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Decoding *Anotylus* (Thomson 1859) Beetle Diversity: DNA and External Morphology Match in Área de Conservación Guanacaste, Costa Rica

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Abstract: Taxonomy underpins biological research because names are needed for comparative analysis, conservation status, and public communication. Despite this, many species remain undescribed and are therefore vulnerable and unprotected, particularly in the tropics. Neotropical Staphylinidae beetles (Coleoptera) are among the insect groups most likely to contain significant unknown and/or cryptic diversity. Here we used an integrative taxonomic framework to conduct a preliminary review of one particularly diverse genus of Staphylinidae (*Anotylus*) in Área de Conservación Guanacaste, northwestern Costa Rica. We began by DNA barcoding novel collections and using Barcode Index Numbers (BINs) as an estimate of taxonomic diversity; we found 18 provisional new species. We augmented this genetic analysis with a morphometric analysis of adult morphological characters and found that we could differentiate most provisional species by external morphology as well as by elevation of collection. All the most abundant species could be differentiated from each other by differences in body size. One BIN included slight (~1%) genetic variation that corresponded with some morphological differentiation suggesting the existence of two species within a DNA BIN. Our results support the efficacy of DNA barcoding collections of tropical insects as an effective biodiversity estimator, one that can be used as a primer for integrative taxonomic studies using BINs as species hypotheses.

Keywords: biodiversity; cryptic species; conservation; BINs; DNA barcode

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1. Introduction

Only a small fraction of global diversity has been described [1]. There are approximately 2.3 million named species [2] from a total diversity that may be as low as 8 million [3] or as high as hundreds of millions [4]. Without knowing what species exist and where, we cannot effectively conserve them [5], manage ecosystem services [6], or monitor biodiversity loss [7], which, if unchecked, can result in dangerous ecological cascades [8]. While fundamental, describing new species is difficult, with many concepts [9], dated revisions [10], and an unfortunate lack of institutional and governmental support for taxonomists [11]. Quite simply, there are not enough trained taxonomists to characterize global biodiversity. This taxonomic impediment is even more alarming, given that we are in the midst of the sixth, and largely anthropogenic, mass extinction [12]. With many limitations to identifying the globe's true biodiversity, and mounting environmental pressures, it is increasingly vital to describe as many species and their natural histories as possible before their extinction [13].

While the actual global number of species is unknown, we know that biodiversity is unequally represented both geographically and taxonomically [14]. Despite containing the majority of species and numerous different major ecosystems, the tropics are underrepresented both in terms of studies and descriptions [15–17]. This has been attributed to historical inaccessibility [18], a lack of scientific infrastructure for accurate species identification [19], and the sheer volume of species [20]. Furthermore, there are inherent biases across taxonomic groups, as larger individuals and more well-studied taxa are disproportionately represented, despite these often being relatively species-poor. For example, despite accounting for less than one-fifth of known biodiversity [21], vertebrates contributed to nearly one-third of species' descriptions between 2006 and 2013 [22]. It is often the hyper-diverse, supposedly "uncharismatic" organisms (such as the invertebrates) that are in most need of taxonomic groundwork [17,23]. These biases place tropical invertebrates among the most poorly understood multicellular taxa in the world [24,25].

Quantifying tropical invertebrate biodiversity is difficult for groups that lack taxonomic infrastructure such as revisions and representative specimens; one such group is the hyperdiverse family of litter-inhabiting small beetles, the Staphylinidae. Due to its species richness, many species with rather high morphological similarity, and relatively unresolved phylogeny, taxonomic revisions are rare and often confined to a single genus or species complex/group [26–28]. At a deeper phylogenetic level, some subfamilies are understood better than others (e.g., the recent movement of the burying and carrion beetles to be a subfamily of Staphylinidae rather than a family (the Silphidae) of its own [29], and several species groups and genera have well resolved phylogenies [30,31]). In addition, there is a large gap between described species and the collection of publicly available standardised DNA barcodes (an average of 0.062 barcodes per described species within the family [32]). Consider the inherent hidden taxonomic scope for tropical staphylinids by comparison to the Canadian fauna (a much more thoroughly studied and less diverse system) where the number of staphylinid species has increased by over 90% within the last 40 years [33] (and achievements made through investment in taxonomic expertise and the advent and integration of molecular techniques with morphology and ecology). Taxonomic revisions are more complicated in the tropics where comprehensive cataloguing of biodiversity is faced with the most species and the fewest research resources. Recent increases in tropical descriptions can be sometimes linked to the development of barcoding initiatives within these regions [34]. With numerous ecosystems and microclimates within a comparatively small area to other biomes, the tropics have developed sharp ecological boundaries associated with (comparatively) stable abiotic regimes of temperature and precipitation. Consequently, these boundaries in the tropics can create a tighter relationship between elevation and diversity than seen in temperate systems. The rapidly changing community assemblages across tropical mountain elevation gradients are known to be some of the most biodiverse systems on the globe [35].

The Staphylindae are amongst the most diverse animal family, with nearly 67,000 species currently described across 32 extant subfamilies [36,37], and a global distribution including arctic [38], tropical [39], taiga [40], and intertidal marine habitats [41]. Notwithstanding that the cryptic diversity of other insect families will eventually make them more diverse (see the hymenopteran parasitoid families of Ichneumonidae and Braconidae [42,43] as well as the small dipteran Cecidomyiidae [44,45]), the staphylinids already have more named taxa than do vertebrates, and, as they also contain much historically cryptic diversity, will likely remain one of the most diverse animal families [46]. Improving the representation, visibility, and understanding of Staphylinidae will only further our understanding of an ecosystem's biodiversity and functioning. Some have argued that they are a good indicator taxon for various ecological processes [47–49] due to the multiple roles that staphylinids play in key ecological processes such as nutrient cycling [50], predation [51,52], and ecological succession [53].

Within the staphylinids, the Oxytelinae is the eighth largest subfamily, with 47 genera containing approximately 2000 species [54]. They are primarily found within leaf litter, with a diet predominantly composed of animal dung and decaying organic matter [55]. One morphologically challenging genus of Oxytelinae, *Anotylus* Thomson, 1859, has been called a taxonomic "left over" since there is no singular morphological character unique to the group [56]. However, Hammond [57] suggested that the genus can be identified by the crest-shaped structure on the scutellum. Whether this applies to all taxa assigned to *Anotylus* to date is unclear. While many beetle taxa may not possess a unique morphological characteristic, not all contain as much undescribed diversity as does *Anotylus* [57]. The 90 currently named species of *Anotylus* have a cosmopolitan distribution, but the greatest concentration of diversity occurs in the Neotropics [58,59]. The last catalogue of staphylinid biodiversity reported two species of *Anotylus* in Costa Rica [58], *Anotylus insignitus* Gravenhorst, 1806 which is an invasive species, and *Anotylus nitescens* Bernhauer, 1942 a species apparently endemic to Costa Rica (Vera Blanca [60]. The former possesses a large distribution throughout the Americas and several Atlantic and Pacific islands [57]; however, like many older species' descriptions, this taxon may be made up of several discreet species with smaller distributions [61]. *A. nitescens* has not appeared in the literature since its original species description [62]. *Anotylus* individuals can range in length from 1 to 6 mm [56], with some species known to be conspicuously sexually dimorphic [63]. Dolson et al. (2021) [64] reported that *Anotylus* made up most of the Oxytelinae recovered from leaf litter sifting across a 1500 m elevational gradient in Costa Rica, being most abundant in cloud forest, with one species apparently restricted to the low-elevation dry forest and one species more widespread across elevation.

In this study, our aim was to integrate morphometrics into the spatial and genetic dataset analysed by Dolson et al. and to provide a preliminary review of Costa Rican *Anotylus* diversity using an integrative approach that combines molecular and external morphological characters to identify provisional species, specifically focusing on characters which can be scored on high-resolution photos of external adult morphology. We did this by first using Barcode Index Numbers (BINs—[65]) to sort samples into provisional species, and then by identifying taxa supported by (i) both molecular and morphological differences, (ii) only molecular variation, and (iii) where morphological variation suggested a valid taxonomic division missed by the BIN algorithm. We found that many, but not all, of the provisional species suggested by DNA barcoding could be differentiated by morphometrics, suggesting that our initial molecular estimate of *Anotylus* diversity is warranted. Rapid identifications (and descriptions), especially in historically overlooked taxa like the Staphylinidae, will better inform conservation management strategies in protected areas as well as our understanding of ecological and evolutionary processes. Rapid descriptions and profound understanding are needed across the tropics, particularly for taxa such as these *Anotylus*, where most of the diversity resides in forests whose future in the climate crisis is in stark jeopardy.

2. Materials and Methods

The integrative taxonomic approach adopted here builds on more than a decade of ongoing (2008–2017) leaf-litter arthropod collections made across a 1500 m elevational gradient in Área de Conservación Guanacaste (ACG) in northwestern Costa Rica [64,66–69].

ACG is a UNESCO World Heritage Site that covers 1470 km^2 of northwestern Costa Rica and three inactive volcanoes, reaching a peak elevation of 1900 m. The collections used here were made principally on the slopes of Volcan Cacao. Across this elevational transect (0–1500 m), there are three distinct forest types (cloud, dry, and rain), and as many as eight Holdrige Life Zones [70]. Dry forests dominate the lower elevations (10–600 m) with warm and dry environments, rain forests reside in mid-elevations (700–1200 m) with predominately warm and wet climates, and, lastly, at the highest elevations (1300–1500 m) are cloud forests that are relatively cool and wet [71,72]. In small isolated tropical mountain systems like Volcan Cacao, relatively stable climatic conditions are common; therefore, the quantitatively small changes in temperature and precipitation across elevations create sharp ecological boundaries across which species are unlikely to pass, offering higher biodiversity among them than in extratropical systems [35]. High-resolution, 360° panoramic images of the forests sampled are available at http://www.gigapan.com/galleries/10092/gigapans (accessed on 23 July 2024).

Staphylinidae were collected using various standardised trapping techniques (see Dolson et al., [64]; Smith et al., for details [66]) across an elevational transect from sea level to the summit of Volcan Cacao (1500 m). Sampling was standardised for site size, time, and intensity, collecting invertebrates using a combination of active searching bait, Davis-sifting (see an example of this search method capturing an *Anotylus* here: https://youtu.be/BKr05x0_d0s?si=ByekGavLQ666LOYy (accessed on 23 July 2024)), pitfalls traps, and mini-Winkler litter extractors. Specimens were preserved in 95% ethanol in the field and later stored at −20 °C. DNA was extracted from one leg per specimen using standardised methods [73], and mitochondrial DNA 5′ COI amplified with primer cocktails outlined in Smith and Fisher [74]. Staphylinidae were identified to subfamily and genera using keys from North America [75] and Mexico [76]. All sequence and collection metadata have been deposited in BOLD (https://doi.org/10.5883/DS-ASANOT (accessed on 23 July 2024)), and physical specimens are stored within the Research Collection of M. Alex Smith at the University of Guelph.

For every *Anotylus* individual ($n = 210$), their COI DNA sequence and any available images from the Barcode of Life Data System (BOLD [77]) were extracted from the public dataset https://doi.org/10.5883/DS-ASSTAPHY (accessed on 23 July 2024). BINs are automatically assigned in BOLD using a five-step process by clustering sequences using their uncorrected pairwise distances in a refined single linkage algorithm (RESL) [65]. A standardised threshold of nucleotide diversity is used to partition sequences and is altered at a later stage to optimise effectiveness. We used BINs as our initial species' hypotheses, utilising the BIN as an epistemological, or operational, criterion of a species. We compared one other method of species delimitation via genetic distance (e.g., Assemble Species by Automatic Partitioning (ASAP) [78]) with the BIN-derived species hypotheses.

From these samples, we selected only those individuals with barcode sequences greater than 300 bp (201) and included one sympatric *Oxytelus* specimen as an outgroup for analysis. Sequences were aligned in MEGA11 [79], using MUSCLE [80]. To identify the best evolutionary substitution model we used MEGA11, which suggested the general time reversible (GTR) model [81], with a gamma frequency distribution and invariant sites (G+I). We produced a Bayesian tree in Geneious 11.0.3+7 [82], with the MrBayes Plugin version 3.2.6 [83]. The tree was then estimated using the Markov Chain Monte Carlo (MCMC) algorithm with a chain length of 12,000,000 and a 1,500,000 burn in and four heated chains at a 0.2 chain temperature, with trees subsampled every 10,000 simulations. A maximum likelihood tree was also generated using the GTR+G+I substitution model with 1000 bootstraps. Trees were rooted on the *Oxytelus* and then visualised using FigTree version 1.4.4 [84].

To estimate the genetic variation and depth of divergence within the sampled staphylinids, we first created a Bayesian inference tree to identify the depth of divergence between individuals and species and then created a pairwise matrix of p-distances between species to estimate nucleotide variation.

To quantify the morphospace for each species, we measured each individual from focus-stacked photographs for 16 different characters (Figure 1): Largest antennal segment length (1), total antennal length (2), body length (3), compound eye area (4), compound eye maximum diameter (5), compound eye maximum width (6), head width (7), temple length (8), pronotum maximum width (9), pronotum maximum length (10), pronotal area (11), elytral maximum width (12), elytral maximum length (13), elytral area (14), pronotal pigmentation (15), and elytral pigmentation (16). While total body length can by affected by the degree retraction of the abdominal segments, we found that it was as useful a measure of an individual's size as adding the lengths of the forebody (i.e., 10 + 13). Measurements 1, 2, 4, 5, and 12–14 have been used previously to delimit species within the subfamily Oxytelinae [85]. The remaining measurements: 3, 6–10, and 15, have also been used as descriptive characters in *Anotylus* [86], since pronotal width, length, and pigmentation have been used previously.

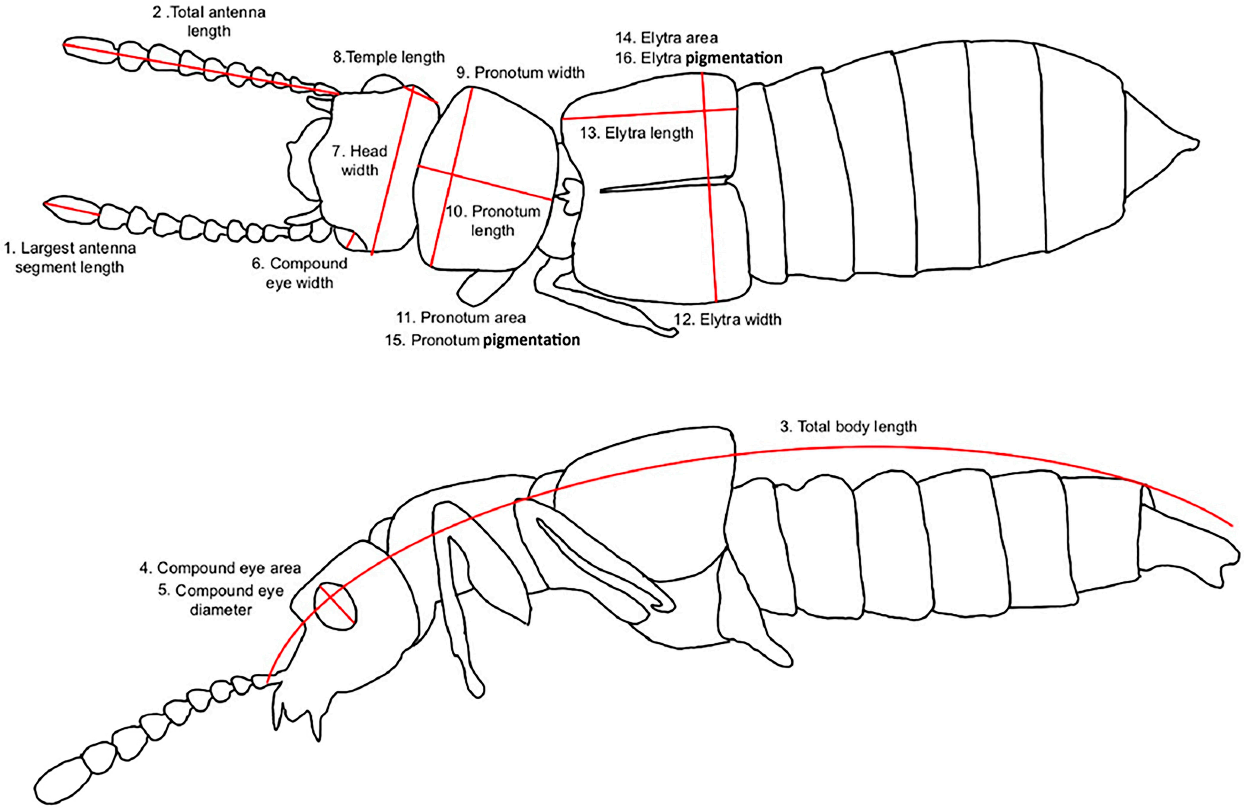

Figure 1. Key morphological measurements are labelled on a sample specimen. Red lines indicate how the measurement was taken. Measurements of areas (4, 11, and 14) were recorded using the area visible in images. Pigmentation (15 and 16) was measured by taking the average of three cross-sections of the character.

Measurements were taken from focus-stacked dorsal and lateral photos using ImageJ [87]. To calculate the area of the elytra and the compound eye, we traced the outline of the structure and used the Measure tool in ImageJ. The measurement is a two-dimensional simplification, as each structure has an irregular, nonflat surface. Elytral and pronotal pigmentation were collected from three dorsal cross-sections of each feature using the "color histogram" plugin for ImageJ 1.54g [88], and later converted from RGB values into HSV using the rgb2hsv function in R [89], with an average of the three values being taken as a representative sample of the character. The species identity for each sample was added to the morphometric data frame post-measurement, to reduce potential confirmation bias. All images are available on BOLD as part of the sample metadata.

We performed a principal component analysis (PCA) in R using the "FactoMineR" package [90]. To maximise the number of specimens in the analysis, we used individuals that possessed measurements for at least 75% of the characters. This consequently increased the number of individuals in the analysis from 127 to 177. Values were generated for absent data using the imputePCA function within the R package "missMDA" [91], by using an iterative PCA algorithm [92], which imputes values based on the similarity of relationships between individuals, and the relationships between variables. The algorithm continues to rerun until the artificial data point converges with the line of best fit for the original data. To avoid overexaggerating relationships, we used a regularised iterative PCA algorithm (rPCA) [93], which assumes consistent and mostly complete datasets. Therefore, before analysis, we calculated the percentage of the complete dataset (96.71%). Furthermore, we used the K-fold cross validation method with 5000 simulations as it identifies the lowest mean square error of prediction in large datasets, when the known percentage of data is missing. PCA plots were visualised using the "ggplot2" package in R [94]. Confidence ellipses around each species were plotted using the geom_mark_ellipse function in R.

To examine variation in the traits determined to be the most important in the PCA across the genus-level phylogeny, we created a boxplot of the variable that contributed the most to PC1 beside the phylogeny and included a specimen photo for each provisional species.

To statistically evaluate interspecific pairwise morphometric differences, we compared values of the dominant PCA axis using an ANOVA with post hoc Tukey tests, and we also completed a phylogenetic PCA using the "phytools" package in R [95]. To compare how genetic and morphometric variation changed amongst these species, we compared pairwise distances of the dominant PCA axis with pairwise p-dist calculations of the DNA barcode region using a Mantel test in the package ncf in R [96].

3. Results

We found 18 putative species of *Anotylus* (BINs) across our elevational gradient in northwestern Costa Rica. While some of these species occurred widely across elevations, others appeared restricted to either dry, rain, or cloud forest (Table 1), and many occurred sympatrically. Diversity in the group increased with elevation, as was shown for Staphylinidae in general by Dolson et al. (2021) [64] and Smith et al. [67]. Provenance metadata for each sample (including collection details, sample images, trace files, and sequences) can be examined at https://doi.org/10.5883/DS-ASANOT (accessed on 23 July 2024) and in Table S1.

Table 1. The number of individuals and the elevational ranges (metres) for each ACG *Anotylus* species in BOLD as of June 2024.

BIN	Count	Mean Elevation (m)	Median Elevation (m)	Maximum Elevation (m)	Minimum Elevation (m)
BOLD:ACZ5516	116	1220.96	1180	1480	304
BOLD:ACZ5742	11	1177.55	1304	1458	304
BOLD:ACZ5987	1	1046.00	1046	1046	1046
BOLD:ACZ6447	10	1009.70	1128	1128	323
BOLD:ADF3772	47	1017.94	1184	1314	700
BOLD:ADF4138	131	850.00	831	1280	820
BOLD:ADF4200	4	1254.25	1262	1304	1189
BOLD:ADF8741	10	1232.20	1247	1313	1050
BOLD:ADG0617	1	1314.00	1314	1314	1314
BOLD:ADG1201	15	1329.20	1310	1482	1000
BOLD:ADH8436	2	1343.50	1343.5	1485	1202
BOLD:ADH9095	2	1187.50	1187.5	1189	1186
BOLD:ADH9620	1	1300.00	1300	1300	1300
BOLD:ADH9622	7	889.29	1011	1011	420
BOLD:ADI3175	4	1216.00	1190	1304	1180
BOLD:ADL5474	2	1232.50	1232.5	1460	1005
BOLD:ADR2790	4	823.75	1047	1190	11

3.1. Genetic Analysis of *Anotylus*

Average pairwise genetic distances between provisional species was large, ranging from 5.03% (between BOLD:ADG1201 and BOLD:ADG0617) to 27.9% (BOLD:ADH9622 and BOLD:ADR2790), with a median of 19.26%. The best ASAP estimate of species via sequence divergence (asap-score of 2.5) estimated 14 species within the 18 BINs by lumping together the four species pairs with less than 5% interspecific sequence divergence. As these four species pairs are characterised by sympatric elevational distributions (Table 1) and marked (if not always significant) PC1 scores of morphometric differentiation (Table S3), we follow the BIN divisions as the first step in this integrative taxonomy. An ASAP partition that agreed with BIN divisions was one of the ten best partitions (asap-score 7.5). Intraspecific variation was low (median variation 0.38% (excluding doubletons and singletons)). The most abundant species (BOLD:ACZ5516) had the greatest intraspecific variation (0.7%).

Bayesian and maximum likelihood phylogenetic examinations of Anotylus provisional species produced nearly identical topologies (Figure 2).

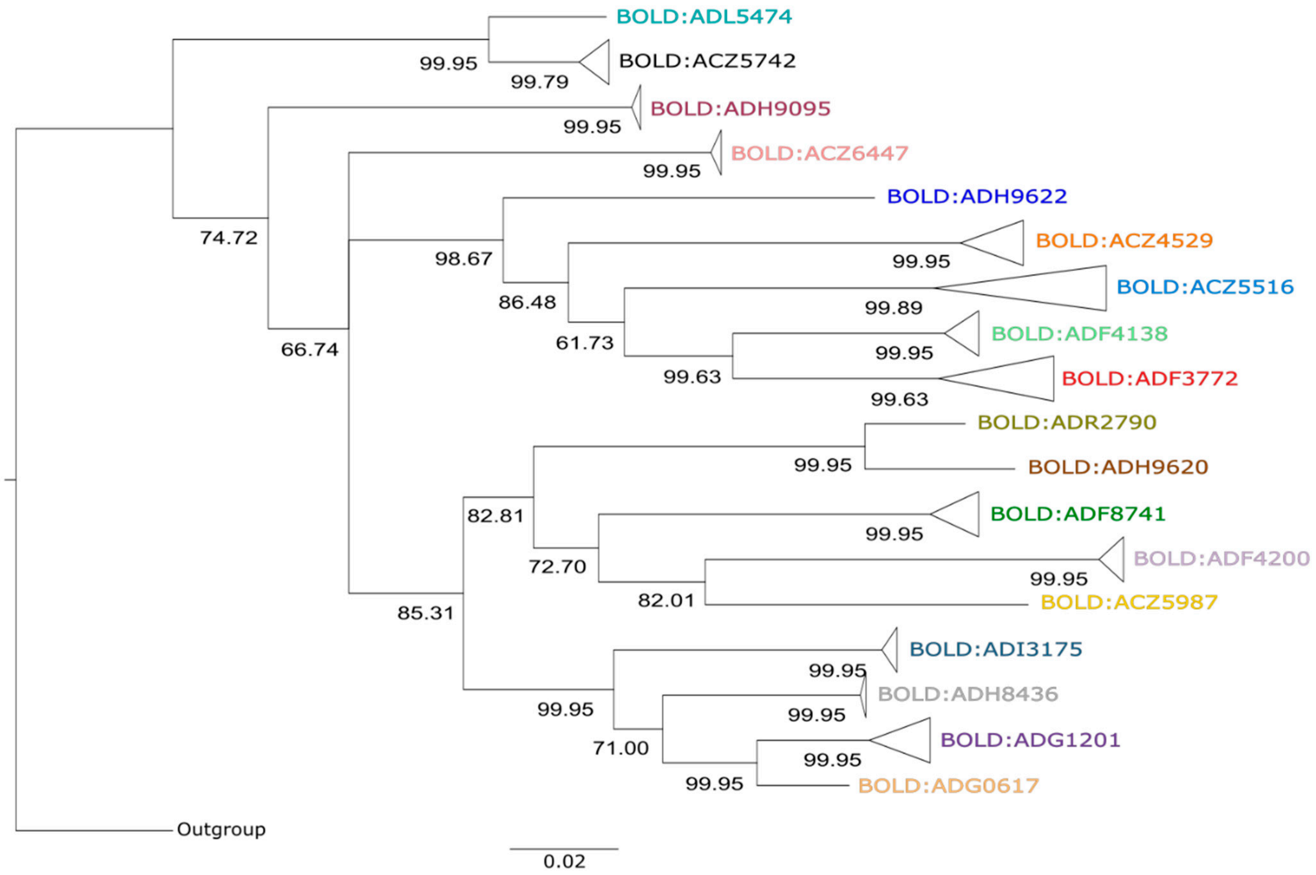

Figure 2. The phylogeny of ACG *Anotylus* calculated using maximum likelihood, with bootstrap support values indicated. Different species indicated in different colours.

3.2. Morphometric Analysis of Anotylus

The cumulative variation exhibited across the first two principal components (PCs) was 78.97%, with PC1 accounting for 68.73% of total variation, which best represented the first 14 morphological characters, all morphometric. Elytral area was the most variable character in PC1. The remaining two characters, pigmentation of elytra and pronotal length, were represented by PC2, capturing 10.24% of total variation. PC1 had the greatest discriminatory power amongst species, while PC2 primarily accounted for variation within species (Figure 3A). While many of the putative Anotylus species exhibited overlapping morphometric variation (Figure 3A), the five most abundant species were all significantly different from each other along PC1 (Figure 3B, Table S3). Specific measurements are available in Table S2, and pairwise interspecific distance across PC1 (and associated Tukey post hoc test values) in Table S3.

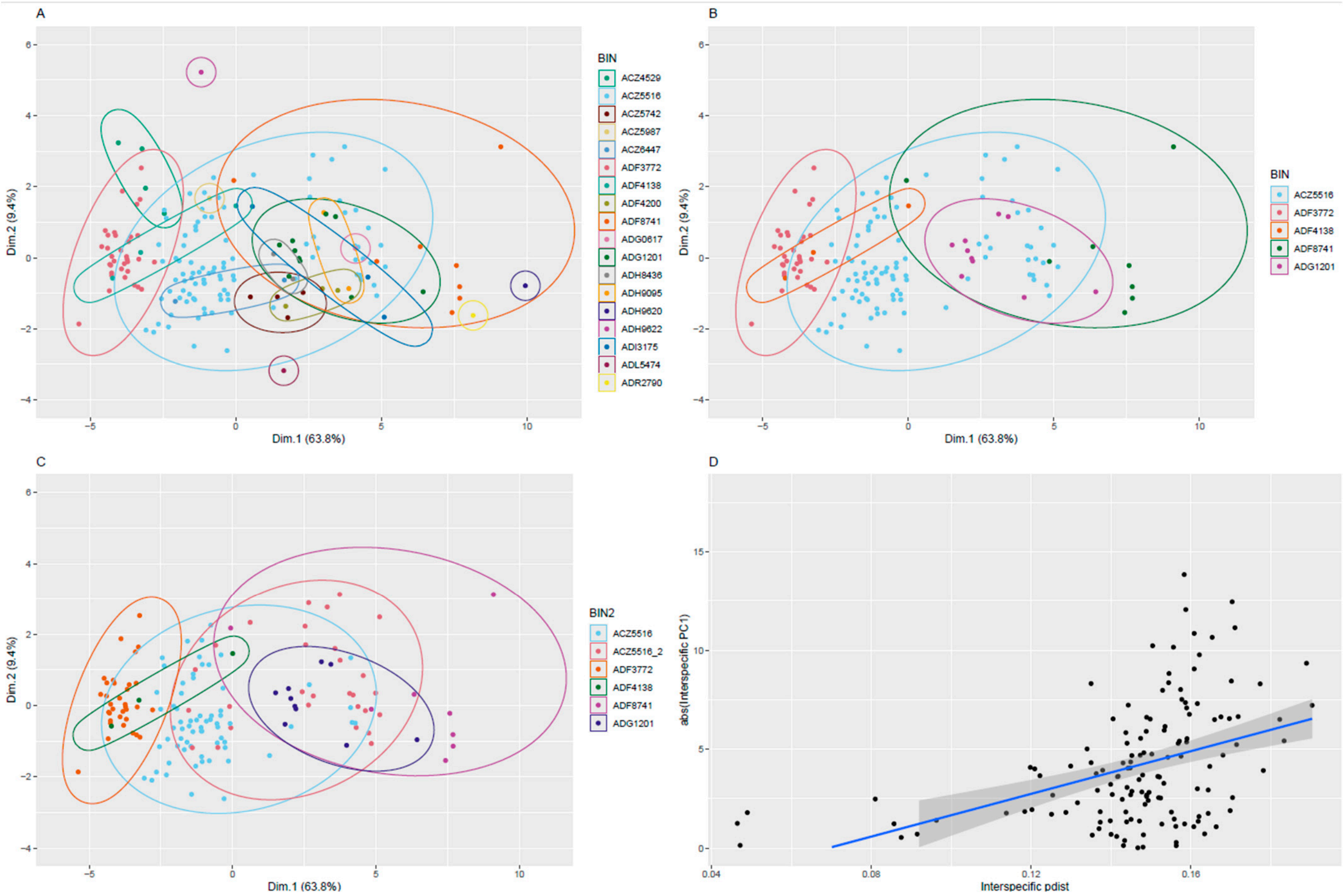

Figure 3. Morphological variation in ACG *Anotylus*. (**A**) PCA of all individuals and all taxa. (**B**) PCA of 5 most abundant taxa. (**C**) PCA of 5 most abundant taxa that includes the BOLD:ACZ5166 split and (**D**) scatter plot of the pairwise relationship between genetic and morphometric variation.

3.3. Comparing Morphometric and Molecular Differences

We identified one putative species with slight (~1%) intraspecific genetic variation whose range extends across nearly the entire elevational gradient (BOLD:ACZ5516). When we explored this genetic variation on the morphometric PCA, we found correspondingly large morphometric variation associated with the genetic differences (Figure 3C). The two intra-BIN genotypes have a nested elevational distribution, with one found between 1000 and 1500 m (with a peak in abundance at 1200), while the more abundant genotype can be found from 300 to 1500 m with a peak in abundance at 1300 m.

Pairwise morphometric variation correlated with genetic variation in the barcode region (Mantel r = 0.387, p = 0.004) (Figure 3D), and a phylogenetic PCA produced similar separation of taxa (Figure S1). While the relationship between genetic and morphometric variation was significant, there were numerous cases where a large genetic difference was associated with only very subtle morphometric differences (Figure 4). Mean interspecific differences in pdist are shown in Table S4.

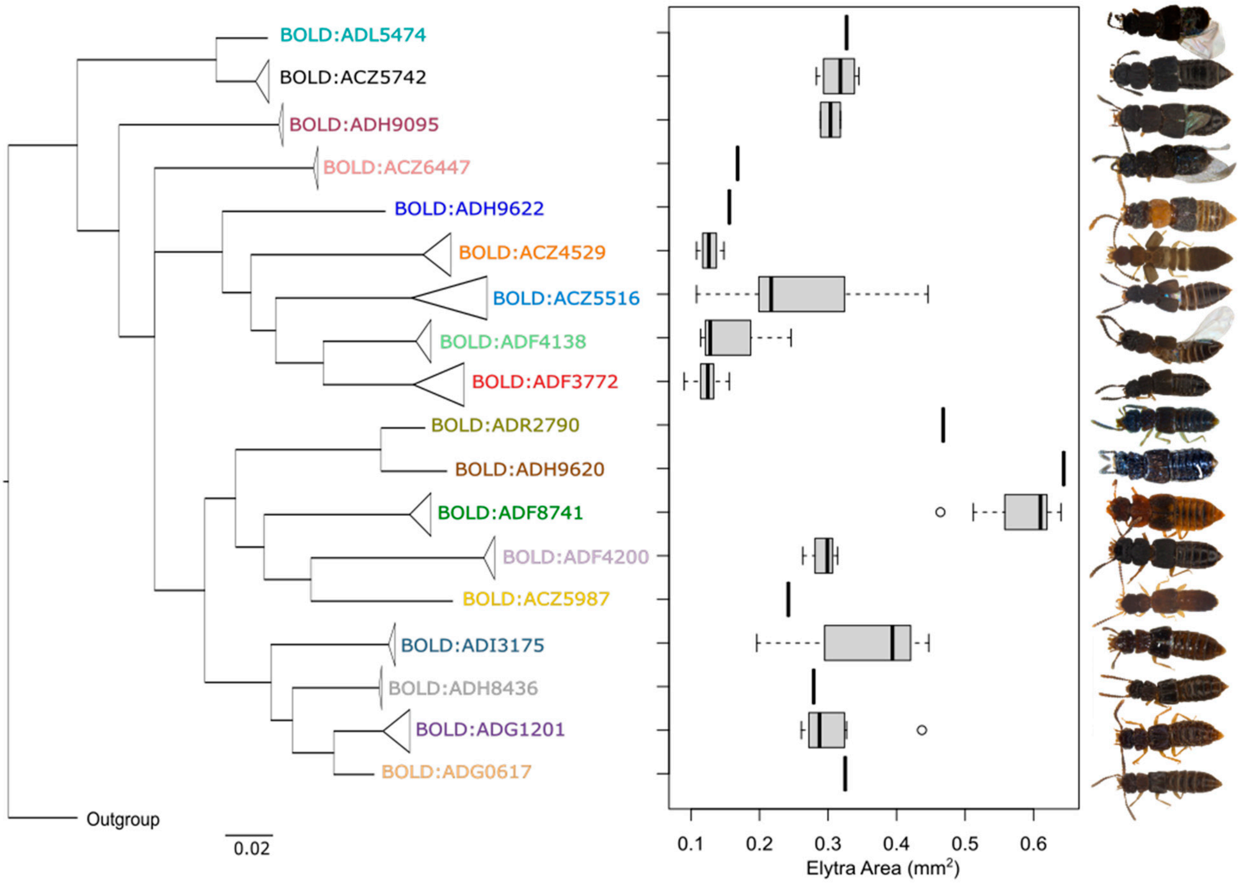

Figure 4. Phylogenetic tree of putative ACG *Anotylus* species with a corresponding boxplot of their elytral area variation (identical topology and bootstrap values to Figure 2) and specimen photos (images not to scale).

4. Discussion

Our results demonstrate that the true diversity of *Anotylus* in Costa Rica is currently severely underestimated. A single elevational transect, on a single volcano, revealed nine times the number of taxa currently listed for the whole country. We believe that none of these new taxa are either of the *Anotylus* known from Costa Rica. While external adult morphometrics did not allow the unambiguous differentiation of all putative taxa identified by barcodes, we did find statistical support for the separation of most of the well-sampled putative species and a significant positive relationship between molecular and morphometric variation within BINs. Due to the, often decoupled, nature of mitochondrial and nuclear molecular evolution [97,98], we would not necessarily expect that morphometric variation would accumulate at the same rate as mitochondrial variation. Finding that, generally, the most divergent putative species suggested by DNA barcoding are diagnosable using external morphometrics supports the use of DNA barcodes as a first-pass assessment of underdescribed diversity in poorly known taxa such as Neotropical *Anotylus*, as has been performed and assumed for other tropical species-rich groups [99–103].

Our study explicitly focused on whether easily quantifiable characters, visible on high-resolution photos of adults, could be used to discriminate putative taxa identified by BINs. This approach was reasonably successful, without the examination of male genitalia, which clearly provide an additional, rapidly evolving character set for the discrimination of closely related taxa [104]. Future work on Costa Rican *Anotylus* will clearly require the integration of morphology and genetics, examination of previously described Neotropical species, and detailed study of genital characters, but our barcode database provides a critical first step. While the informative variation present in male genitalia may help eventually describe some taxa elucidated here, this would not remove the subsequent bias inserted by using a morphological character present in only ~50% of the adults collected to differentiate taxa (i.e., identifying females and immature individuals will not be possible based on characters on the male genitalia). In this case, a method that includes measures across intraspecific variation inherent to sex and age (such as DNA) will perform best across all individuals.

4.1. *Anotylus*—Diverse and Variable across Elevation

In many ways, the putative *Anotylus* species recognised here are exemplars of understudied Neotropical diversity—and the broader taxonomic group to which they belong is characterised by insufficient taxonomic resources (or at least resources that are incommensurate with its enormous diversity). The genus has also been an acknowledged taxonomic "receptacle" [57] for species that lack the discriminating features of other groups, meaning that it is likely to be paraphyletic. In the Neotropics, *Anotylus* is diverse [56,59]; they can be locally abundant and their abundance and diversity change rapidly across abiotic gradients associated with elevation [64]. Individual taxa are, therefore, prone to being overlooked and may be particularly vulnerable to isotherms shifting upslope in the current climate crisis [67]. We note a high degree of sympatry amongst these provisional species. Of our five most frequently collected species, four can be observed at the lower-elevation cloud forest site at 1300 m on Volcan Cacao, while the remaining common species is known only from a different volcano across (approximately) the same elevational range. These observations speak to known elements of *Anotylus* ecology—namely, that species have small geographic distributions [57] and are often found in sympatry [57]. In other tropical montane systems, Hammond [57] observed that there was a reduction in elytral size that was associated with a reduced capacity for flight with increased elevation. We found that elytral area was one of our most variable morphometric measurements and speculate that while all these species are not likely to move far or frequently, the species with the smallest elytra will be the taxa most restricted to their current elevational band of abiotic conditions, and, therefore, the first that we would lose to approaching high temperatures.

4.2. Cryptic Diversity within Cryptic Diversity

Two distinct morphological groups within a single DNA BIN may point to the existence of recently diverged taxa which are cryptic in a molecular sense. No other BINs demonstrated an intra-BIN division in their morphospace or phylogeny. Such a morphological split might be intraspecific and caused by sexual dimorphism (as has been observed in other *Anotylus* [59,105]), but this would not explain the corresponding genetic differences. Intraspecific genetic and morphological differences might be explained by separation along a strong elevational gradient, but while the groups exhibit a somewhat nested distribution, each group can be found at largely the same elevations (particularly in the upper elevations; thus, geography is unlikely to be the factor driving divergence. Thus, we feel that this is likely a case where a BIN contains more than one species, as has been observed in other insect groups [106,107]. The discovery of more than one species in one BIN, as demonstrated by their elevation and natural history, is no surprise. For example, in the *Astraptes fulgurator,* where 10 species were found in one name [108], six of these ten exist in a single BIN, where each can be distinguished by larval color, microecosystem, and barcode cluster within a BIN. Confirming the split we have documented here (and supporting the other provisional species we have erected) would ideally be followed by additional study (e.g., male genitalia and additional molecular markers, as in [61,102,109,110]). While description of these species was beyond the scope of this work, we do note that, from a morphotaxonomic perspective, these external measurements are often included in descriptions but frequently are not definitive. Despite this historic trend, our work highlights the utility that the combination of natural history (often elevation) with a DNA barcode and (comparatively) easy to acquire external morphometrics can offer diagnostic taxonomy [111].

4.3. Rarity

While our collections span a significant elevational transect and time period, half the provisional species identified here (9/18) are represented by fewer than four samples. In tropical ecosystems, it is not uncommon for species to be sampled, and often subsequently described from single specimens (e.g., [112–115]), and in a recent review, Deng et al. [116] found that more than 20% of the insect species described in a decade were singletons. Such a preponderance of singletons is likely due to a combination of natural rarity and small population sizes and geographic ranges. Singletons can also be a signature of incomplete sampling, and it is therefore possible that some of the 18 provisional species may reflect a limit of sampling and currently unknown estimates of intraspecific variation. However, in cases such as this, where we know where a species lives along a significant abiotic gradient (such as temperature) and we know that they differ markedly in their genetics, it is prudent to adjust our default statistical null expectation from one where we treat samples as being from the same species, until shown otherwise, to where we treat samples that differ in ecology, genetics, and morphology as separate until we collect some new evidence that unifies them. i.e., we accept a larger statistical Type I error (failing to reject a null hypothesis when it is true) by "splitting", because "lumping" will minimise the Type II error of accepting a null hypothesis when it is, in fact, false. This leads to an underestimation of diversity; furthermore, it fosters an underappreciation for the magnitude of importance for the Type II error (of accepting the same when they are separate) [117]. Doing so improves the visibility of rare species that would historically be overlooked and can help mitigate the bias of underestimating the true species richness within tropical regions.

4.4. Cryptic Species and Protected Areas

In a recent review, Li and Wiens [4] reported that incorporating morphologically cryptic insect species (delimited using molecular methodology) would realistically increase estimated global biodiversity by at least two orders of magnitude—and perhaps more. Our study deals with one genus of small rove beetles on one Costa Rican volcano but shows that even in relatively well-studied tropical areas such as ACG, (semi-)cryptic staphylinid diversity is extensive. What these putative beetle species should be called, how widespread they are in the Neotropics, and how they are differentiated ecologically are all critical questions which remain unresolved—as they are for much of tropical invertebrate biodiversity. Thus, while formal description is beyond the scope of this work, but we anticipate will follow it, we have helped to demonstrate that surveys and subsequent protected area management decisions that utilise DNA-barcode-catalysed inventories of provisional species (such as BINs) are using biologically meaningful (and transparent) units of diversity that, in many cases, will eventually correspond with formally described species.

4.5. Elevational Patterns and Vulnerability in the Climate Crisis

Along tropical montane slopes, there exist a myriad of stable microclimates (until climate change happens)—and species that live here are often not adapted to moving among these microclimates or between adjacent peaks by passing through the other microhabitats separating them [35]. The result is often that the biodiversity of each mountain is unique, and many tropical mountain peaks house endemic species. This is certainly the case of Volcan Cacao, where the higher-elevation sites are characterised by (comparatively) stable cool and wet weather while the sites at lower elevations experience higher variation associated with long hot periods without rain and then relatively wet and warm rainy seasons [67,70,72,118]. At least, this is how temperature variation has divided itself historically across elevation [118]. In the current climate crisis, hot temperatures are occurring more frequently at higher elevations, and precipitation patterns are less dependable, as historic norms show [72]. The provisional species we identified here exist along this finely divided volcanic slope—and their future is likely in unavoidable jeopardy with ongoing climate change in Costa Rica. The diversity of these *Anotylus* increases into the cloud forest. These historically cold and damp locales likely foster many microhabitats and many opportunities for niche division amongst detritivores and dung eaters like these *Anotylus*. What can we expect with increased heat and decreased dependence on historical precipitation regimes but reduced opportunities for species that are likely decomposers of decaying plant material and dung? We fear that our elucidation of this diversity may have an elegiac element where future work will struggle to find some of these species again. ACG has experienced declines in insect diversity and abundance over the past two decades, occurring in varied taxa and habitats, and there is no other overarching causative agent that can be implicated beyond the myriad effects of climate change [118]. While these *Anotylus* are likely to experience such declines, we hope they will be among the survivors within the protected forests of ACG, where contiguous forests may provide sufficient movement opportunity for those species that can move.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16080441/s1, Table S1: Sample information and metadata for the specimens analysed here; Table S2: Morphological measurements; Table S3: Pairwise matrix of interspecific distances of PC1 (beneath diagonal) and Tukey post hoc test values (above diagonal); Table S4: Pairwise matrix of estimated interspecific distances between BINS (pdist). Figure S1: Phylogenetic PCA of BINs.

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Data Availability Statement: All sample metadata, including collection information, specimen images, and DNA sequences and trace files are available here: https://doi.org/10.5883/DS-ASANOT (accessed on 23 July 2024).

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