding exist, the most straightforward one is objective clustering, which groups sequences into units based on a specified threshold of uncorrected pairwise distances between sequences. These MOTUs not only provide species approximations, but also pre-sort specimens such that the rate of taxonomic identification and description could be greatly accelerated (Lee, 2016). Molecular analysis has the advantage of revealing patterns of regional genetic divergence and allowing biodiversity comparisons at larger geographic and taxonomic scales and even enable the estimation of faunal overlap with other countries (Ashfaq *et al.,* 2017).

In this respect, this study determined species diversity via DNA barcoding of Diptera in the Island of Bohol, Philippines.

Information obtained may also aide in the development of tailor-fit conservation programs for important species.

Materials and methods

Sampling area

The study was conducted in Bohol Island, Philippines: Magsaysay Park, Bilar, Bohol- Trap 1 (9.70431°N, 124.1239°E); Trap 2 (9.70359°N, 124.1252°E) from June to August 2016 (Fig. 1).

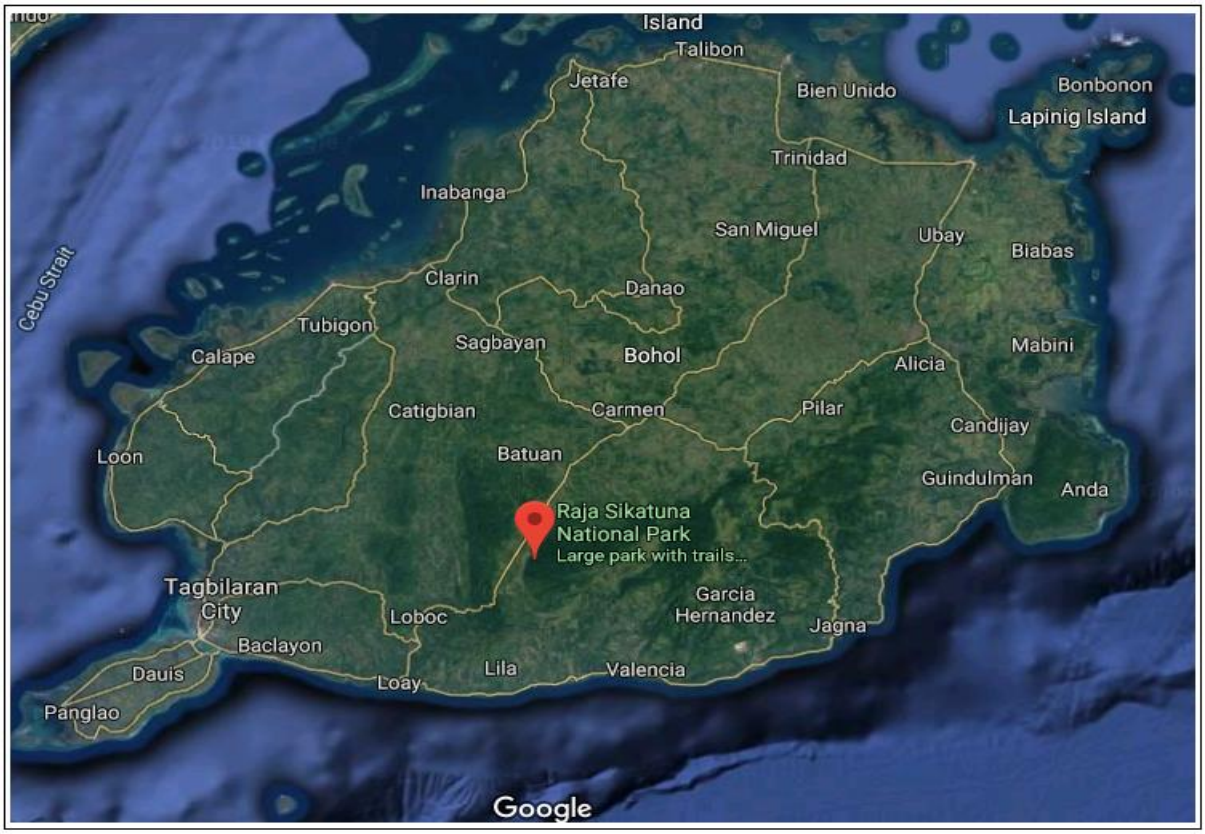

Fig. 1. Map of Bohol Island: showing the sampling site (Magsaysay Park/Rajah Sikatuna National Park) source: google maps.

Collection and Pre-sorting of samples

Malaise traps were deployed in areas of minimal disturbance. One or two traps were set at 20-100m intervals in each location and placed for one to three months depending on the location. Traps were open during the entire sampling period and bottles containing 70% unmethylated ethanol alcohol were changed every week.

Collected specimens were sorted into various families and placed in separate vials with screw caps then labelled and coded for molecular work. Sorting of specimens was based on *"The Families of Diptera of the Malay Archipelago"* (Oosterbroek, 1998) and

verified by taxon-specific experts from the National University of Singapore (NUS).

Molecular analysis

All sorted samples were processed using direct-PCR (Wong *et al.,* 2014; Lee, 2016). A portion of a leg was cut from each specimen and placed into the well, with the tissue submerged in the reaction mixture. DNA leaching out from the tissues provided the starting template for further DNA amplification (Table 1). PCR conditions were set based on specimen family: Family Dolichopodidae, its initial denaturation at 95°C (3 mins); 1 cycle of 94°C (1 min), annealing 47°C (1 min), and 72°C (1 min), followed by 40 cycles; Final extension at 72°C (5 mins). For other families e.g. Families Mycetophilidae, Culicidae and Stratiomyidae, initial denaturation at 94°C (5 mins); 1 cycle of 94°C (1 min), annealing 47°C (2 mins) and 1 hr for mixed Diptera, and 72°C (1 min), followed by 35 cycles; Final extension at 72°C (5 mins). PCR products were then purified by Bioline's Sure Clean, according to the manufacturer's instructions.

Next generation sequencing

Next-Generation Sequencing PCR products were then quantified in equimolar ratios, and pooled before being sent for library preparation and Next-Generation Sequencing, using the Illumina MiSeq and HiSeq 2500 sequencing platforms. Sequencing libraries were prepared by AITbiotech, using the TruSeq Nano DNA Library Preparation Kit (Illumina), according to the manufacturer's protocol. Illumina MiSeq runs were provided by AITbiotech with the use of MiSeq Reagent Kit v3 (2 X 300 bp read lengths) and HiSeq runs were provided by SCELSE with HiSeq 2500 System and Rapid SBS Kit v2 (2 X 250 bp read lengths).

Digital reference collections

A digital reference collection is a physical reference collection of specimen and is used for taxa that need to be identified routinely by biologists from different backgrounds and for up-to-date identification tools for dipterans, thus, confirming identifications by comparing undetermined specimens with specimens that have been identified by taxonomic experts (Ang *et al.,* 2012). All images generated were archived in an image database hosted at NUS.

Specimen images were taken using Dun Inc. Passport II imaging system (using a 65mm MPE lens) and processed via Adobe light room. Images at different focal lengths were taken and then compiled into a fully resolved image using Zerene Stacker, and then digitally processed for publication using Photoshop CS5.

Data analysis

Data analysis pipeline was performed according to Meier *et al.* (2016). Paired-end reads were merged using PEAR 0.9.6 (Zhang *et al.*, 2014). The reads from each PCR product were assigned to their corresponding specimen using the uniquely labelled primer pair combinations, and the dominant read was identified as the specimen barcode. This was done using a Python script (Srivathsan and Meier, 2012) in order to demultiplex the data, count the number of reads per sample, identify and group identical reads of these amplicons into sets, identify the dominant set of reads and combine it with otherwise identical length-variants, and counting the number of reads in the largest identity set, and comparing it with the count of the set with the second-highest number of reads (Meier *et al.,* 2016).

As a means of quality control, barcoding of a particular sample was only considered to be successful if (i) the total read count was > 50×, (ii) the total barcode count was > 10×, and (iii) and the most dominant read was at least five times that of the second most dominant read (Meier *et al.*, 2016).

Quality control is essential to ensure that individual barcodes had sufficient coverage (> 50×) and were not simply the result of experimental error (e.g. sequencing primer dimers or random DNA fragments), as well as to reject dominant sequences resulting from PCR amplification error (if PCR amplification error occurs early in the PCR cycles, the first and second most dominant read will be more

similar proportionally). Basic Local Alignment Search Tool (BLAST) was used to search for sequences that were matching (> 97% identity) to taxa.

This was done to identify contaminated sequences from the dataset. Post-QC sequences were then aligned using the online version of MAFFT v7, adjusting the direction of nucleotide sequences according to the first sequence (Katoh and Standley, 2013).

Alignment was also checked for stop codons in MEGA version 6 (Tamura *et al.,* 2013), with appropriate gaps added at the beginning of the sequences to account for different sequence length.

Another Python script was used to cluster sequences using uncorrected pairwise distances, at varying threshold levels from 0% – 10% (Meier *et al.,* 2008; Srivathsan and Meier, 2012).

Results and discussion

Faunistic composition

A total of 367 samples were successfully sequenced, yielding 75.61% (248/328) sequence recovery rate from Bohol samples. In addition, a total of 94 MOTUs were identified at 3% threshold (Table 2 and 3).

Table 1. List of reagents (and quantities) for the different groups of specimens in direct-PCR reaction.

Reagent	Dolichopodidae Specimen		Mycetophilidae, Culicidae and Stratiomyidae Specimen		Mixed Diptera Specimen	
	Per rxn	MM (x53)	Per rxn	MM (x53)	Per rxn	MM (x53)
H_2O	13.0	689	10.3	535.6	13.3	691.6
Buffer	2.0	106	2.0	104	2.0	104
dNTPs	1.5	79.5	1.5	78	1.5	78
F primer	1.0	53	2.0	104	1.0	52
BSA	1.25	66.25	2.0	104	1.0	52
Taq	0.25	13.25	0.2	10.4	0.2	10.4

Eight families of Diptera were identified morphologically and based on the molecular data these include: Dolichopodidae, Culicidae, Stratiomyidae, Tephritidae, Empididae, Mycetophilidae, Tabanidae and Sepsidae. Other Families identified but not included in the analysis were: Tachinidae, Asilidae, Xylomyidae, Platystomatidae, Drosophilidae, Ephydridae, Chloropidae, Rhajionidae and Rhiniidae. The specimen sequence success rate of DNA samples recovered in DNA barcoding workflow can be associated with various factors, such as failure to amplify and sequence degraded DNA, the method of preservation, and the age of samples (Hajibabaei and McKenna, 2012). For the purpose of this study, a clustering threshold of 3% was chosen as this threshold has been widely used for species delimitation in the literature (Hebert *et al.,* 2003; Greve *et al.,* 2012).

Species diversity based on MOTUs

All specimens in this study were assigned interim names (presumptive species, also called MOTUs) in order to perform species diversity analysis. There were sixty-nine (69) presumptive Diptera species were identified in Bohol based on the number of MOTUs at 3% clustering threshold. In addition, the family Mycetophilidae showed the highest number of species at 49.28% total species, respectively.

Moreover, diversity of presumptive species of Diptera is high in Bohol (H'=3.70) and possibly due to habitat heterogeneity, or perhaps because Bohol sampling locality was relatively an undisturbed rainforest.

Table 2. COI sequence recovery success rate in Diptera from Bohol Island, Philippines.

Sampling Location	Total No. of Specimen	Total No. of Successfully Sequenced	No. of MOTUs at different Clustering threshold		
			3%	4%	5%
Bohol	328	248	94	92	92

The observed dominance (D=0.04) of the presumptive Diptera species belonging to families Mycetophilidae (R.A=9.85%) and Stratiomyidae (R.A=8.37%), implies that possible habitat heterogeneity between sampling sites plays a role, and subsequently contributed in the evenness distribution of species found in Bohol (Didham, 1996; Tews *et al.*, 2004; Novotny *et al.*, 2006). Moreover, the accumulation curve of Bohol showed a defined plateau (Fig. 2) suggesting the rich abundance of species in the area. Magsaysay Park in Bohol, offer environmental differences such as the type of microhabitats for dipterans (Thompson, 2003). In addition, 87.5% of the presumptive species belonging to the eight families of diptera identified were found only in Magsaysay Park, Bohol.

Table 3. Diversity indices of Diptera found in Bohol Island, Philippines based on the number of MOTUs.

Biodiversity Indices	Sampling Sites
	Bohol
Taxa_*S*	69
Total No. of Individuals	203
Dominance_D	0.04
Shannon_*H*	3.70
Evenness_*e^H/S*	0.58

The sampling site in Magsaysay park is part of Bilar Peak and is situated in Rajah Sikatuna Protected Landscape of Bilar town in the island-province of Bohol. The Park covers 9,000 hectares of karst forest with old-growth trees and undergrowth (Ella, 2017).

In Magsaysay Park, the malaise traps were deployed in a closed canopy area because it has been reported that the canopy cover influences the species richness and composition of understory plants (Siefert, 2005). Subsequently, it affects the species composition of Diptera in the said park of Bohol (Ivković *et al.*, 2015).

The distribution of mycetophilid species has been reported to be influenced by the characteristics of the habitat (Økland, 1994). In addition, species diversity of Mycetophilidae was reported higher at the undisturbed sites, and some species are dependent on the availability of wood-growing fungi and on decaying wood (Jakovlev *et al.*, 2008).

Furthermore, species from family Dolichopodidae develops in various habitats and is often cursorial on foliage, tree trunks, mud flats, and river rocks and abundant in warm moist habitats. Adults are predators on soft-bodied invertebrates while the larvae are found in soil, rotted vegetation, mud, under bark, and tree holes (Bickel, 2013). These accounted differences in habitat preferences and the possible effects of disturbances of adults and larvae may have influenced the distribution of these two dipteran families (Borkent, 1981; Guernaoui and Boumezzough, 2009). Their presence and abundance in Magsaysay Park are expected since the area is relatively undisturbed by anthropogenic activities. Important species were recommended for conservation. In this respect, important species were identified effectively through DNA barcodes and aide in conservation strategies.

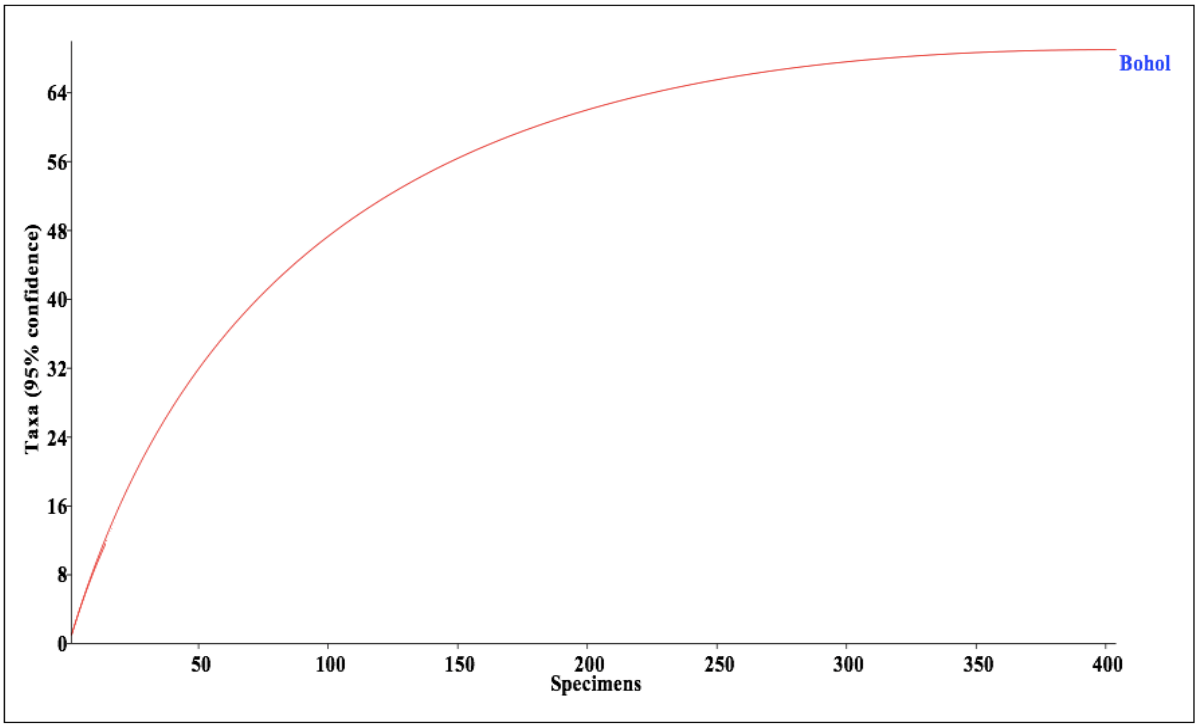

Fig. 2. Species accumulation curve for Bohol Island, Philippines generated by PAST v2.17c.

Conclusion

The utility of DNA barcoding provides another way to estimate diversity based on the number of MOTUs at different clustering threshold values. The MOTUs at 3% threshold identified eight (8) families of Diptera that complements with the morphology-based and molecular approach. The diversity of presumptive species of Diptera in Bohol tend to be influenced by the heterogenous microhabitats. Most of the presumptive species collected were found only in Magsaysay Park, Bohol, hence, the place should serve as a conservation site.

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References

Ang Y, Lim GS, Meier R. 2008. Morphology and DNA sequences confirm the first Neotropical record for the Holarctic sepsid species Themira leachi (Meigen)(Diptera: Sepsidae). Zootaxa **1933,** 63-65.

Ang Y, Puniamoorthy J, Pont AC, Bartak M, Blanckenhorn WU, Eberhard WG, Meier R. 2013. A plea for digital reference collections and other science-based digitization initiatives in taxonomy: Sepsidnet as exemplar. Systematic Entomology **38,** 637-644. http://dx.doi.org/10.1111/syen.120.15

Ashfaq M, Akhtar S, Rafi MA, Mansoor S, Hebert PD. 2017. Mapping global biodiversity connections with DNA barcodes: Lepidoptera of Pakistan. PloS one **12,** e0174749. http://dx.doi.org/10.1371/journal.pone.0174.749

Bickel DJ. 2013. The family Dolichopodidae (Diptera) of the Pilbara region, Western Australia in its Australasian biogeographic context, with the description of 19 new species. Records of the Western Australian Museum **83,** 291-348. http://dx.doi.org/10.18195/issn.0313122x.83.2013.291-348

Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K., Ngram KK, Das I. 2007. Cryptic species as a window on diversity and

conservation. Trends in ecology & evolution **22**, 148-155.
http://dx.doi.org/10.1016/j.tree.2006.11.004

Borkent A. 1981. The distribution and habitat preferences of the Chaoboridae (Culicomorpha: Diptera) of the Holarctic region. Canadian Journal of Zoology **59,** 122-133.
http://dx.doi.org/10.1139/z81-019

Didham RK, Ghazoul J, Stork NE, Davis AJ. 1996. Insects in fragmented forests: a functional approach. Trends in Ecology & Evolution **11**, 255-260.
http://dx.doi.org/10.1016/0169-5347(96)20047-3

Ella DS. 2017. PEAKWARE: World Mountain Encyclopedia. Date accessed: April 27.

Esselstyn JA, Oliveros CH, Moyle RG, Peterson AT, McGuire JA, Brown RM. 2010. Integrating phylo- genetic and taxonomic evidence illuminates complex biogeographic patterns along Huxley's modifica- tion of Wallace's Line. Journal of Biogeography **37,** 2054–2066.
http://dx.doi.org/10.1111/j.1365-2699.2010.02378.x

Greve C, Gimnich F, Hutterer R, Misof B, Haase M. 2012. Radiating on oceanic islands: patterns and processes of speciation in the land snail genus Theba (Risso 1826). PLoS One, *7*e34339.
http://dx.doi.org/10.1371/journal.pone.0034339

Guernaoui S, Boumezzough A. 2009. Habitat preferences of phlebotomine sand flies (Diptera: Psychodidae) in southwestern Morocco. Journal of medical entomology **46,** 1187-1194.
http://dx.doi.org/10.1603/033.046.0529

Hajibabaei M, McKenna C. 2012. DNA mini-barcodes. In *DNA barcodes*. Humana Press, Totowa, NJ. p 339-353.
http://dx.doi.org/10.1007/978-1-61779-591-6_15

Hall R. 1998. The plate tectonics of Cenozoic SE Asia and the distribution of land and sea. Pp. 99–132 *in* R. Hall and J. D. Holloway, eds., Biogeography and geological evolution of SE Asia. Backhuys Publishers, Leiden, p 417.

Hebert PD, Cywinska A, Ball SL, Dewaard JR. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences **270,** 313-321.
http://dx.doi.org/10.1098/rspb.2002.2218

Inclan DJ, Stireman III JO. 2011. Tachinid (Diptera: Tachinidae) parasitoid diversity and temporal abundance at a single site in the northeastern United States. Annals of the Entomological Society of America **104,** 287-296.
http://dx.doi.org/10.1603/AN10047

Ivković M, Miliša M, Baranov V, Mihaljević Z. 2015. Environmental drivers of biotic traits and phenology patterns of Diptera assemblages in karst springs: The role of canopy uncovered. Limnologica **54,** 44-57.
http://dx.doi.org/10.1016/j.limno.2015.09.001

Jakovlev J, Kjærandsen J, Viklund B. 2008. Fungus gnats (Diptera: Bolitophilidae, Diadocidiidae, Ditomyiidae, Keroplatidae & Mycetophilidae) from Tyresta National Park and Nature Reserve in Sweden. Sahlbergia **14,** 29-52.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular biology and evolution **30,** 772-780.
http://dx.doi.org/10.1093/molbev/mst010

Lee T, Ming T. 2016. Next-Gen barcoding reveals overwhelming species diversity in two insect groups: Implications for morphology-based taxonomy (Thesis). Department of Biological Sciences National University of Singapore.

Meier R, Zhang G, Ali F. 2008. The use of mean instead of smallest interspecific distances exaggerates

the size of the "barcoding gap" and leads to misidentification. Systematic biology **57,** 809-813. http://dx.doi.org/10.1080/10635150802406343

Meier R, Wong W, Srivathsan A, Foo M. 2016. $1 DNA barcodes for reconstructing complex phenomes and finding rare species in specimen-rich samples. Cladistics **32,** 100-110. http://dx.doi.org/10.1111/cla.12115

Novotny V, Drozd P, Miller SE, Kulfan M, Janda M, Basset Y, Weiblen GD. 2006. Why are there so many species of herbivorous insects in tropical rainforests? Science **313,** 1115-1118. http://dx.doi.org/10.1126/science.1129237

Økland B. 1994. Mycetophilidae (Diptera), an insect group vulnerable to forestry practices? A comparison of clearcut, managed and semi-natural spruce forests in southern Norway. Biodiversity & Conservation **3,** 68-85. http://dx.doi.org/10.1007/BF00115334

Oosterbroek P. 1998. The families of Diptera of the Malay Archipelago **1,** Brill.

Ricklefs RE, Bermingham E. 2007. The causes of evolutionary radiations in archipelagoes: Passerine birds in the Lesser Antilles. The American Naturalist **169,** 285–297. http://dx.doi.org/10.1086/510730

Ryberg M. 2015. Molecular operational taxonomic units as approximations of species in the light of evolutionary models and empirical data from Fungi. Molecular ecology **24,** 5770-5777. http://dx.doi.org/10.1111/mec.13444

Sánchez-González LA, Navarro-Sigüenza AG, Krabbe NK, Fjeldså J, García-Moreno J. 2015. Diversification in the Andes: the Atlapetes brush-finches. Zoologica Scripta **44,** 135-152. http://dx.doi.org/10.1111/zsc.12088

Scotland RW, Olmstead RG, Bennett JR. 2003. Phylogeny reconstruction: the role of morphology. Systematic Biology **52,** 539-548. http://dx.doi.org/10.1080/10635150390223613

Siefert A, Green T. 2005. Effects of changes in canopy cover on understory vegetation in the Long Island Pine Barrens. In *Brookhaven (NY):* Pine Barrens Research Forum.

Srivathsan A, Meier R. 2012. On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. Cladistics **28,** 190-194. http://dx.doi.org/10.1111/j.1096-0031.2011.00370.x

Stork NE. 1997. Measuring global biodiversity and its decline, pp. 41Ð 68. In M. L. Reaka-Kudla, D. E. Wilson, and E. O. Wilson [eds.], Biodiversity II: understanding and protecting our biological resources. Joseph Henry Press, Washington, DC.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution **30,** 2725-2729. http://dx.doi.org/10.1093/molbev/mst197

Tan DS, Ang Y, Lim GS, Ismail MRB, Meier R. 2010. From 'cryptic species' to integrative taxonomy: an iterative process involving DNA sequences, morphology, and behaviour leads to the resurrection of Sepsis pyrrhosoma (Sepsidae: Diptera). Zoologica Scripta **39,** 51-61. http://dx.doi.org/10.1111/j.1463-6409.2009.00408.x

Tews J, Brose U, Grimm V, Tielbörger K, Wichmann MC, Schwager M, Jeltsch F. 2004. Animal species diversity driven by habitat heterogeneity/diversity: the importance of keystone structures. Journal of biogeography **31,** 79-92. http://dx.doi.org/10.1046/j.03050270.2003.00994.x

Thompson FC. [Ed.]. 2008. Biosystematic database of world Diptera. Accessed on 12 November 2016). http://www.sel.barc.usda.gov/Diptera/biosys.htm

Villegas RN. ed. 2003. Tubod: the heart of Bohol. Manila: National Commission for Culture and the Arts. ISBN 9718140360.

Vogler AP, Monaghan MT. 2007. Recent advances in DNA taxonomy. Journal of Zoological Systematics and Evolutionary Research **45,** 1-10. http://dx.doi.org/10.1111/j.1439-0469.2006.00384.x

Wong WH, Tay YC, Puniamoorthy J, Balke M, Cranston PS, Meier R. 2014. Direct PCR'optimization yields a rapid, cost-effective, nondestructive and efficient method for obtaining DNA barcodes without DNA extraction. Molecular Ecology Resources **14,** 1271-1280. http://dx.doi.org/10.1111/1755-0998.12275

Zhang J, Kobert K, Flouri T, Stamatakis A. 2013. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics **30,** 614-620. http://dx.doi.org/10.1093/bioinformatics/btt593