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Species richness estimates of Blattodea s.s. (Insecta: Dictyoptera) from northern Guyana vary depending upon methods of species delimitation

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Abstract. Cockroaches (Order: Blattodea) comprise a taxon that, although very abundant in tropical forests, remains largely unstudied. Making sense of the diversity of species is a challenging task hindered by the large numbers of species and the abundance of cryptic or polymorphic forms. Here, we estimated species richness of cockroaches (s.s.) from northern Guyana while applying a method to deal with these confounding factors. We utilized two interpretations of abundance data, the first using only morphological information, and the second using both morphological and genetic barcode information. The two methods of species delimitation greatly influenced the resulting estimates of species richness. When incorporating genetic barcodes, our total species richness estimate decreased by 26%. Our results emphasize the importance of using independent datasets to delimit species boundaries and expert identification of specimens when possible.

Introduction

Describing diversity is fundamental to progress in taxonomy, conservation biology, ecological modelling and other fields. Unfortunately, measuring the total number of species of a particular taxon or the total number of species in an area is usually biased by species abundance patterns and sampling effort. There are numerous methods used to estimate the total number of species in an area (e.g. using distribution and abundance, species accumulation curves, species description curves or ecological models). Ultimately, however, these all depend on how species boundaries are defined and how species concepts are applied to problems.

Biology is built on a scaffolding of the concept of a 'species' but the delimitation of species boundaries is difficult. This is compounded by the fact that there are many definitions of a species. Some definitions may be crafted to reflect practicality of use (Mishler, 1985; Mallett, 1995), biological theory (Mayden, 1997; Mayr, 1942) or both in an attempt to balance these two ideals which are sometimes at odds (Sperling, 2003; De Queiroz, 2007). The problems with many species definitions are the result of the reality of biological complexity. For example, many kinds of morphological crypsis, intraspecific polymorphism and hybridization are culprits in confounding species concepts (Hebert *et al.*, 2004; DeSalle *et al.*, 2005; Kuchta *et al.*, 2009; Lumley & Sperling, 2010; Cerutti-Pereyra *et al.*, 2012). This can greatly influence our perceptions of diversity. However, one can achieve a higher quality of species delimitation by adopting a robust species concept, such as the phylogenetic species (Mishler, 1985; Mayden, 1997; De Queiroz, 2011), as the definition of use and apply it with multiple independent datasets (Lumley & Sperling, 2010).

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Diversity, ecology and species delimitation in cockroaches

Cockroaches (Order: Blattodea) currently comprise ∼4500 species (s.s.) plus ∼2700 termite species (Beccaloni & Eggleton, 2011). The known diversity of cockroaches is two orders of magnitude lower than that of the hyper-diverse insect orders such as Lepidoptera (>157 000 species described; Van Nieukerken *et al.*, 2011), or beetles (> 350 000 species described; Maddison, 2000). Yet, even modestly speciose taxa like the

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Blattodea present a 'taxonomic impediment', a problem where the amount of diversity and lack of taxonomists prevents us from describing species before their extinction (Giangrande, 2003). An additional complicating factor is the uneven taxonomic distribution of insect specialists. While Blattodea is a relatively small order there are fewer researchers studying them than other orders of similar size (e.g. dragonflies, which have national and international organizations devoted to their study, as well as an abundance of nonprofessional enthusiasts).

Cockroaches have not been commonly utilized in biodiversity studies, despite their ecological importance. They are large consumers of both plant and animal detritus (Bell *et al.*, 2007; D.A. Evangelista, M.M. Wilson and J.L. Ware, in preparation) and may represent the largest proportion of biomass among insects in tropical canopies (Basset, 2001). Some species are indicators of ecological variables (Fisk, 1983). Two major studies have been carried out which focus solely on the total regional diversity of cockroaches (Fisk, 1983; Wolda, 1983). Although other studies have included cockroaches in diversity samples (Paoletti *et al.*, 1991; Basset, 2001; Basset *et al.*, 2012), little focus has been given to this cryptic group of insects.

From all systematic perspectives these insects are inherently difficult to assess. Comparable individuals of closely related species may have highly conserved external morphology and thus may be difficult to distinguish. Individuals may be highly polymorphic over the course of development and adults are often significantly different from juveniles (Hebard, 1920; Rehn & Hebard, 1927). This is very important when considering that juveniles may comprise up to 90% of individuals in cockroach surveys (Fisk, 1983). Cockroaches also have high levels of developmental stochasticity, resulting in great variation in external spination, setation and coloration (Bell *et al.*, 2007; D.A. Evangelista, personal observation). Sexual dimorphism can also exaggerate male–female differences enough that the sexes may appear to be entirely different species (Hebard, 1920; Roth, 2003; Bell *et al.*, 2007). With the dearth of experts and keys in Blattodea many adults cannot be identified and certainly the identification of immatures is nearly impossible.

Genetic barcoding as an alternative to traditional identification

Genetic barcodes (cytochrome oxidase I or *COI* haplotypes) are useful pieces of information that can be used for both identifying (Hebert *et al.*, 2003, 2004) and defining the boundaries of species (Blaxter, 2004). Recent studies have shown that other genes can be equally or more effective in these roles (Dupuis *et al.*, 2012). Regardless, there may be an important role for barcoding in many applications (e.g. see – Hajibabaei *et al.*, 2007; Steele & Pires, 2011).

There are many criticisms of the process of barcoding. *COI* sequences (i.e. barcodes) may not track species lines because of the presence of pseudogenes (Song *et al.*, 2008), hybridization, introgression (Schmidt & Sperling, 2008) ancestral polymorphism and recent evolutionary divergence (Moritz & Cicero, 2004; DeSalle *et al.*, 2005). Finally, substitution rate seems to not be an inherited trait (Kumar, 2005; Yi, 2007). This lends support to criticisms against using rules defining species based on percentage differences in nucleotide substitutions, which have expectedly been demonstrated to be violated for many taxa (Cognato, 2006). These are certainly not inconsequential problems, but one solution to these issues is the use of multiple independent datasets to delimit species (Zhou *et al.*, 2007; Dupuis *et al.*, 2012).

These issues highlight the need for a different approach to the identification of species. Given this, we explore how species richness is affected by two methods of species delimitation: (1) defining morphological types based on overall similarity and the presence of shared monomorphic traits, (2) defining phylogenetic types using mitochondrial *COI* haplotypes and morphological groupings as a guide for delimiting species in the case of ambiguities. Using method (2), *COI* haplotypes will reconstruct a tree topology but taxa will be divided into species only with support from our morphological evidence (Fig. 1).

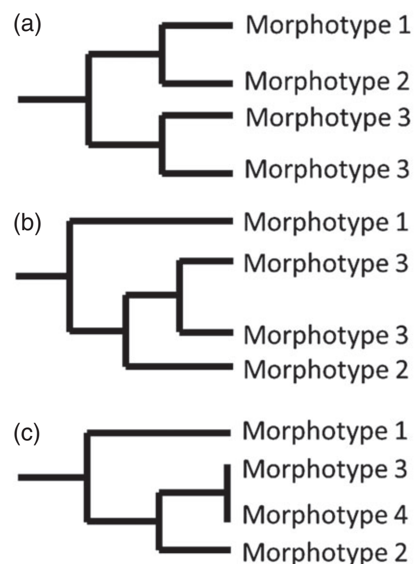

Fig. 1. These three trees partially exemplify how we analyze our tree. Tip labels indicate morphotype designations of each specimen. Branch lengths indicate genetic distance. In 1a and 1b, morphotype 3 is confirmed to be a valid species because both individuals' COI haplotype is most similar to that of its own morphotype. We also determine that morphotype 1 and 2 are separate species in both of these because they have both separate morphology and COI haplotypes. Part 1c shows that morphotypes 3 and 4 have no genetic difference between them. In this case we reexamine their morphology. For example, if all individuals of morphotype 3 are female and all individuals of morphotype 4 are male we will assume that these are actually the same species and were inappropriately split because of sexual dimorphism.

Methods

Specimen collection

We collected specimens from two sites in northern Guyana: CEIBA Biological Station in Madewini, Guyana (6°29′N, 58°13′W) and Kamuni River near Santa Mission, Guyana (6°33′N, 58°18′W). Along the Kamuni River, we sampled only from within bromeliads. At CEIBA we collected most specimens in cups baited with beer, light traps and bromeliads. We used the baited cups both as pitfall traps and to sample the canopy by tying them to tree trunks at various heights (0, 2, 4.8, 9.2, 13.8, 17 and 21.3 m). To supplement these methods, we performed manual and visual searches of the local environment and collected cockroaches by hand. We stored all specimens in 139-proof vodka (locally sold as 'High Wine') temporarily and then transferred to them to 70% ethanol in the lab. These specimens are temporarily stored in the Rutgers-Newark insect collection but are considered to be the ultimate property of the government of Guyana.

Morphological types

We defined our morphological types based on ∼5–10 standard external morphological characters (spination on anterior-ventral margin of fore-femur, sub-genital plate shape, frons coloration, cerci shape, overall body shape, overall body color, supra-anal plate morphology, pronotal shape and coloration). We chose these characters because they are variable, easy to discern, and used in other literature (P.M.A. Choate, unpublished data; Rehn & Hebard, 1927; Helfer, 1953). We first categorized all the types into general body forms and then further delimited them into specific morphotypes.

Species richness estimates

We used three methods to estimate total species richness: bootstrapping (100 000 replicates), bias corrected Chao-1 and the abundance-based coverage estimator (ACE). These were implemented on the software Mathematica v9.1 (Wolfram Research, Champaign, IL, U.S.A.). Bootstrapping is a random sampling of data that estimates the level of inherent bias. Total species richness estimates are obtained in this case by assuming that the difference between the resampling richness and the sample richness is equal to the difference between the sample richness and the total richness (Smith, 1984). Chao-1 is a nonparametric richness estimator that uses a modified ratio of singletons to doubletons as an estimate of the number of species unsampled (Chao, 1983). ACE is a similar nonparametric method that takes into account other abundance classes (Chao & Lee, 1992). We calculated cockroach richness using all metrics for the total dataset and for ecological subsamples (e.g. bromeliad fauna, leaf litter fauna and other fauna). We defined our dataset solely by morphological grouping and redefined it using congruent morphological and barcoding data, as explained below.

Within each morphotype we chose a few individuals for barcoding (at least four whenever possible). The individuals chosen were usually the same ones used to define morphological types and were in good condition. We attempted to sample different variants of the same types in order to allow the genetic data to recognize separate species if possible. Due to the volume of samples we did not genetically sample each individual. In using the morphotype variants as the base unit of variation, we are assuming that there is no variation within these groups. It is possible that this is not the case and that by genetically sampling all individuals we could uncover new diversity.

COI sequencing

For DNA extraction we used QIAGEN DNeasy extraction kits (Qiagen Group, Venlo, Limburg) and their standard tissue extraction protocol. Once extracted, we amplified the *COI* fragment using a nested PCR with primers and to minimize the probability of *COI* pseudo-genes being amplified and prevent artificially increasing our total number of species (Song *et al.*, 2008). We chose a 600 nucleotide length fragment of the *COI* mitochondrial gene as our barcode sequence. Our nested PCR used universal primers LCO and HCO followed by a PCR cycle using primers 1709 and 21921 (Simon *et al.*, 1994). Amplification of sequences was confirmed by gel electrophoresis. We only sequenced samples showing bands that were obviously more intense than the second band of shorter DNA to prevent amplifying the pseudo-gene region. We sent all amplified samples to MacroGen, NY for sequencing and used Sequencher (Gene Codes Corporation, Ann Arbor, MI, U.S.A.) for contig assembly and to resolve ambiguities.

We also used utilized other selected mantis, termite, and corydiid sequences as outgroup taxa, which were either sequenced using the above protocol or downloaded from Genbank.

Tree generation and evaluation

We compiled all sequences, aligned them with the software CLUSTALX2, and then manually refined the alignment in Mesquite (Maddison & Maddison, 2011). Sequences that were difficult to align or that seemed to have improbable structure (insertions/deletions not in multiples of three, or multiple stop codons) compared to the majority of other sequences were assumed to be pseudo-gene replicates and were excluded from the analysis. We recoded third codon position nucleotides as R-Y to decrease the probability of homoplasy affecting tree topology.

We then generated a maximum likelihood (ML) tree using GARLI v2.0 (Zwickl, 2006) that was the consensus of 500 bootstrap pseudo-replicates. The replicate trees were summarized to compose our final tree using DendroPy

(Sukumaran & Holder, 2010). We deposited all sequences in GenBank.

We used the resulting barcode tree in concordance with morphological data to determine congruence for support of 'true' species delimitations. A basic explanation of this process is given in Fig. 1 and further explained in the File S1.

Evaluating the two methods

We made an initial estimate of species richness using only morphological type information and then recalculated all richness metrics (as explained above) once we revised our list of relative species abundances with the barcoding data (Fig. 2).

Results

In total, 740 individuals were collected from the field. These were separated into 77 morphological types (Table 1 and File S1). Of these, we obtained and analysed sequences from 64 out of the 77 types. An example of the process used to revise our species list is given in Fig. 3. Revisions to the original list of species based on our tree are summarized in Table 1 and further explained in the File S1.

Sample richness was greatly affected by revising the type list with the data in our tree (Table 1 and Fig. 4). The entire dataset exhibited a 25% reduction in total species count and leaf litter taxa showed the greatest discrepancy among the subsets with a 22% reduction. This may indicate that leaf litter taxa may show polymorphism more often than other taxa; however, this should be explored further in future studies.

The estimates of total species richness showed similar trends. Total richness estimates using bootstrapping were 26% lower for the entire dataset, 20% lower for the leaf litter subset and 22% lower for the 'other' subset (Fig. 4). The differences in bootstrap total richness estimates are significant ($\alpha = 0.05$) for the full dataset and for the 'leaf litter' and 'other' subsets.

Figure 5 illustrates how the two methods of species delimitation can differentially affect separate methods of total species richness estimates. In particular, unbiased Chao-1 estimates of total richness were affected differently by the addition of genetic data. This stems from the different sensitivity of the Chao-1 metric to sample richness and species of different abundance classes.

Discussion

Delimiting species with two independent datasets

The *COI* was largely polytomous but was highly informative in revealing morphotype associations. Using ecological collection information we can see that some previously unassociated groups found in similar habitats are likely to be closely related taxa, if not the same species. This is true for the epilamprine morphotypes and their juvenile instars, which we were unable to associate to adults based solely on morphology. This was also true for unusual color morphs of the most common species, 'Blattodea sp. 1'.

Differing estimates of species richness

There was a significant difference in species richness estimates between the two methods. This was also true for two out of the three subdivisions of the data (Fig. 4).This shows that, without expert identification of specimens, i.e. without morphological expertise, richness estimates may have been erroneous. Even with expert identification, however, many of the originally incorrectly categorized specimens still may not have been associated with their proper morphotypes. This is particularly true with immatures as taxonomic literature is scarce with descriptions of juveniles, save for only the most common species (Rehn, 1903; Hebard, 1920).

By associating our morphotypes to one another or splitting morphotypes, we greatly affected the abundance profile of the data. Some abundant species became more abundant and the number of rare species was reduced. This is relevant because richness estimators (Chao-1 and ACE) are more sensitive to changes in the number of rare species than they are to the sample richness. Similarly, bootstrapping relies heavily on the abundance of the species from which it samples.

Ecological relevance of cockroach diversity

A literature review of the cockroach fauna of Guyana shows that 88 species have been recorded from the country (Evangelista *et al.*, unpublished data). This is roughly on a par with our projected richness of 91 (Fig. 4) for the fauna of our two northern sites. Our sampling does not reach any significant representation of the geographical heterogeneity of the larger region, although we did not attempt to quantify this. Given this, we would most certainly assume that the total diversity of the countries' fauna is greater than what has been recorded thus far, a result that would have been predicted based on general knowledge of neotropical diversity and lack of prior sampling of cockroaches in Guyana.

Conclusions

On the value of the morphotype

Other studies which primarily use morphotypes counts for richness estimates (e.g. Stuntz *et al.*, 2002; Stork & Grimbacher, 2006; Coddington *et al.*, 2009; Donoso *et al.*, 2010) may be adversely affected by problems associated with morpho-identification. Our results show that polymorphisms and variation in individuals create a potential of error in associating individuals to the correct types. If measuring β diversity, one can reduce these effects by keeping morphotype definitions and sampling conditions consistent across plots.

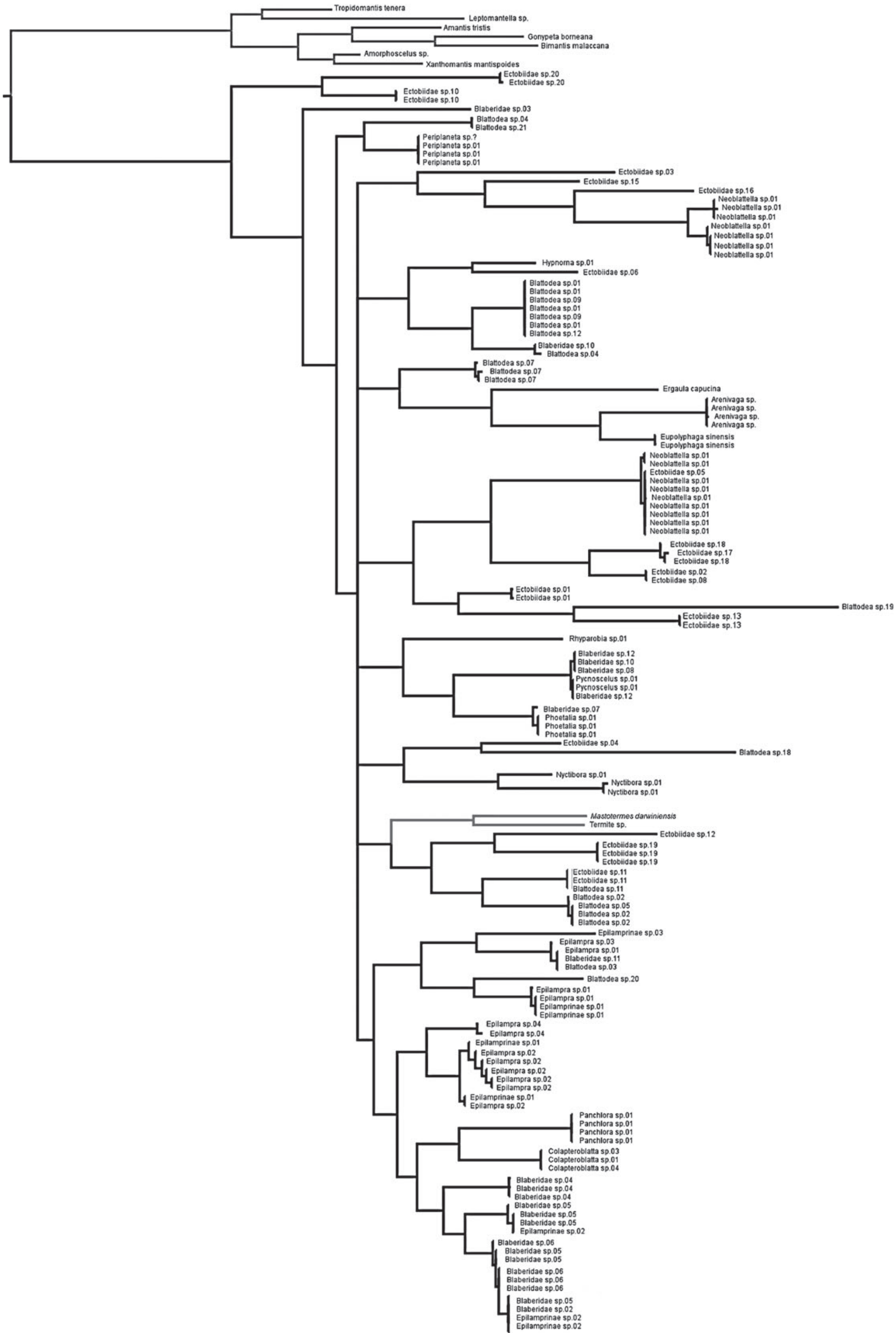

Fig. 2. Maximum likelihood tree of the COI gene extracted from the Blattodea of Guyana and other identified cockroach, termite and mantis specimens. Consensus of 500 bootstrap replicates.

Table 1. A summary of the abundance profile for the data on cockroaches collected from Guyana. This also shows subsections of the data divided by specimens found in any of three ecological habits. Abbreviations M – refer to a morphological interpretation of species, M + G – refers to an interpretation of species using both morphological and genetic data and Δ – refer to the difference between M and M + G.

				Number of Species in ecological division . . .		
	Species	Singletons	Doubletons	Bromeliad	Leaf litter	Other
M	77	33	13	17	32	44
M + G	58	23	7	16	25	37
Δ	19	10	6	1	7	7

M, morphological interpretation of species; M + G, interpretation of species using both morphological and genetic data; Δ, difference between M and M + G.

However, if a certain morph of a given species is differentially prevalent across sites then improper association of this morph could result in erroneous conclusions. Another way to avoid these pitfalls – without using genetic information – would be to only sample certain morphs of all species, as is sometimes done with ants (Longino *et al.*, 2002). However, this will severely reduce sample size in many cases and may fundamentally change the distribution of species abundances due to sex ratio skews or age stage biases. This would thereby affect species richness estimates. We intentionally did not eliminate any types from our sample.

One of the major problems with the genetic barcode comes from the presence or absence of the so-called 'barcode-gap'. The barcode-gap is the point where one can distinguish intraspecific variation from interspecific variation (Wiemers & Fiedler, 2007). We found a similar problem in finding the 'morphological-gap' when comparing among life stages and sexes. However this was due to the fact that we were unable to utilize genital morphology, which has been proven to be more effective in diagnosing taxa for the Blattodea (McKittrick, 1964; Grandcolas, 1996; Roth & Gutierrez, 1998; Klass & Meier, 2006). Indeed, from a systematic perspective, genital characters can be very useful in delimiting and defining closely related taxa and when considering only these characters, the 'morphological-gap' should be easier to identify. Yet, this was not useful for us because genitalia are effectively irrelevant in the association of juveniles to adults. Similarly, it is extremely difficult to make reliable associations of males to females using genital morphology.

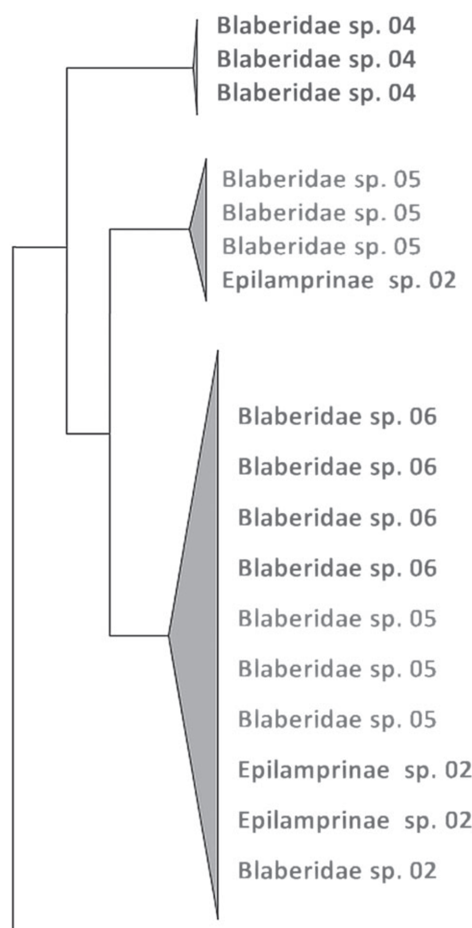

Fig. 3. As an example of the species delimitation process we include this clade of our ML tree showing the relationships between a few Blaberids from Guyana. If we start with the taxon Blaberidae sp. 5 as a morphological type we can see that specimens of this type have non-identical COI haplotypes, indicated by the branch length separating them. Then we see that these haplotypes cannot be grouped monophyletically. The next step would be to match the morphologies of the "alien taxa", in this case Blaberidae sp. 2, sp. 6 and Epilamprinae sp. 2, with the morphology of Blaberidae sp. 5. When looking at the morphologies we determined that the type Epilamprinae sp. 2 is the only winged morph. Blaberidae sp. 5 and 6 are indistinguishable except for the shape of the subgenital plate. Blaberidae sp. 2 is much smaller than all the other types but has significant morphological similarities with all other types, despite superficial dissimilarity. Therefore we determine that the alien taxa are of compatible types and therefore one species. Blaberidae sp. 04 is a much simpler case where we have non-identical COI haplotypes but it is possible to group them monophyletically.

How to delimit species

The current body of information about how many species there are in the various families of cockroaches (Beccaloni & Eggleton, 2011) is highly dependent on the subjectivity and limitations of taxonomists who described them. The literature is abundant with examples of authors expressing their loss at adequately describing groups (Rehn, 1903; Shelford, 1909, 1911; Rehn & Hebard, 1927). Homoplasy and pleisiomorphy can be greatly confounding. Species richness in an ecological context is different from species richness in a taxonomic context, but clearly there is a connection between the two. Although presented in an ecological context, we believe our study represents an attempt to independently verify where

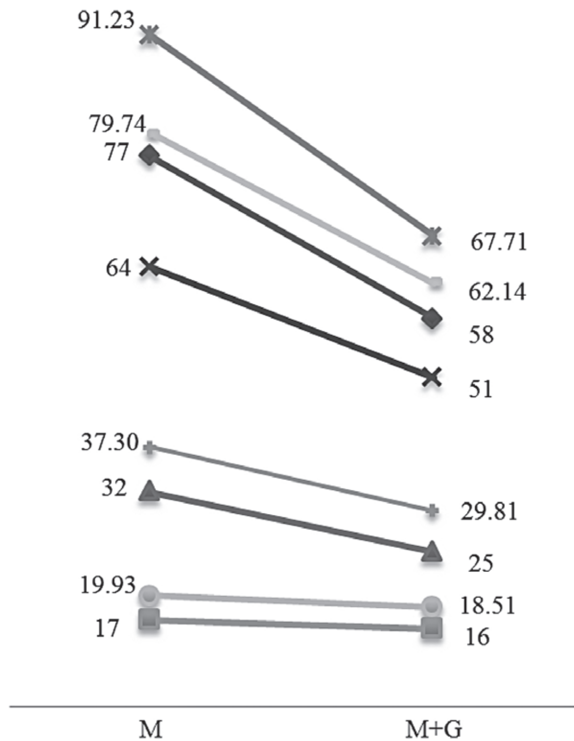

Fig. 4. We calculated the sample richness and total estimated richness of our sampled guyanese Blattodea and subsections of the data divided by microhabitat. We show the change in these calculations in using two interpretations of the data: M, morphological interpretation of species; M + G, interpretation of species using both morphological and genetic data. Sample richness of full set; Sample richness of bromeliad subset; Sample richness of leaf litter subset; Sample richness of 'other' subset.

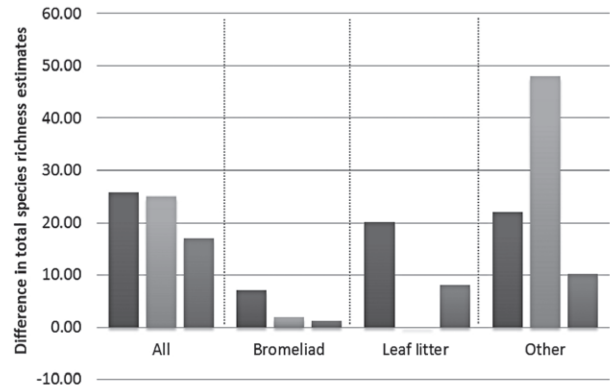

Fig. 5. Differences in estimates of total richness between both interpretations of the data (M and M + G) as compared between three methods of calculating richness (bootstrap, unbiased Chao-1, and ACE). This is shown for the full set of cockroaches collected and for three pseudo-replicate subsets divided by ecological realm from our site in Guyana. Because of unique taxon assemblages in various ecological realms, the effects of error in morphological type assignment may not vary uniformly, as can be seen here. ■ (left) Bootstrap; ■ (middle) Unbiased Chao-1; ■ (right) ACE.

species reside in evolutionary space using novel data. Although the morphological forms in this study were relatively limited when compared to the greater diversity of Blattodea or all insects, we exemplify a procedure that may prove useful when applied more widely.

It is true that the single mitochondrial barcode region may be insufficient to delimit species boundaries but this is true for many morphological markers as well. Clearly, having more independent data to verify species delimitation is better than having less, no matter if those data are genetic, morphological, ecological or behavioural (Moritz & Cicero, 2004; Schmidt & Sperling, 2008). Morphological divergence in genitalia can be direct evidence of secondary reinforcement; yet, in the case of species clines this may be as arbitrary as a genetic distance between lineages and, as mentioned previously, is only useful when looking at adults of a single sex.

We should not take species for granted as their definition is tenuous. If less stringent methods (e.g. single dataset, few characters) are used to define species these are subject to the tendency of the taxonomist for lumping or splitting taxa. Even if stringent methods (e.g. multiple independent datasets, many characters) are used, new geographic sampling may yield unexpected variability which may make for ambiguous cases. In truth, we can never know with absolute certainty what a species is, considering the probability of missing data or ongoing evolutionary novelty.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12043

Figure S1. Tree evaluation method. These two decision trees visually display the algorithm by which we delineated species. Part 1 starts using morphological groupings and part 2 starts using genetic groupings. The same outcome should be reached regardless of starting point. The line marked by * indicates a case where multiple morphotypes have identical COI sequences.
Part 1 shows how one would start evaluating species boundaries based on morphological groupings and part 2 shows how one would start evaluating species boundaries based on monophyletic groupings in the COI tree. If COI haplotypes are identical across multiple morphotypes we would follow the dotted line on Part 2. Following the process in this figure we started from part 1. It is also possible to start at part 2 but this seemed impractical in most cases.

Table S1. This table lists each morphotype (named for lowest taxonomic designation ascertained) their abundance and their relative ecologies. Ecologies were determined by collection method. Individuals collected in bromeliads are designated with "bromeliad" ecology. Individuals collected

in pitfall traps or manually collected in litter are designated "leaf litter species". Individuals collected in the canopy are designated as "canopy" species. Individuals collected using other methods are designated as "other".

Table S2. This table lists the updated list of types based on independent data from morphology and COI barcoding. Rows in bold indicate changes to original abundances because of association or splitting of groups. Numbers in the Δ column represent changes in abundance of that species due to association or splitting. Types with an abundance of zero are omitted.

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