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A preliminary integrated-taxonomic review of a staphylinid genus (*Anotylus* Thomson 1859) of the Area de Conservacin Guanacaste, Costa Rica.

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**UNIVERSITY OF
PLYMOUTH**

**A preliminary integrated-taxonomic review of a staphylinid genus
(*Anotylus* Thomson 1859) of the Área de Conservación
Guanacaste, Costa Rica**

by

KEELAN DANTE UNDERWOOD

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

RESEARCH MASTERS

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Acknowledgements

For my late grandmother, Patricia Underwood. An artist, confidant, globetrotter, mentor, nurturer, historian, to name only a few of her traits. Gran, you instilled your curiosity into me from a young age as my second parent, for you I will always be grateful. You may vanish from my eyes but never from my heart.

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Author's Declaration

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

This thesis has been proofread by a third party; no factual changes or additions or amendments to the argument were made as a result of this process. A copy of the thesis prior to proofreading will be made available to the examiners upon request.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

A programme of advanced study was undertaken, which included the taught modules; ANIM5007 (Small Population Conservation), and BIO5131 (Postgraduate Research Skills & Methods).

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Date 26th of February 2024

Abstract

Keelan Dante Underwood.

A preliminary integrated-taxonomic review of a staphylinid genus (*Anotylus* Thomson 1859) of the Área de Conservación Guanacaste, Costa Rica.

Taxonomy underpins biological research, names are needed for comparative analysis, conservation status, and even public communication. Yet many species remain undescribed and are therefore vulnerable and unprotected. Whilst the true number of species is unknown, the underrepresented taxonomic groups and geographical regions are, with Neotropical Staphylinidae being amongst the most poorly represented. Barcoding initiatives highlight taxa in need of revision and act as effective estimators of biodiversity using Barcode Index Numbers (BINs) as prospective species. This study acts as a preliminary review of a Staphylinid genus (the *Anotylus*) in Costa Rica as they have not been revised using modern taxonomy. Here using an integrative taxonomic framework utilising simple morphometric characters and the COI gene, I identified five distinct Evolutionary Significant Units (ESUs). With only two species of *Anotylus* formally identified in Costa Rica this presents a significant increase in the believed biodiversity of the genus within the region. This will ultimately only increase as more comprehensive characteristics are used that facilitate formal species' descriptions, likely further subdividing the ESU's into putative species. These subdivisions may already be evidenced by a BIN within this study that possessed distinct morphological variation with genetic support. Despite being unable to differentiate individuals to a species level, there is still an increase to the previously believed biodiversity of Costa Rican *Anotylus*. Here barcoding demonstrates its effectiveness as a simple biodiversity estimator whilst also displaying its use as a primer for integrative taxonomic studies using BINs as species hypotheses. The easy identification of taxa for review may help mitigate the taxonomic impediment and usher in a wave of rapid species' descriptions, especially in historically overlooked taxa like the Staphylinidae. This in turn better informs conservation management strategies and our understanding of biological processes.

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Foreword

The term “integrative taxonomy” was coined independently at the same time (Dayrat, 2005; Will et al., 2005). It is the use of numerous characters such as DNA, morphology, and more to delimit, define, and identify species. Integrative taxonomy was proposed in response to the advent of DNA identification techniques such as barcoding (Will et al., 2005). It was feared that barcoding encouraged the return to a single typological form of taxonomy (Lipscomb et al., 2003; Will and Rubinoff, 2004). The use of single typology in taxonomy is archaic as it fails to truly represent a species’ evolutionary lineage and ecological role (Sober, 1980).

Taxonomy as a science is rooted in the idea of testable species hypotheses. Depending on what species concept the author uses, this will determine what criteria are deemed important for species delimitation (Sites and Marshall, 2003). Revision of species hypotheses is difficult when the reviewer uses alternate criteria to distinguish a species than those used for the original species description (Agapow et al., 2004), especially as they are rarely stated (Schlick-Steiner et al., 2010). Integrative taxonomy allows rigorous testing of species hypothesis against numerous criteria and can either further support a species’ place in a taxonomic hierarchy or indicate taxa in need of taxonomic revision. Validation and/or review of species ultimately creates a more accurate reflection of true biodiversity.

I will discuss what integrative taxonomy is and if it works, before exploring the disproportionate use of operational criteria used to delimit and define species and whether criteria should be used more equally. Lastly, I will provide a short review on the developments within integrative taxonomy since the term’s introduction as well as potential future developments.

Integrative taxonomy is the use of numerous lines of evidence from independent operational criteria to support or oppose species hypothesis. The criteria can vary depending on the organism being tested. Although the term was introduced in 2005 (Dayrat, 2005; Will et al., 2005), the principle has long been practised (Blackwelder, 1967; Templeton, 1980). A major constraint of species delimitation resides in what species concept is used; different species concepts will result in different species boundaries. Operating off what would later be described as the universal species concept (De Queiroz, 2007), a species is defined as a lineage, the operational criteria used to delimit these lineages have previously been used as species concepts in the past. In integrative taxonomy it does not matter what operational criteria are used to separate and identify species, as long as they are independent and clearly stated. These criteria will provide evidence for or against a species hypothesis. However, no species hypothesis can truly be confirmed or denied, only the results of numerous operational criteria can provide levels of confidence to a species hypothesis (Schlick-Steiner et al., 2010).

Numerous accounts of how integrative taxonomy should be conducted exist (Dayrat, 2005; Evenhuis, 2007; Yeates et al., 2011), the simplest description of integrative taxonomy is as a three point process (Pante et al., 2015). First, a variety of independent characters must be collected from a population with taxonomic uncertainty. Secondly, a species hypothesis is tested that places the individuals sampled at a species level in consistently clearly defined groups. Finally, a species description and name are created for any newly identified species. The success of integrative taxonomy is clear as it is only sensible that confidence in a hypothesis will increase when numerous lines of independent evidence support it. A review of species delimitations using a single character had a mean failure rate of 30% but dropped to 9% and 2.7% when two or three characters were used (Schlick-Steiner et al., 2010). That

said, initial species delimitations and descriptions are predominantly conducted using two lines of evidence with only 17.4% of studies using three or more criteria (Pante et al., 2015). This suggests that whilst confidence is higher the more criteria are used, there is an imbalance in which criteria are used to delimit and define species.

Reviews of methods used in integrative taxonomy clearly outline a focus on morphological and molecular based approaches (Page et al., 2005; Schlick-Steiner et al., 2010; Pante et al., 2015). Molecular methods predominantly focus on the COI gene for metazoans (Andújar et al., 2018), but many other genes are also used independently or in conjunction (Shaffer and Thomson, 2007; Dupuis et al., 2012; Schoch et al., 2012). Usage of non-traditional operational criteria such as bioacoustics (Seger et al., 2021), coalescent theory (Knowles and Carstens, 2007), chemical analysis (Seppä et al., 2010), elevational distribution (Smith et al., 2014), and more, have grown since the turn of the century. Though they are often used as a supporting criteria to more morphological or molecular centred approaches (Bertsch et al., 2005; Heethoff et al., 2011).

With molecular and morphological operational criteria dominating integrative taxonomy, it is important to ask if other lines of evidence should be used more? It has been suggested that a species hypothesis tested using integrative taxonomy is treated as secure when three different disciplines agree: morphological, genetic and another criteria (Schlick-Steiner et al., 2010). This has been criticised for being restrictive as all good science is based in a hypothetical deductive framework (Yeates et al., 2011). No hypothesis can ever be confirmed and to discourage collecting further supporting or contrasting evidence is unwise as it restricts scientific progression of underutilised methods, consequently discouraging their use in favour of more established methods. However, in a world with taxonomists

themselves becoming extinct and funding for the field diminishing (Pearson et al., 2011), further research often favours the cheaper and easier approaches such as the molecular and morphological, giving little opportunity to develop alternate methods. Another argument on why these secondary characters are scarcely used is because we cannot observe them naturally (Bickford et al., 2007; De Queiroz, 2007; Goulding and Dayrat, 2016). I would agree that it is hard to establish defining characteristics for species when they cannot be easily identified, however we have seen DNA (a historically unobservable character) become a backbone of modern taxonomy in only two decades. That said, the principle of integrative taxonomy is to use multiple lines of evidence to support a hypothesis. Ironically molecular advocates predominantly use a single gene alongside non DNA based criteria to support their species hypothesis (Butcher et al., 2012; Sharkey et al., 2021). In the spirit of integrative taxonomy any molecular characters should be multi-locus to ensure that the lineages are monophyletic. This is not an attempt to undermine the work of the molecular revolution but to reiterate integrative taxonomy's core principle.

Morphological and molecular studies used together create a positive feedback loop that better develops, and answers questions generated by each other (Page et al., 2005). I would argue that this is true for all lines of evidence used in delimiting taxa, and more broadly a core tenant of scientific research. This is central to the work of taxonomists as scientists, to grow from and sustain neighbouring scientific fields. Especially when our understanding of the evolutionary drivers for a species' divergence may be unknown. Taxonomic conflicts concerning morphology and molecular must not be abandoned but justified with evolutionary history (Yeates et al., 2011). This can only truly be understood by using numerous alternate disciplines that frame these disparities (Page et al., 2005). For example, cryptic species have become well supported using auditory or chemical analysis when

morphological differences are difficult to identify (Funk et al., 2011; Kather and Martin, 2012). This provides insight into the evolutionary history of these organisms and why species divergence occurred. Although, as previously discussed the initial delimiting factor is often molecular (Bertsch and Schweer, 2011), and in some cases the secondary supporting criteria is morphological (Juste et al., 2019). Regardless of which operational criteria are used to delimit species, confidence can only be held when their assumptions and limitations are known (Heethoff et al., 2011). A golden age of taxonomy in the next few decades is possible only if we acknowledge and develop improvement in the whole field, and not devoting to a bicharacter system rooted in the molecular and morphological.

An early but important development was motioned by DeSalle et al., (2005), with their “taxonomic circle” (Figure 1). A species hypothesis is formed based on describable character variations seen in a population, this and another independent character from the same population are tested and if both assessments divide the population in the same manner, then the circle is broken, and a new species can be described. If the analysis disagrees, then another assessment using an alternate criterion can be conducted. Until two assessments agree, no new species can be described. Another development was the division of the practise into two pathways, integration by congruence and integration by culmination (Padiál et al., 2010). Integration by congruence is a discovery-based approach which supports species hypothesis rooted in lineage divergence as opposed to character identification. Primarily utilised in molecular studies which easily identify deeply diverged lineages. Whereas integration by culmination is a hypothesis driven approach where variation in any character can be used to create a species hypothesis. Further characters can be added to support or oppose this hypothesis and when evidence disagrees an evolutionary justification is sought. These methods have been in use before the term’s

introduction (Blackwelder, 1967; Hebert et al., 2004). Yeates et al., (2011), criticised the term integrative taxonomy and recommended that it be succeeded by “iterative taxonomy” under the philosophy that species hypothesis should be continually challenged as new data surfaces. Arguably this is what integrative taxonomy already is (Gullan et al., 2010; Lumley and Sperling, 2010). The field of integrative taxonomy has not truly progressed as such, just further clarified and defined. Whilst this does not directly contribute to the advancement of the field it does improve its accessibility, something that should not be underestimated, especially as taxonomy’s current decline/stagnation will impact many other scientific fields.

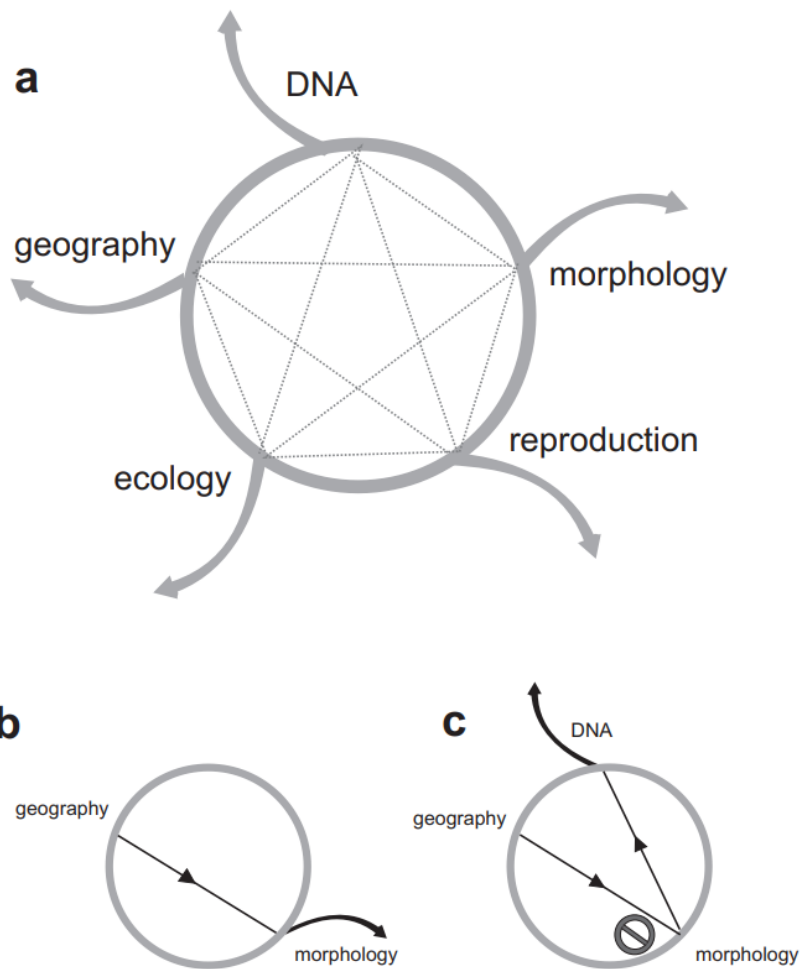


Figure 1. The taxonomic circle DeSalle et al., 2005, redrawn and quoted by Padial et al., 2010. “Dotted lines in (a) connect lines of evidence used to discover species or support previous hypotheses. The recognition of a species is considered when congruence between a taxonomic character and geography allows breaking out of the circle (arrows). For example, in classical taxonomy (b) the occurrence of morphologically distinct specimens at different locations can be used to propose and support a species hypothesis. In the case of cryptic species (c), morphology fails to support the hypothesis but other characters (e.g. molecular) do provide support.”

Integrative taxonomy’s existence is to aid in identifying and describing what species exist, not how many there are (Goulding and Dayrat, 2016). Species’ descriptions needs specialists, resources, and time, all of which is in short supply (Pearson et al., 2011; Tancoigne and Dubois, 2013). In a review of integrative taxonomy’s influence by one of the original advocates a key concern is that the rapid data collection is outpacing the taxonomic process needed to describe species (Goulding and Dayrat, 2016). Whilst this is a valid

concern that candidate species can be left for years or potentially decades until they are described with taxonomic rigor (Fontaine et al., 2012), it appears that the ratio of described to undescribed species is relatively even at 1.11:1 (Pante et al., 2015), although this does vary significantly between taxa with ranges from 0.44:1 (molluscs) to 3.00:1 (crustacea). The primary restraint for candidate species failing to be described in their initial delimitation is that the publishers are likely unable or unwilling to name and adequately describe species (Satler et al., 2013). It is also possible that they may be unfamiliar with the details of the organisms they have collected and do not know if what they have sequenced is a new or already established species.

The Barcode Index System has helped contribute to quick species identification (Ratnasingham and Hebert, 2013), but many entries have yet to be identified to a species level and it is a slowly developing process that is dependent on taxonomists verifying species identification (Lopez-Vaamonde et al., 2021). Though a risk resides with incorrect identification of organisms as new species, which are in fact pre-existing species that have yet to be sequenced and inputted into barcoding databases. Furthermore, the detachment of an organism from its name also detaches it from its literature and makes conservation and understanding the organism exceedingly difficult (Tan et al., 2010). Arguably, the majority of unidentified species delimited via this method are new and do not have any existing literature (Butcher et al., 2012; Sharkey et al., 2021). As research progresses any species sequenced and correctly identified will highlight any synonymy.

Integrative taxonomy is the use of numerous operational criteria to support or oppose species hypothesis regardless of species criteria. The simultaneous use of various criteria creates greater confidence in a species hypothesis than any single typological system would.

Although integrative taxonomy is dominated by two criteria, a combination of morphological and molecular characters. This is likely because of their effectiveness and well-established methods. Although other operational criteria are underutilised as a result, greater focus should be placed on these alternate criteria as they help frame our understanding of a species evolutionary history. Integrative taxonomy as a science has barely developed since its introduction nearly two decades ago, most “developments” are just explanations of already established practices, though this does improve ease of access to a waning scientific field. For integrative taxonomy to become more impactful the data processing and description must catch up to the explosive rate of data collection from barcoding initiatives. A golden age of taxonomy is on the horizon but only with an equalisation of the underappreciated taxonomists and underutilised disciplines.

A preliminary integrated-taxonomic review of a staphylinid genus (*Anotylus* Thomson 1859)
of the Área de Conservación Guanacaste, Costa Rica.

Introduction

Determining this planet's true biodiversity is a fundamental question, vital to our understanding of the world we reside in. Estimates range between 5 million and 30 million species (Costello et al., 2013; May, 2002), and with only a maximum of 2.3 million described (Bánki et al., 2021), we have only described a fraction of it (Mora et al., 2011). Without knowing what species exist and where, we cannot effectively conserve them (Deichmann et al., 2017), manage ecosystem services (Suzán et al., 2009), or monitor biodiversity loss (Noss, 1990), which if unchecked can result in dangerous ecological cascades (Bortolus, 2008). However, while fundamental, describing new species is difficult; with many species concepts (Mayden, 1997), dated revisions (Padial and De la Riva, 2006), and a lack of institutional and societal support for taxonomists (Packer et al., 2009), there simply are not enough trained taxonomists to characterize global biodiversity. This taxonomic impediment is even more dire considering the epoch of the sixth mass extinction (Barnosky et al., 2011). With many limitations to identifying the globe's true biodiversity, and mounting environmental pressures, it is increasingly vital to describe as many species as possible before their extinction (Wagner et al., 2021).

Whilst the true number of species is unknown, we know that global biodiversity is unequally represented both geographically and taxonomically. Despite containing the majority of species and numerous different ecosystems, the tropics are highly underrepresented (Giam et al., 2011; Titley et al., 2017; Vieites et al., 2009). This has been attributed to historical inaccessibility (Godfray et al., 2007), a lack of scientific infrastructure for accurate species

identification (Wheeler et al., 2004), and the sheer volume of species (Moura and Jetz, 2021). Furthermore, taxonomic groups possess inherent biases in diversity, as larger, and more well-studied taxa are disproportionately represented. For example, vertebrates contributed to nearly a third of species' descriptions between 2006 and 2013 (Pante et al., 2015), despite accounting for less than a fifth of known biodiversity (Zhang, 2011). It is often the hyper-diverse "uncharismatic" organisms that are in dire need of taxonomic revision, such as the invertebrates (Clark and May, 2002; Titley et al., 2017). These biases makes tropical invertebrates being one of the most poorly understood taxa in the world (Cardoso et al., 2011; Wilson, 1985).

Quantifying tropical invertebrate biodiversity is difficult for groups that lack taxonomic infrastructure such as revisions and representative specimens; one such group is the hyperdiverse beetle family, the Staphylinidae. Due to its species richness, many species with high morphological similarity, and unresolved phylogeny, taxonomic revisions are rare and often confined to a single genus or species complex/group (Brunke and Chatzimanolis, 2018; Janák and Bordoni, 2015; Liu et al., 2021). The Staphylinidae have undergone several revisions at various scales but further research is needed as the complete evolutionary history of the Staphylinidae remains unclear (Mckenna et al., 2015a). At a deeper phylogenetic level some subfamilies are well understood (some better than others) and several species groups and genera have well resolved phylogenies (Brunke et al., 2021; Chatzimanolis, 2008). However genetic representation is poor with an average of 0.062 COI sequences per described species within the family (Gusarov, 2018). Another example of staphylinid underrepresentation occurs in Canada, a temperate and northern nation comparatively rich in staphylinid taxonomists. However, even here the number of staphylinid species has increased by over 90% within the last 40 years, with more species

expected to be described (Brunke et al., 2019). If this is the rate of discovery in the north in the first decades of the 21st century, consider what proportion of diversity remains undescribed in the tropics. Taxonomic revisions are harder in the tropics where comprehensive cataloguing of biodiversity has only recently begun. Recent accelerations in tropical descriptions can be (at least in part) due to the development of barcoding initiatives within these regions (Basset et al., 2004). With numerous ecosystems and microclimates within a comparatively small area to other biomes, the tropics have developed sharp ecological boundaries. Consequently, these boundaries in the tropics can create a tight relationship between elevation and diversity in a manner unlike temperate systems. In the tropics, biodiversity is generally correlated with elevation (Pianka, 1966), and endemism concentrates towards higher elevations often 1,000-2,000 MASL (Betz et al., 2020; Fu et al., 2006; Musthafa et al., 2021). The community assemblages of tropical mountain ranges have long been hypothesized to be some of the most biodiverse systems on the globe (Janzen, 1967).

With over 63,000 species of Staphylinidae currently described across 32 extant subfamilies (Irmiler et al., 2018; Tihelka et al., 2020), with a global distribution including arctic (Lohse et al., 1990), tropical (Sakchoowong et al., 2008), taiga (Belskaya and Kolesnikova, 2011), and intertidal marine (Moore and Legner, 1976), habitats, and more (Irmiler et al., 2018).

Improving the representation and visibility of Staphylinidae will only further our understanding of an ecosystem's biodiversity and health as they have been argued as good indicator taxon (Anderson and Ashe, 2000; Bohac, 1999; Klimaszewski et al., 2018). This is attributed to the multiple roles that staphylinids play in key ecological processes such as nutrient cycling (Makranczy, 2006), pest control (Kishimoto and Adachi, 2008; Kollat-Palenga and Basedow, 2000), ecological succession (Pohl et al., 2008), edge effects (Tóthmérész et

al., 2014) and have even been used in forensic entomology (Mađra et al., 2014). The Oxytelinae is the eighth largest subfamily with 47 genera containing 1,975 species (Gusarov, 2018). Primarily found within leaf litter with a diet predominantly composed of dung and other decaying organic matter (Lü and Zhou, 2012). One genus of Oxytelinae, the *Anotylus* Thomson 1859, have been called a “left over” genus since no singular morphological character was unique to the group (Herman, 1970). However, more recent research indicates that they are best identified by the crest shaped structure on their scutellum (Hammond, 1976a). The 90 members of the *Anotylus* have a cosmopolitan distribution but the greatest concentration of diversity is seen in the Neotropics (Herman, 2001; Makranczy, 2011). The last catalogue of Staphylinidae biodiversity reported two species of *Anotylus* in Costa Rica (Herman, 2001), *Anotylus insignitus* (Gravenhorst, 1806) which is an invasive species, and *Anotylus nitescens* (Bernhauer, 1942) an endemic species. The former possesses a large distribution throughout The Americas and several Atlantic and Pacific islands (Blackwelder, 1943; Hammond, 1976a), however like many older species’ descriptions it is likely several discreet species with smaller distributions (Burns et al., 2009) Whilst the latter has no reference in scientific literature besides brief acknowledgement in reviews and its original species description with its distribution restricted exclusively to Costa Rica (Bernhauer, 1942). Individuals within the genus range in length from 1-6mm (Herman, 1970), but species and subfamilies are known to be sexually dimorphic regarding conspicuous characters (Makranczy, 2017). As our understanding of the genus has improved, alongside the establishment of integrative taxonomy, the *Anotylus* are now primed for taxonomic revision with barcoding highlighting where the most progress can be made.

One solution to the taxonomic impediment was the advent of barcoding, whereby a short standardised fragment of DNA is used to identify and describe species (Hebert et al., 2003). One key element of DNA barcoding is that the DNA fragments are stored and available in a publicly accessible database (The Barcode of Life Datasystem (BOLD)) (Ratnasingham and Hebert, 2007), often prior to publication. An invaluable function of BOLD is the automatic generation and assignment of a provisional molecular operational taxonomic unit (MOTU) (Blaxter et al., 2005), dubbed a Barcode Index Number (BIN) which clusters sequences (Ratnasingham and Hebert, 2013). This allows swift species identification when sequences from specimens have been previously identified within the database. Therefore rapidly produces preliminary, and testable estimations of biodiversity, and additionally identifies uncatalogued Evolutionary Significant Units (ESUs) especially within a region or taxa where taxonomic framework is poor or non-existent (Bergsten et al., 2012; Ortiz et al., 2017). The BIN algorithm in BOLD can highlight taxa in need of revision and consequently enables rapid species' descriptions where needed (Hebert et al., 2004; Pentinsaari et al., 2019). However, there are not enough taxonomists to process the sheer volume of existing data (Pante et al., 2015), nor are they sufficiently funded to do so (Engel et al., 2021). It is therefore not uncommon for undescribed species to wait decades between their discovery and formal description (Fontaine et al., 2012). Clearly there is a need to examine candidate taxa from provisional species like BINs to identify those that warrant formal descriptions and further research. Without such an approach, undescribed species will remain unprotected and neglected in conservation management strategies (Deichmann et al., 2017), thus increasing their vulnerability and likelihood of extinction (Liu et al., 2022).

My aim in this study was to provide a preliminary review of Costa Rican *Anotylus* using an integrative approach combining molecular and morphological characters to identify

potentially undescribed species. I did this by first using BINs as putative species hypotheses, and then identifying where molecular and morphological variation both supported the existence of ESUs.

Methods

Using this integrative taxonomic approach of both molecular and morphological characters testing predesignated BINs as species hypotheses, was possible because of a decade of collections made across a 1,500 m elevational gradient in the Área de Conservación Guanacaste (ACG) in north-western Costa Rica, 360° images of sample sites are available on each specimen's BOLD page.

The ACG is a UNESCO World Heritage Site that covers 1,470km² of north-western Costa Rica and three inactive volcanoes reaching a peak elevation of 1,900MASL. Across this elevational transect three distinct forest types are found (cloud, dry and rain), and up to eight Holdridge Life Zones (Janzen et al., 2011). Dry forests dominate the lower elevations (10-600MASL) with warm and dry environments, rainforests reside in mid-elevations (700-1200MASL) with predominately warm and wet climates, and lastly at the highest elevations (1300-1500MASL) are cloud forests which are cool and wet (Dolson et al., 2021). In small isolated tropical mountain systems like the ACG stable climatic conditions are common and therefore sharp changes in temperature and precipitation across elevations are expected, consequently creating sharp ecological boundaries and higher biodiversity than temperate systems (Janzen, 1967). It is estimated that 4% of global terrestrial biodiversity can be found here (Janzen, 2004).

To identify the genetic variation and depth of divergence within the community of staphylinids I first estimated the group's phylogeny using the COI gene to create a Bayesian

inference tree to identify the depth of divergence between individuals and BINs and then created a p-distance pairwise matrix between BINs to estimate the nucleotide variation. To estimate the morphological variation occupied by these genetic clusters, I measured 16 characters ranging from body size to cuticle lightness and visualised these using Principal Components Analysis (PCA), to assist in the identification of key characteristics that differentiate. This exploratory analysis will help identify potential candidate species in need of further investigation as well as delimiting characteristics capable of assisting formal descriptions for this diverse assemblage of tropical staphylinids in a global biodiversity hotspot.

Staphylinidae were collected over a nine-year period using various trapping techniques across an elevational transect from sea level to the Cacao volcano summit at 1,500MASL. Briefly, sampling was standardised for site size, time, and intensity, collecting invertebrates using a combination of active searching, peanut butter cookies and canned tuna bait, Davis-sifting, pitfalls traps, and Winkler extractors. More detailed methods are described elsewhere (Dolson et al., 2021; Smith et al., 2014). Specimens were preserved in 95% ethanol and later stored at -20°C. DNA was extracted from one leg/ specimen using standardised methods (Ivanova et al., 2006), and mitochondrial DNA 5' COI was amplified with primers designed by Smith and Fisher (2009). Staphylinidae were identified to subfamily and genera using keys from North America (Brunke et al., 2011), and Mexico (Navarrete-Heredia et al., 2002). When possible, samples were identified to a finer taxonomic scale by A.J. Brunke. All sequence and collection metadata have been deposited in BOLD, whilst physical specimens are stored within the Research Collection of M. Alex Smith at the University of Guelph.

For every individual identified as a member of the genus *Anotylus* (210), their COI DNA sequence and any available images from BOLD were extracted (<https://doi.org/10.5883/DS-ASSTAPHY>). Provisional MOTU's are automatically generated in BOLD using a five-step process by clustering sequences using their uncorrected pairwise distances in a Refined Single Linkage algorithm (RESL) (Ratnasingham and Hebert, 2013). A standardised threshold of nucleotide diversity is used to partition sequences and is altered in a later stage to optimise effectiveness. I used BINs as my species' hypotheses utilising the unified species concept (De Queiroz, 2007), in which species are primarily defined as lineages and the operational criteria used to distinguish these lineages is secondary. I chose this species concept for its practical flexibility, and shared values with the ideals of integrative taxonomy.

Only sequences with a base pair length greater than 300 were used, resulting in 201 individuals including the outgroup (one sympatric *Oxytelus* specimen) for the analysis. Sequences were aligned in MEGA11 (Tamura et al., 2021), using the MUSCLE alignment algorithm (Edgar, 2004). To identify the evolutionary substitution model of best fit I used MEGA 11 (Tamura et al., 2021), which suggested the General Time Reversible (GTR) model (Tavaré, 1986), with gamma frequency distribution and invariant sites (G+I), which has been suggested as the standard in phylogenetic reconstruction (Abadi et al., 2019). A Bayesian tree was produced in Geneious 11.0.3+7 (Kearse et al., 2012), with the MrBayes Plugin version 3.2.6 (Huelsenbeck and Ronquist, 2001). 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 utilised 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 7,500 bootstraps. Trees were rooted on this individual

and then visualised in FigTree version 1.4.4 (Rambaut, 2018), then further developed in Inkscape (Inkscape Project, 2022).

To quantify each BIN's morphospace, I made measurements of each individual from z-stacked photographs for 16 different characters (Figure 2): Largest antennal segment length (1), total antenna length (2), body length (3), compound eye area (4), compound eye diameter (5), compound eye width (6), head width (7), temple length (8), pronotum width (9), pronotum length (10), pronotum area (11), elytra width (12), elytra length (13), elytra area (14), pronotum lightness (15), and elytra lightness (16). Measurements 1, 2, 4, 5, and 12-14 have been used previously to delimit species within the subfamily Oxytelinae (Hammond, 1976b). The remaining measurements; 3, 6-10, and 15 have also been used as descriptive characters in the *Anotylus* genus (Wang and Zhou, 2020), since pronotum width, length and lightness have been used previously the same measurements on the elytra were also used to differentiate BINs. Individual size was not accounted for in data collection as the PCA code used later automatically standardised the data set.

Measurements were taken from focus-stacked dorsal and lateral photos using ImageJ (Schneider et al., 2012). Elytra and pronotum lightness were collected from three dorsal cross sections of each feature using the “color histogram” plugin for ImageJ (Prodanov, 2010), and later converted from RGB values into HSV using the “dplyr” R studio plugin (Wickham et al., 2009), in R (R Core Team, 2021). Lastly, an average of the three values were taken as a representative sample of the character. Each sample’s BIN was added to the data frame post-collection to reduce potential confirmation bias.

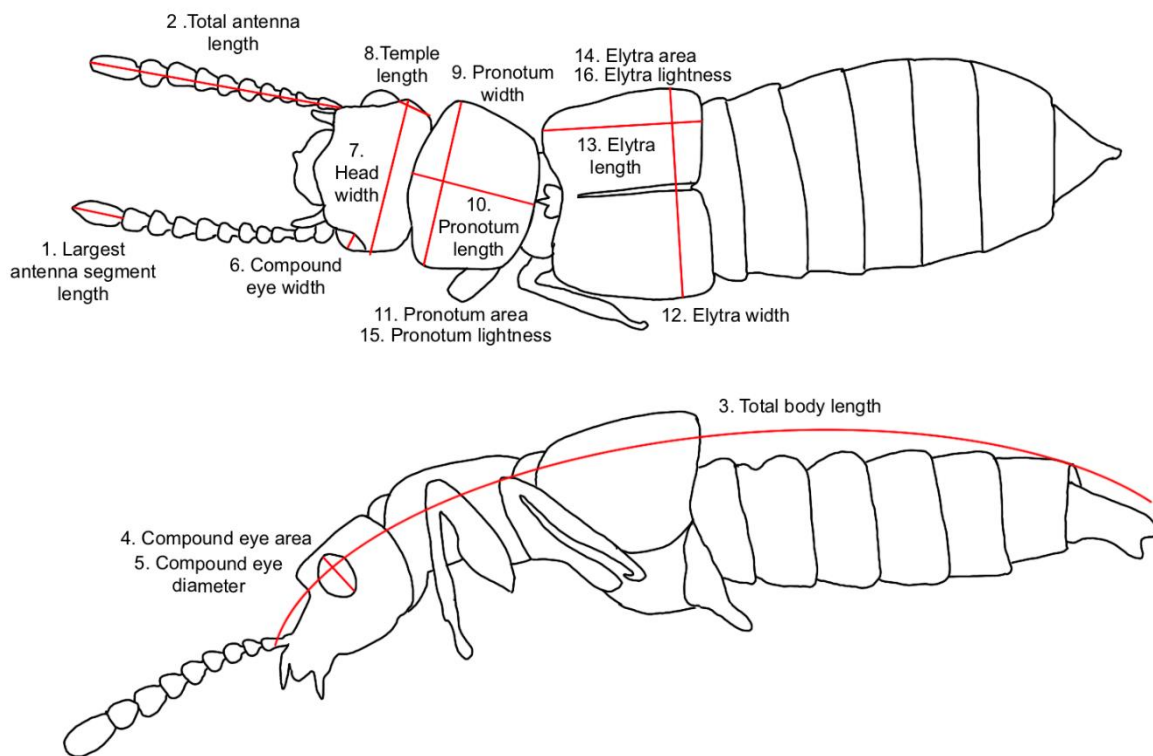


Figure 2. Key morphological measurements labelled on a sample specimen. Red lines indicate how the measurement was taken. Measurements of areas (4, 11, and 14) were recorded using the area visible in images. Lightness (15 & 16) were measured by taking the average of three cross-sections of the character.

A Principal Component Analysis (PCA) was performed in R (R Core Team, 2021), and RStudio version 4.1.2 (RStudio Team, 2020) using the “FactoMineR” package (Lê et al., 2008). To maximise the number of specimens in the analysis, I used individuals which possessed measurements for at least 75% of the characters. This consequently increased the number

of individuals in the analysis from 127 to 177. Since PCAs require complete data sets, values were generated for absent data using the `imputePCA` function within the R package “`missMDA`” (Josse and Husson, 2016). This works by using an iterative PCA algorithm (Kiers, 1997), 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 I used a regularised iterative PCA algorithm (rPCA) (Verbanck et al., 2015), which assumes consistent and mostly complete data sets. Therefore, before analysis I calculated the percentage of the complete data set (96.71%). Furthermore, I used the K-fold cross validation method with 5,000 simulations as it identifies the lowest mean square error of prediction in large data sets when the known percentage of data is missing. PCA plots were visualised using the “`ggplot2`” R package (Wickham et al., 2009), and further refined in Inkscape (Inkscape Project, 2022).

Here I use the definition of ESUs as used by Fraser and Bernatchez (2001), in which an ESU is a lineage with restricted gene flow displaying neutral or adaptive variation that differs from organisational groups below the species level. To identify any ESUs in this study I created a figure that identified if molecular and morphological variation between BINs coincided. To achieve this, I created a boxplot in R for the most variable morphological character from the PCA analysis, I then aligned this alongside the BI phylogeny so that the morphological character of each BIN was adjacent to its position in the phylogeny. This allowed any correlated variation between different characteristics to be quickly identified. Finally, a high-quality photo of a specimen from each BIN was placed at the end of the figure as a visual representative.

Results

A 658bp fragment of the COI gene from 200 *Anotylus* specimens were downloaded and aligned. Some individuals possessed incomplete sequences, however 159/200 sequences possessed more than 650bp, and 178/200 with more than 600bp. Individuals were assigned to their predesignated BINs from BOLD (Table 1).

Table 1. The number of individuals and the elevational ranges (metres above sea level) for each Barcode Index Number (BIN).

<u>BIN</u>	<u>Elevational Range (MASL)</u>	<u>Number of Individuals</u>
BOLD:ACZ4529	0-100	4
BOLD:ACZ5516	300-1,500	112
BOLD:ACZ5742	300-1,350	4
BOLD:ACZ5987	1,000-1,050	1
BOLD:ACZ6447	300-750	2
BOLD:ADF3772	750-1,350	38
BOLD:ADF4138	950-1,300	3
BOLD:ADF4200	1,150-1,300	3
BOLD:ADF8741	1,050-1,350	10
BOLD:ADG0617	1,300-1,350	1
BOLD:ADG1201	1,000-1,500	10
BOLD:ADH8436	1,150-1,500	2
BOLD:ADH9095	1,150-1,200	2
BOLD:ADH9620	1,300-1,350	1
BOLD:ADH9622	750	1
BOLD:ADI3175	1,150-1,350	3
BOLD:ADL5474	1,450-1,500	2
BOLD:ADR2790	1,000-1,050	1
Total		200

Corrected average pairwise distances between BINs ranged from 5.03% (between BOLD:ADG1201 and BOLD:ADG0617) to 27.9% (BOLD:ADH9622 and BOLD:ADR2790), with a median of 19.26%. Whilst the intra-BIN variation remained low, BOLD:ACZ5516 was the largest BIN (N=96) and expectedly had the greatest intra-bin variation at 0.75%. The three least abundant BINs (not including those represented by singletons or doubletons), had an intra-BIN variation of <0.01%, 0.09%, and 0.15%. The median intra-BIN variation was 0.38% (excluding doubletons and singletons). Meanwhile uncorrected pairwise distances within each cluster ranged from 0.08% to 0.72% (median 0.38%), and from 4.5% to 18.8% between clusters (median 13.0%).

The cumulative variation exhibited across the first two principal components (PC) was 78.97%. With PC.1 accounting for 68.73% of total variation which best represented the first 14 morphological characters which were all morphometric. Elytra area was the most variable character within the most variable PC, and therefore best represented the variation amongst individuals. The remaining two characters, lightness of elytra and pronotum were represented in PC.2 with 10.24% of total variation. From the dispersion of individuals in the PCA, PC.1 had the greatest discriminating power amongst BINs whilst PC.2 primarily accounted for variation within BINs (Figure 4.A). Some variables were best represented alongside other characters but possessed an independent element of variation expressed in different PCs. These included largest antennae segment which was slightly more represented in PC.1 than PC.3, temple length, which was represented in PC.1, PC.5, and PC.4. and lastly eye width was strongly attributed to PC.1 but was not fully represented amongst the first five PCs (Supplementary figure 1). Confidence ellipses require a minimum of four data points, of the 18 BINs only six met this criterion. Whilst some overlap was visible between most BINs, others (BOLD:ADL5474 & BOLD: ADH9622) clearly rested on the

periphery of the PCs and were easily differentiated using the morphological characters measured. For example, BOLD: ADH9622 was distinguishable by its extremely light cuticle, whilst BOLD:ADL5474 possessed an overtly dark cuticle. The overlapping BINs could be differentiated, BOLD:ACZ4529 and BOLD:ADF3772 showed extremely little variation across PC.1 and overlap, but was easier to differentiate using the cuticle lightness associated with PC.2. Both BINs could be differentiated from the other abundant BINs with confidence ellipses. Of which BOLD: ACZ5742 & BOLD:ADG1201 heavily overlapped in morphospace but could be distinguished from BOLD:ADF8741, especially BOLD:ACZ5742. However, it is difficult to differentiate individual BINs when visualising BOLD:ACZ5516 as well, since it occupied a large central position in the PCA, with the largest confidence ellipses which overlapped all but two of the other BINs (Figure 4.B). To allow easier interpretation BOLD:ACZ5516 was visualised alongside all other BINs (Figure 4.B), and independently (Figure 4.C). When visualised independently, the large variation was due to a molecular intra-BIN division of two groups (Figure 4.D). No other BINs in the analysis demonstrated a similar split in the PCA.

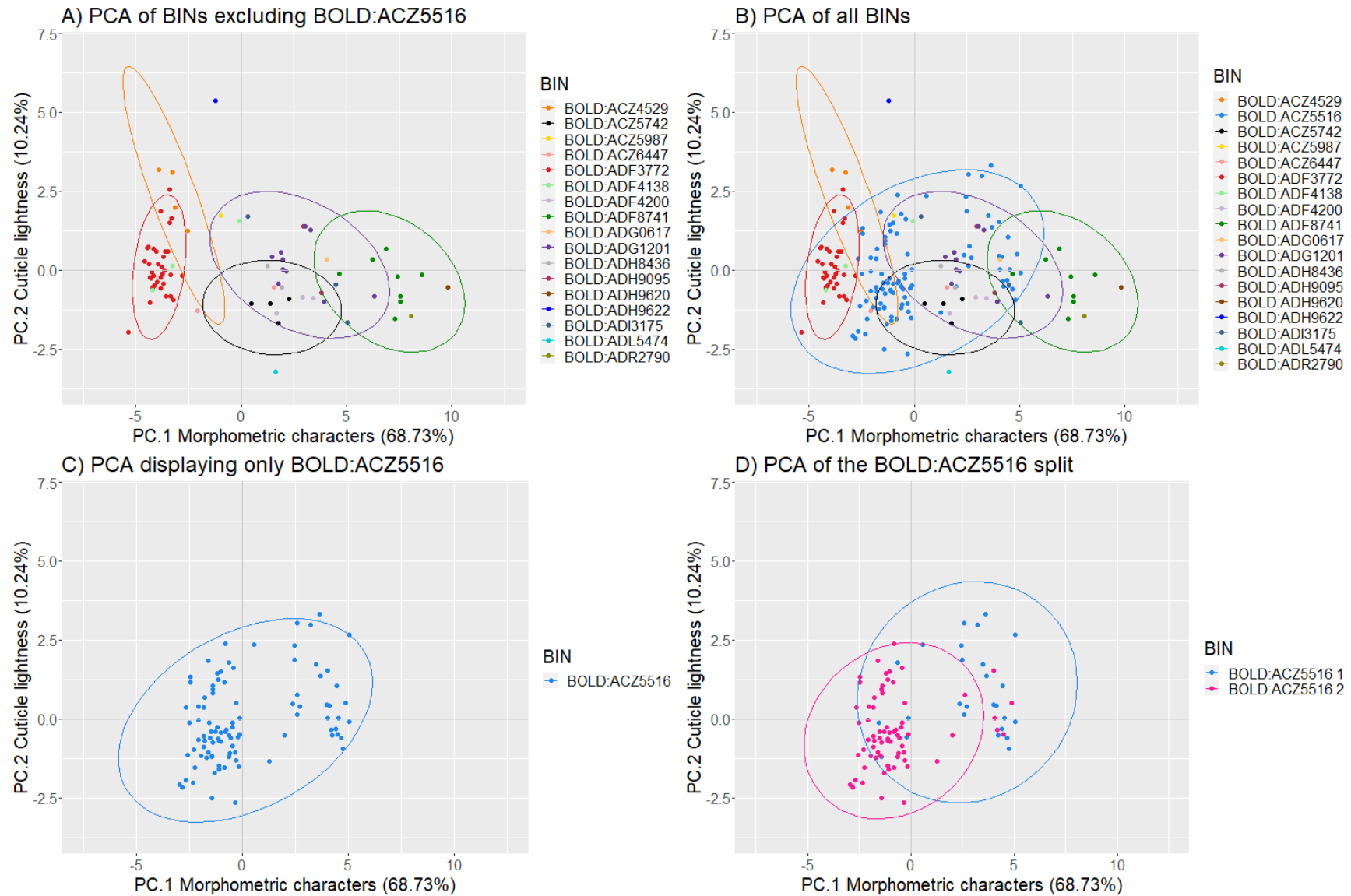


Figure 4. Principal component analysis of A) all Barcode Index Numbers (BINs) except BOLD:ACZ5516. B) All BINs. C) Just BOLD:ACZ5516. D) The split of BOLD:ACZ5516 using molecular sub clusters to differentiate individuals. PC. 1 best represents morphological characters 1-14 whilst PC. 2 best represents characters 15-16. Subfigures were all created with the same data output and only the BINs visualised differ.

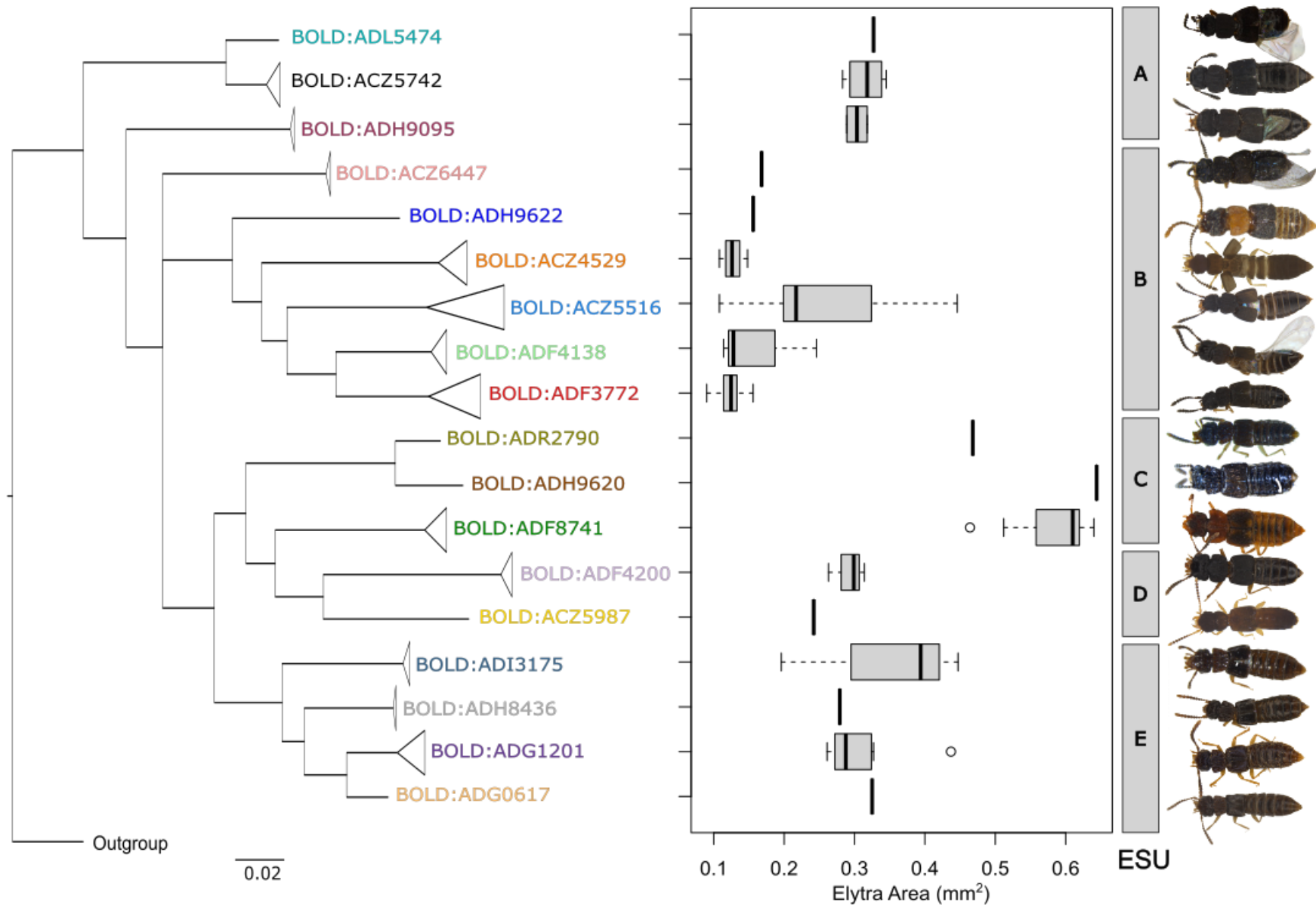


Figure 5. Phylogenetic tree of Barcode Index Numbers (BINs) with boxplot of elytra area variation (identical topology and bootstrap values to figure 3). Bars besides the boxplot denote Evolutionary Significant Units (ESUs)/groups in which molecular and morphological variation coincide. Example photo of a specimen from each BIN is included to allow visual comparison (images not to scale).

The BINs generated by BOLD yielded valid support in the phylogenetic analysis to be treated as ESUs. When combined with the morphological characters used here it is difficult to differentiate individual BINs. However, morphological and molecular variation coincides for five distinct groupings, which indicate the presence of putative species (Figure 5). Group A has the deepest divergence within the phylogeny and is composed of BOLD:ADL5474, BOLD:ACZ5742, and BOLD:ADH9095. All three BINs lack variability of elytra area. Group B has a clear morphological grouping with the smallest elytra area which correlates strongly with the phylogenetic clade. Notably, the Bayesian phylogeny suggests a non-nested community, however as mentioned earlier the Maximum likelihood's topology included BOLD:ACZ6447 within the BOLD:ADH9622 cluster. The variation of morphological characters within the group supports the inclusion of BOLD:ACZ6447 within the genetic cluster. This phylogenetic clade has a clear correlation with small elytra areas however BOLD:ACZ5516 had the largest variation both phylogenetically and morphologically. As seen in the PCA (Figure 4.C) it possessed two distinct groupings. When retroactively compared to the phylogeny, one of the morpho groups corresponded highly with a genetic sub cluster nested within the BIN, suggesting a split (Supplementary figure 3, Figure 4.D). This divided the morphospace into two groups, BOLD:ACZ5516_1 had larger morphometrics whilst BOLD:ACZ5516_2 had smaller morphometrics (Supplementary figure 4, Figure 4.D). Group C is clearly differentiated from the other groups by possessing the largest elytra areas. With BINs BOLD:ADR2790, BOLD:ADH9620, and BOLD:ADF8741, the group is paraphyletic due to the contrasting elytra areas of Group D which are nested within Group C's phylogenetic clade. Group D was the smallest group that can be

distinguished from the others with only BOLD:ADF4200 and BOLD:ACZ5987. This group cannot be easily distinguished morphologically from group E, yet its position in the topology indicates a recent divergence from group C. Group E, possess similar elytra areas to Group A but had a much greater variation within individuals BINs and is the most genetically distant clade from Group A.

Discussion

My results imply that there is a clear association between the morphological and molecular variation of most clades, conservatively indicating the presence of at least five ESUs. While unable to be identified to a species level, gross molecular and morphological boundaries can be identified, and in the absence of formal species' descriptions these groupings are best described as ESUs (Vogler and Desalle, 1994). The phylogenies varied slightly in topology but were well resolved with the only difference being the removal of a polytomy, whose relocation is supported by morphological characters. The deeper phylogeny expectedly had weaker node support due to the genetic markers used. Group A cannot be distinguished morphologically from groups D and E, since group A has the earliest divergence in the phylogeny it indicates that this is possible that the plesiomorphic state that has been retained in groups D and E. With tropical mountain ranges possessing a myriad of stable microclimates the biodiversity of each mountain is likely different (Janzen, 1967), with mountain peaks themselves housing endemic species. The ACG has at least two other volcanoes within the protected area which have yet to be investigated, let alone the expansive mountain ranges in the rest of the country. However, the split within BOLD:ACZ5516 suggests a greater level of variation within ESUs and BINs than we have identified.

The two distinct morphological groupings that occur in BOLD:ACZ5516 are interesting and suggestive of possible unidentified species that fall below the genetic variation threshold used to generate a BIN. No other BIN demonstrated an intra-BIN division in their morphospace or phylogeny. Sexual dimorphism has been observed in other *Anotylus* (Makranczy, 2011; Yue et al., 2012). If this was an example of dimorphism or potential speciation with a genetic cut-off lower than that used to calculate BINs, we might expect clustering within the BIN and such variation was evident in the phylogenetic tree. I re-ran the PCA treating these subclusters as separate BINs, and found that these strongly correlated to the separate morphospace clusters (Figure 4.D). This is likely a morphological division with subtle genetic support less than the generated BIN cut-off. Indeed, finding valid species within a BIN has previously been observed in both Coleoptera and Lepidoptera (Hendrich et al., 2015; Janzen et al., 2011). It is possible that the BIN is experiencing an ongoing sympatric speciation event. The groups occupy nearly identical elevations and geography and are unlikely to be factors driving divergence, and it is currently unclear what is. The presence of genetic individuals in each other's morphospace may be a result of mtDNA introgression, a not uncommon circumstance with young species and one that has been observed in staphylinids (Assing, 2017; Audisio et al., 2009). Furthermore, in some morphologically distinct invertebrates genetic differentiation can only be achieved using a single diagnostic nucleotide (Burns et al., 2007; Smith et al., 2008, 2007). Ultimately BINs are provisional species and their generation algorithm can both over and under split known species. The results suggest that BOLD:ACZ5516 is likely an example of the latter. It is worth noting that the smaller of the BOLD:ACZ5516 subgroups becomes the third largest group in the sample. Therefore, other potential candidate species may be hiding in the other BINs with smaller sample sizes, this may

also explain the large intra-BIN variation seen in some groups. Whilst the point at which speciation is complete is difficult to define, separating the BIN into two taxonomic units is supported under the unified species concept.

Whilst clear divisions between groups can be seen, this study does not possess the power to differentiate individuals to a species level. This has likely been a combination of limited statistical power from low sample sizes, and a lack of species differentiating characters used in the morphological analyses. Whilst spanning a large transect over a nine-year period, the sample set has yielded few samples for some BINs, with 9/18 BINs represented by singletons or doubletons. However, in tropical ecosystems this is not uncommon with traditionally taxonomic distinct species often only sampled or described with a single specimen (Escobar et al., 2005; Lim et al., 2011; Stork and Grimbacher, 2006). This is likely due to small population sizes and/or geographic ranges that result in few specimens especially from indiscriminate sampling methods. It is therefore possible that BOLD may have over split BINs due to limited samples, especially when the intraspecies variation exceeds the artificial barcode variance threshold. Increasing sample sizes by broadening geographic ranges may consequently merge BINs (Brunke et al., 2019). Alternatively, it is sometimes unfeasible to expand the data set, in such cases adjusting the default species hypothesis so that species are treated separately until evidence supports otherwise improves the visibility of singleton species that would historically be overlooked (Smith et al., 2011). This mitigates the bias towards underestimating the true species richness within tropical regions as a result of under sampling (Coddington et al., 2009). Furthermore It is known for singleton species to receive vindication decades after their formal description (Amato et al., 1999). The second limitation within this study is that the

morphological characters used provided limited differentiating power, this was due to a lack of species differentiating characters. Investigating alternate characteristics such as behaviour, ecology, elevation, and food sources may define clearer species' boundaries. However, even with the basic morphometric characters used here, clear groupings are visible and exceed the number of known species of *Anotylus* in Costa Rica. Even with limited sample sizes and species defining characters the presence of distinct ESUs can be seen and indicate a greater species richness for Costa Rican *Anotylus* than previously believed, further research with broader geographic samples sizes and descriptive characters will only increase the groups representation and number.

Here we see five separate ESUs which doubles the previously thought *Anotylus* biodiversity in Costa Rica. This coincides with research that *Anotylus* diversity is greatest towards the Neotropics (Herman, 2001; Makranczy, 2011), and that species within the genus are known to exist in sympatry despite possessing small distributions (Hammond, 1976a). Future studies that use more descriptive characters will likely facilitate formal species description and further divide the ESUs found here. Studies in China and Korea on the Micropeplinae subfamily have reported high congruence of species boundaries between barcoding and traditional morphological characters (Lee et al., 2020; Tokareva et al., 2021). If these relationships extend to the ACG, then there is a wealth of diversity yet to be identified, as a recent study reported 380 staphylinid BINs within the region (Dolson et al., 2021). The underrepresentation of the Staphylinidae in the ACG may now potentially be quantified. Ultimately the identification of more taxonomic units expands our understanding of community structure, regional biodiversity and enables future research on the organisms

themselves. This better informs conservation management strategies as improving the taxa's visibility raises attention to their conservation status and ecological importance. This is especially important as invertebrates are often overlooked in the management strategies of protected areas (Chowdhury et al., 2023). However, this is impossible without a name, and like many previous studies on the Staphylinidae, clear distinct taxa are visible but formal description is beyond the scope of this work.

Whilst molecular and morphological variation coincide to identify ESUs, the ability to differentiate morphospecies between individuals BINs is not possible with the characters used here. To better differentiate morphospecies future research should look at alternate characters. Deeper ancestral nodes in the phylogeny possessed low node support, expected in phylogenies using rapidly evolving genes like COI. Reliable phylogenetic studies utilise numerous different genetic markers (Cryer et al., 2019; Leavitt et al., 2013). Genetic markers such as 18S and 28S have shown promise in staphylinid phylogenies (Chatzimanolis, 2014; Mckenna et al., 2015b), and may be useful for future work in the *Anotylus*. Additionally this may help identify if the BOLD:ACZ5516 split is undergoing mitochondrial introgression as eight individuals resided in the alternate sub-groups morphospace. Morphological characters such as the shape and structure of both the genitalia (especially the endophallus) and the crest-shaped impression on the scutellum have previously illuminated species level variations (Herman, 1970; Makranczy, 2011), due to limited access to the physical specimens this data was unavailable for this study. The genitalia have been argued to evolve as fast as the COI gene in invertebrates (Ortiz et al., 2017), and therefore act as an effective character for differentiation in congruence with barcoding. Furthermore, this would provide the ability to identify if these BINs are described or undescribed

species, since traditional *Anotylus* species' descriptions rely heavily on these characters (Makrarczy, 2011; Wang et al., 2017). The two known species of *Anotylus* in Costa Rica are not represented in the BOLD database, and their identification will help improve our understanding of the regions biodiversity, especially if neither are present in this study's dataset. Expanding the dataset so that smaller BINs are better represented should be considered, a targeted sampling method focusing on Staphylinidae may not only improve statistical power but may merge or identify new BINs. Furthermore, future study on the taxa should utilise a serialised PCA method to identify the delimiting characters between individual BINs, this method has shown promising results in identifying separate species whilst also acting as an effective aid for constructing identification keys (Kucharczyk et al., 2012). Ultimately all the above should provide the adequate tools to allow new formal species' descriptions and improve the visibility of this overlooked taxa.

Using an integrative taxonomic framework of molecular and morphological characters I have identified five ESUs that possess clear partitions. This contributes to resolving a facet of the taxonomic impediment, by improving the visibility of Neotropical Staphylinidae which are severely underrepresented in global biodiversity. Whilst a larger investigation using alternate diagnostic characters is needed, this project has identified over double the previously known number of taxonomic units within the *Anotylus* genus in Costa Rica, and laid the first brick on the road to fully review Costa Rican staphylinid biodiversity. At the very least I hope this work inspires future research on these overlooked invertebrates and that others may see the unique individuality they possess before they become another casualty to anthropogenic influences.

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Appendices

Appendix 1: R code and data for analysis

See attached files

“Appendix 1.1 R Code.r”

and

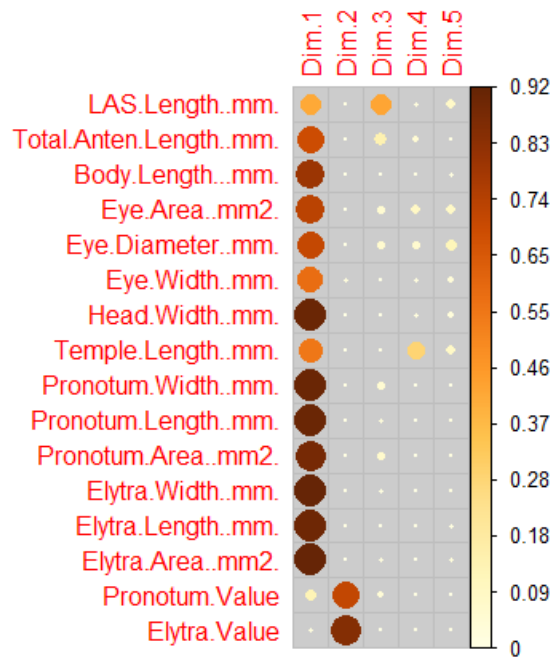
“Appendix 1.2 Data for R analysis.csv”

Appendix 2: Eigenvalues and Corrplot for Principal Component Analysis

Supplementary table 1: List of eigenvalues for each principal component/dimension in the analysis

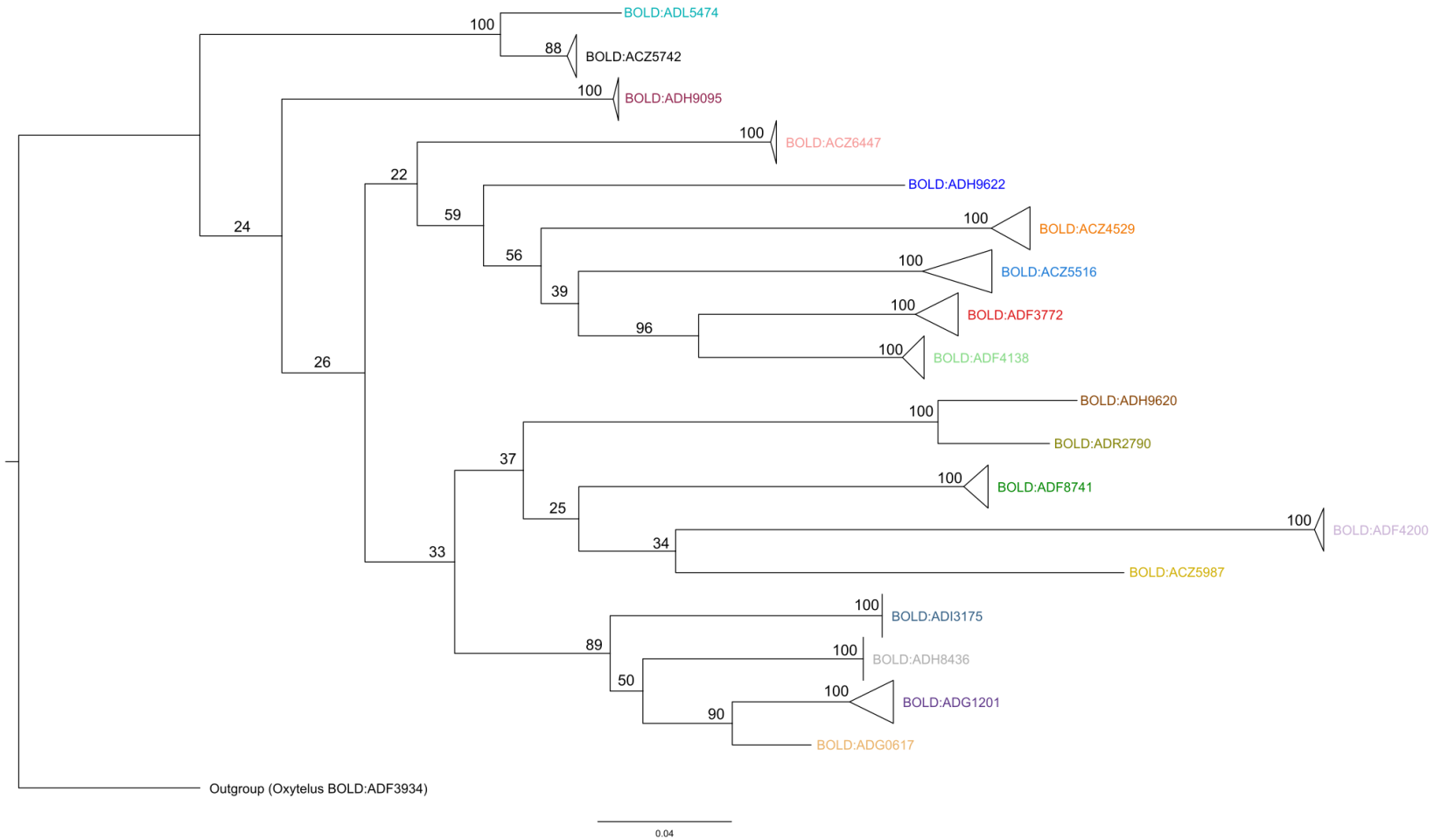
	Eigenvalue	Variance percent	Cumulative variance percent
Dim.1	10.997	68.729	68.729
Dim.2	1.639	10.244	78.973
Dim.3	0.920	5.752	84.726
Dim.4	0.583	3.646	88.371
Dim.5	0.555	3.469	91.840
Dim.6	0.437	2.729	94.569
Dim.7	0.243	1.522	96.091
Dim.8	0.195	1.219	97.310
Dim.9	0.145	0.905	98.215
Dim.10	0.080	0.503	98.718
Dim.11	0.061	0.384	99.101
Dim.12	0.057	0.354	99.455
Dim.13	0.039	0.246	99.701
Dim.14	0.028	0.178	99.879
Dim.15	0.013	0.081	99.960
Dim.16	0.006	0.040	100.000

Cos2 of variables for each Principal component



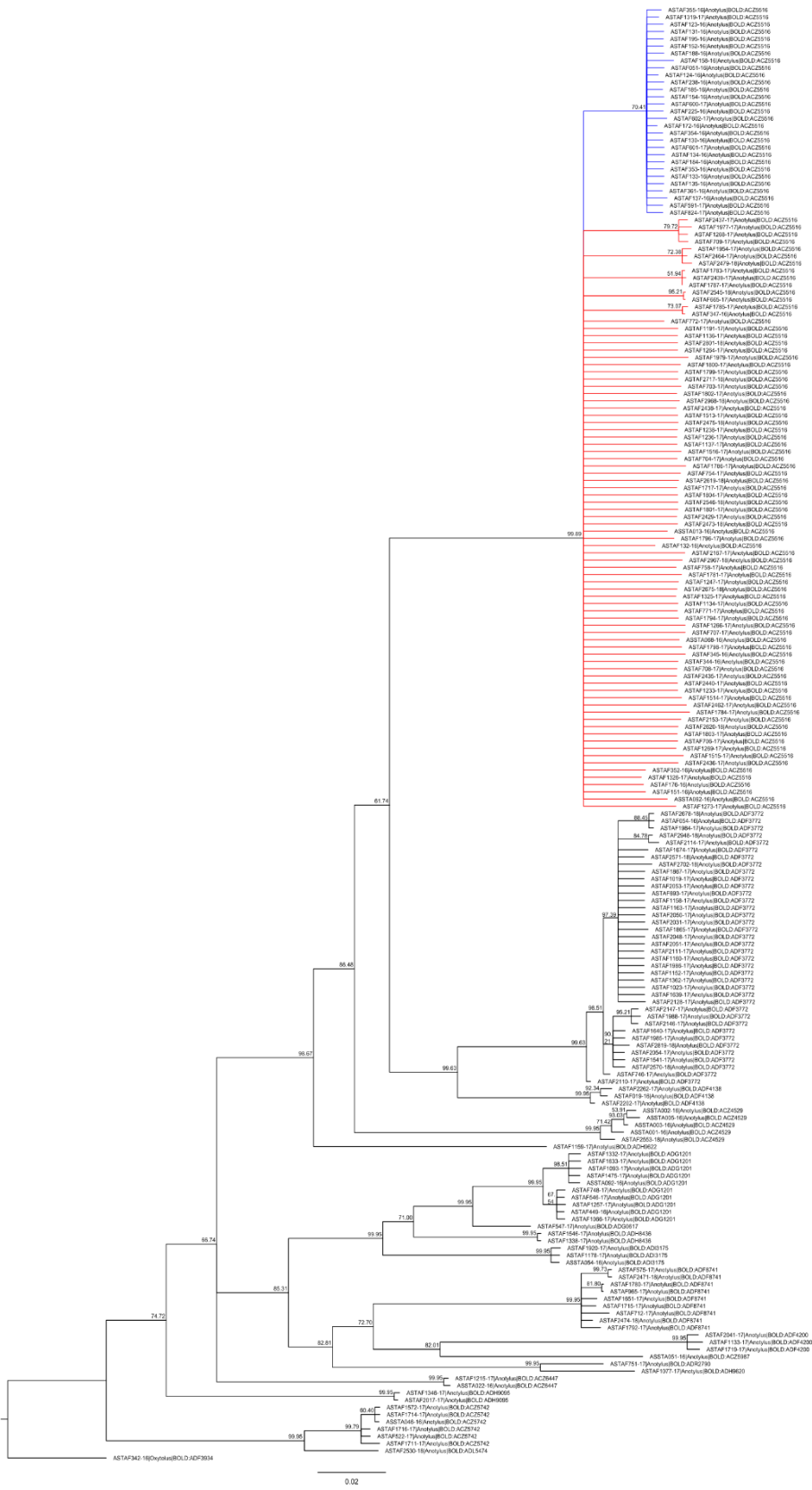
Supplementary figure 1: Corrplot with the proportional representation of each morphometric character within each principal component/dimension. Larger and darker circles demonstrate higher values within that dimension.

Appendix 3: Maximum Likelihood phylogeny

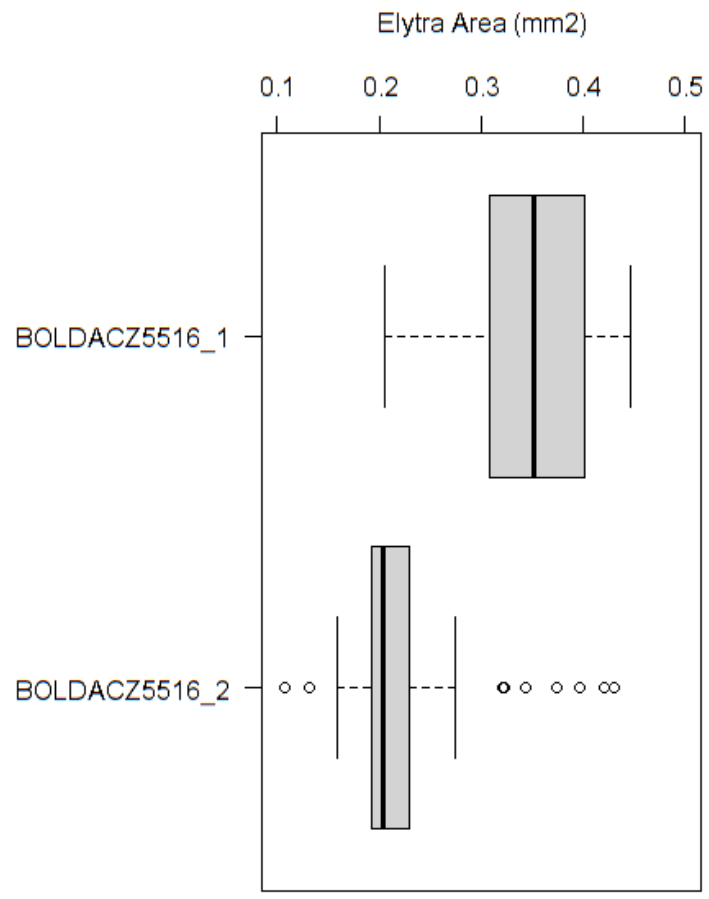


Supplementary figure 2: Maximum Likelihood phylogeny of *Anotylus* Barcode Index Numbers (BINs). Node support provided and individual BINs coloured separately. Topology identical to that of the Bayesian phylogeny except the polytomy involving BOLD:ACZ6447 is resolved.

Appendix 4: Evidence of a potential split within BOLD:ACZ5516



Supplementary figure 3. Complete uncompressed phylogeny of all individuals in the analysis. The largest BIN (BOLD:ACZ5516) is coloured in red and the distinct genetic sub cluster within the BIN coloured blue.



Supplementary figure 4. Box plot of elytra area, the most variable character within the Principal Component Analysis. Individuals were separated into the molecular subgroupings within the BOLD:ACZ5516 phylogeny.

Appendix 5: List of samples and affiliated data

See attached Microsoft Excel document titled “Appendix 4 Meta data.xlsx”