ABSTRACT

SORGER, DANIELA MAGDALENA. On Ants, Islands and Evolution. (Under the direction of Dr. Robert Dunn).

Islands have long inspired ideas of evolution. Both Darwin and Wallace developed their seminal works by studying islands. Here, I step into their footsteps and explore divergence and diversification on islands by considering non-traditional islands such as sand ridges, mountains, and forest patches. I explore how isolation influences populations and species in the light of modern integrative approaches that include natural history, morphology, behavior, and genetics. I study these questions through the lens of ants (Hymenoptera: Formicidae) as my study system, a diverse and almost ubiquitous insect group that is dominant in most ecosystems. My research operates at the interface of micro- and macroevolution where I document differences at the population-level that intersect with species-level divergence.

The first set of islands I explore are ancient inland sand ridges in Central Florida, about one million years old, that harbor a unique flora and fauna. A charismatic ant species (*Odontomachus relictus*) that is already considered one of the rarest ants in North America occurs exclusively on two of these ridges. I reveal genetic divergence of populations between ridges that points to species-level differences which suggests that these ants should be a conservation priority. I further document additional genetic isolation at the population-level within ridges.

Next I consider isolation and divergence along a climatic gradient on a tropical mountain in Borneo where diversity is vast and understudied. I consider morphological, chemical and genetic variation within a widely distributed trap-jaw ant species (*Odontomachus rixosus*). I discovered that it actually consists of a cryptic species complex

with two new species that I describe and name. My results show that only one of these species truly has a wide elevational distribution. In a next step I investigate divergence within this species (*Odontomachus saltans* sp. nov.) along the gradient using population genetic tools. While I did not find genetic structure, I found evidence of morphological structure consistent with macroecological theory. I also document a new behavior in these species, a leg-jumping behavior, in addition to the well-studied jaw-jumping behavior.

Lastly, I study sacred church forests in Ethiopia's highlands, forest islands of varying size in an otherwise barren landscape. Here, I encounter an ant species (*Lepisiota* sp.) that conspicuously behaves like an invasive species, defying the natural boundaries of these forest islands and instead using urban development and disturbance to spread. Despite exhibiting invasive ant characteristics similar to global tramp species like Argentine ants, my genetic results suggest that this species is native and is likely a "local invasive".

On Ants, Islands and Evolution

by
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APPROVED BY:

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DEDICATION

I would like to dedicate this work to those who are following their passion and to my fellow ant lovers who have managed to already do just that.

BIOGRAPHY

In 2006, I was just starting my last year at the University of Economics and Business Administration in Vienna, Austria, my hometown. I was focused on my goal to have a career in business as a management consultant in a big company and earn tons of money. – And then I went on my exchange semester to the University of Illinois Urbana-Champaign and decided to take a biology class in addition to my business classes, just out of curiosity, to get a glimpse of a different field since that is not something the Austrian university system typically encourages.

I graduated from business school with an MSc in International Business Administration in 2008. Three years later with a great deal of passion and determination to study ants for the rest of my life, two years of learning and working at the Natural History Museum in Vienna, a year of basic biology classes at the University of Vienna, a trip to Guatemala and Borneo…I am a biologist, exploring the secrets of nature, and looking for answers to exciting research questions.

What happened? – Honestly, I don't know. I fell in love with ANTS, randomly. No background in biology, no particular science exposure when growing up, nothing that could have suggested this sudden change in life focus.

And now I am the happiest person in the world – and I did not even know that a profession like this would exist for me; growing up and being taught that a job is something you need in order to survive and earn money, not necessarily something that should be fun. But let's face it – if you do something you love, there is a different energy to it with much more power and motivation that almost inevitably leads to success (because giving up is not an option anyways).

ACKNOWLEDGMENTS

First of all I would like to acknowledge the ones who have supported me from the very beginning when I was armed with nothing but my passion for ants and when pursuing this career seemed like a crazy idea. I had amazing support from people who believed in me, facilitated, encouraged and taught me: Dr. Chris Smith (Earlham College, Indiana), Dr. Herbert Zettel (Natural History Museum Vienna), Dr. Martin Pfeiffer (National University of Mongolia), Dr. Andy Suarez (University of Illinois Urbana-Champaign), Dr. Rob Dunn (North Carolina State University). Words cannot express how thankful I am for helping me make my (ant) dream come true.

Many more have offered emotional, intellectual, and technical support throughout this journey, to name just a few: Dustin Dowless (!), Christine Brown, Lauren Nichols, Jessica Stocking, Justa Heinen-Kay, and, especially in these last few months, Megan Thoemmes.

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CHAPTER 1: Cryptic divergence of native species among Florida's inland sand ridges: the case of trap-jaw ants (Hymenoptera: Formicidae: *Odontomachus*)

Prepared for publication

D.M. Sorger, W. Booth, and R.R. Dunn

Abstract

Florida's inland ridges originated as sand islands at a time when sea levels were significantly higher than they are now. These ridges (some up to 100 miles long) are home to endemic animals like the charismatic trap-jaw ant *Odontomachus relictus*. This ant occurs on only two of these sand ridges and lives in sand hill scrub habitat. Besides its high habitat-specificity, little is known about the life history of this ant or whether and how its life history differs between ridges. The two ridges have been separated for up to one million years, sufficient time for divergence to occur. We tested the hypothesis of divergence using genetic, morphological, and color data from a large sample of *O. relictus* from both ridges. Our results show mitochondrial genetic divergence at the species-level between ridges as well as between smaller geographic groups within each ridge. This genetic pattern was also present in the color analysis but not when considering morphometrics. Our genetic results suggest limited maternal dispersal. We discuss these results in light of conservation implications as these ants might represent two of America's rarest ant lineages each living in a highly threatened habitat.

Introduction

Islands have long inspired ideas of evolution. Both Darwin and Wallace developed their seminal works by studying the Galapagos Islands and the Malay Archipelago, respectively. Islands share a suite of characteristics that make them suitable for studying evolution and diversification, such as their relatively small size and distinct boundaries, the ability to serve as replicates, and, most importantly, geographic isolation (Losos & Ricklefs, 2009). These traits are not unique to water-locked islands but also apply to islands in a broader sense such as trees, lakes, cities, or mountain tops. Biologically, islands are then, not only land masses surrounded by water but, more generally, "any discrete habitat isolated from other such habitats by an inhospitable matrix" (Gillespie & Roderick, 2002). Another distinction relates to the origin of such islands, whether they are formed de novo with immigration as the primary agent of colonization ("Darwinian" islands) or if they have been separated from a mainland with a full complement of species intact (fragment islands). Over evolutionary time, both island types will result in endemics due to isolation (Gillespie & Roderick, 2002).

A prominent (and highly endangered) fragment island system is found in Florida's inland sand ridges (Fig. 1.1). These ridges are relictual shorelines and beach dunes (White, 1970). The youngest ridge (Atlantic Coastal Ridge) roughly corresponds to the current shoreline while the larger and older ridges are located in the center of the state (Opdyke, Spangler, Smith, Jones, & Lindquist, 1984). These ridges date back to the Pleistocene or possibly earlier (Opdyke et al., 1984; Weekley, Menges, & Pickert, 2008) and went through rounds of isolation from the mainland and intra-ridge fragmentation due to fluctuations in the sea level (Lamb & Justice, 2005; Webb, 1990). The ridges feature unique habitats such as sandhill scrub and are home to over 50 known endemic animal and plant species (M. Deyrup,

1990; Lamb & Justice, 2005), some of which are federally endangered such as the Florida scrub jay (*Aphelocoma coerulescens*), eastern indigo snake (*Drymarchon couperi*), sand skink (*Neoseps reynoldsi*), and plants such as the scrub palm (*Sabal etonia*) and Florida ziziphus (*Ziziphus celata*). The ridges are also highly threated by anthropogenic change as they are considered to be the best land for citrus orchards and prime land for urban development, particularly as sea levels rise (Sallenger, Doran, & Howd, 2012). While parts of the ridges are protected, the great majority (almost 90 %) are not. Similarly, many, perhaps most, of the species on these ridges have never been studied. As a result, some of the endemic species on these ridges may go extinct before they are discovered and named (M. Deyrup, 1989) like a recently named flightless mole cricket (M. Deyrup, 2005) and a colletid bee (M. A. Deyrup & Deyrup, 2011).

Another endemic animal recorded from Florida's ridges is the charismatic trap-jaw ant *Odontomachus relictus*. The genus *Odontomachus* has a worldwide distribution in the sub-tropics and tropics (69 extant species) and is represented by six species in the United States, four of which occur in Florida. All species in this genus possess peculiar mandibles that they can shut very rapidly (up to 60 m/s) and which allow them to propel themselves backwards to escape threats (Patek, Baio, Fisher, & Suarez, 2006). *O. relictus* lives in sandhill scrub habitat and occurs on only two sand ridges: the Lakes Wales Ridge and the Brooksville Ridge. These two ridges are the two largest and oldest ridges. Each ridge is about 180 km long and 100 m in elevation. The Brooksville Ridge is divided into Northern and Southern parts separated by the Withlacoochee River. *O. relictus* has only been recorded from the Southern Brooksville Ridge. The species was only recently described and the authors suggested that it might be a relict species from dry periods in the Pleistocene (M.

Deyrup & Cover, 2004). Besides its high habitat-specificity, little is known about the life history of this ant or whether and how its populations and biology differ between ridges. The Lake Wales and Brooksville Ridges have been separated for about one million years, sufficient time for divergence to occur among populations of *O. relictus* and other endemic species if dispersal among ridges is rare. On the other hand, the ridges are geographically close (40 km) and differ little (at least superficially) in the selection their environments are likely to pose such that the opposite is also possible, no isolation between ridges or geographic structure within them.

Here, we seek to test the hypothesis that genetic divergence exists between ridges and that, if such divergence exists, additional genetic structure exists within ridges. We examine species- as well as population-level differentiation in the mitochondrial gene Cytochrome Oxidase Subunit I (COI). The barcoding region of COI is often used to characterize species-level divergences in ants. A difference of 5 % or more is considered major and as a result lineages separated by this level of sequence divergence are often considered distinct species (Fisher & Smith, 2008). In addition, we consider a suite of morphological and color features to test if genetic divergence (if present) is accompanied by phenotypic differentiation.

Methods

Sampling

All *Odontomachus relictus* specimens were collected by DMS in June, July and November 2011, June and July 2012, and July 2015. Ants were collected during evening and night time via hand collecting using an aspirator. Specimens were stored in 99% ethanol for molecular and morphological analysis. In total, samples were collected from 118 locations, 35 on the

Brooksville Ridge and 85 on the Lake Wales Ridge (see Fig. 1.1 and Appendix A). Sampling locations did not always correspond to nest locations and no complete nests were collected. However, sampling locations were usually near nest sites and workers collected at one location likely belonged to the same nest.

Morphometrics & Color Analysis

100 specimens from 10 locations (10 workers each) were used for morphometric analysis (Fig. 1.2). Ten standard morphometric characters were measured and three indices were calculated (Table 1.1). All specimens were dry mounted on card triangles. Measurements were taken with a Dino-Lite AD-4113T digital microscope at 60x by help of the software Dino-Capture 2.0. Digital photographs for color analysis were taken with a Canon EOS 7D SLR digital camera (Canon EFS 18-55mm lens) in a box lined with white paper at standardized light conditions. Color information from three color channels, red (R), green (G), and blue (B), was extracted from six body parts, antennae (Ant), head (Head), thorax (Tho), anterior legs (AntLeg), posterior legs (PostLeg) and abdomen (Abd) from a subset of samples (32 individuals from 11 locations, 2-3 workers per location). Color data was digitalized by help of the program AntColorGrabber (developed by Eric Butler, Shaw University, North Carolina). Images were processed in Adobe Photoshop CS6; body parts were selected by use of the magic wand tool (tolerance: 15) with multiple grabs so that the majority of the body part was colored. Color and morphometrics data were scaled and centered and linear discriminant analyses (LDA) were conducted in the program R using the packages MASS (Fournier et al., 2012) and Lattice (Sarkar, 2008). LDA visualizations were done with the R package ggplot2 (Wickham, 2009).

DNA extraction and genetic analysis

For phylogenetic analysis a fragment of 638 base pairs of the mitochondrial gene COI was amplified and sequenced from 50 workers from both ridges; 13 workers from the Brooksville Ridge, 37 workers from the Lake Wales Ridge (Fig. 1.2).

Total genomic DNA was isolated from whole specimens using the Qiagen DNeasy blood & tissue extraction kit (QIAGEN, Valencia, CA). From each specimen, a 638 bp fragment of the mitochondrial Cytochrome Oxidase subunit I (COI) gene was amplified by PCR using primers LepF1 (5' -ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Hajibabaei, Janzen, Burns, Hallwachs, & Hebert, 2006; Hebert, Penton, Burns, Janzen, & Hallwachs, 2004). Polymerase chain reactions were performed in 20 µl volumes containing: 1X PCR buffer, 2 mM MgCl2, 100 mM dNTPs, 2 pM of each primer, 0.5 U Taq DNA polymerase (Apex, Genesee Scientific, San Diego), ~50 ng DNA template, and ddH2O to 20 µl. PCR cycling conditions were comprised of an initial denaturation stage of 3 min at 94 °C, followed by 35 cycles each consisting of 30 sec at 94 °C, 30 sec at 45 °C, and 1 min at 72 °C. PCR products were visualized on a 2.5 % agarose gel to confirm samples contained a single band, and they were subsequently purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, CA). Purified products were then bi-directionally sequenced on an ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were visualized and edited in CLC Main Workbench (CLC bio, Aarhus, Denmark). Sequence variation was calculated with the Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software (Tamura, Stecher,

Peterson, Filipski, & Kumar, 2013). Diversity parameters, including nucleotide diversity (π) and haplotype diversity (h), were computed with DnaSP 5 (Librado & Rozas, 2009) for each ridge separately. Genetic distances between haplotypes were reconstructed using a minimum-spanning network algorithm implemented in PopART 1.7 (epsilon = 0) (Bandelt, Forster, & Röhl, 1999). Trends in the demographic history of *Odontomachus* spp. were investigated using Tajima's D and Fu's Fs statistics. A significant negative value in these two tests indicates a recent population expansion event.

Phylogenetic trees were constructed using Bayesian Markov Chain Monte Carlo (MCMC) simulations with BEAST v1.8.2 (Drummond, Suchard, Xie, & Rambaut, 2012) and a maximum likelihood approach using MEGA software version 6.0 (Tamura et al., 2013) with 1000 bootstrap replications. Both programs used the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution identified by jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012) as an appropriate model based on Bayesian Information Criterion (BIC) values. The Bayesian analysis used the default priors on branch lengths, rate parameters, and tree topology with four chains of 50 million generations sampled every 5,000 generations. The first 10 % of trees (4,000) were discarded as burn-in. TaxonDNA software version 1.6.2 (Meier, Shiyang, Vaidya, & Ng, 2006) and MEGA software version 6.0 (Tamura et al., 2013) were used to calculate genetic divergences using uncorrected p-distances.

A linear regression of COI divergence and geographic distance (km) was constructed using the software R (R Development Core Team, 2011). The response variable was transformed using an Arcsine * square root transformation and the predictor variable was log transformed. In addition, a curve was fitted using a loess regression (locally weighted scatterplot smoothing).

Results

Distribution

The distribution of *Odontomachus relictus* along the two ridges was not continuous. The species was found on the southern part of the Brooksville Ridge in three areas that we divided into a northern (BR-N), central (BR-C), and southern (BR-S) part. The furthest distance between these three areas was 32 km (Fig. 1.1). On the Lake Wales Ridge, the species was also found in three general areas, the northern part (LWR-N), the central part (LWR-C) and, at its greatest density, the southern part (LWR-S). The furthest distance between *O. relictus* sampling locations on the Lake Wales Ridge amounted to 242 km (Fig. 1.1). The collections in this study largely correspond to historic and more recent collections (M. A. Deyrup, 2011; M. Deyrup & Cover, 2004); *O. relictus* was not collected at two sites where it has been previously documented: Saddleblanket Lake Preserve (The Nature Conservancy) and Carter Creek North (Florida Fish and Wildlife Conservation Commission).

Phylogenetics

Our phylogenetic analysis revealed two lineages, each corresponding to one ridge (Fig. 1.3). Average sequence divergence between the two ridges was 4.8 % (also see Table 1.2). The Brooksville Ridge (BR) clade is further divided into three clades that correspond to the geographic locations of the three main sampling locations (BR-N, BR-C, BR-S) (Fig. 1.2). Average sequence divergence within the BR clade amounts to 2.1 %. The structure of the Lake Wales Ridge (LWR) clade also corresponds to the geographic locations of samples. The largest clade is formed by the southern LWR (LWR-S), the location with the highest density of collections. The two locations in the center of the LWR (LWR-C) were not monophyletic,

instead they represent distinct lineages, with an average divergence of 3.2 %. The more southern LWR-C clade is most closely related to LWR-S, with the northern LWR-C clade being the next closest one. The LWR-N clade is monophyletic and represents the sister clade to all locations south of it. Average sequence divergence within the LWR clade is 1.9 %. Branch support was high between major clades (>85 bootstraps / > 0.90 post. probability) except for the split between the two LWR-C locations (74 / 0.42), the split between LWR-N and the rest of LWR (0.59 post. prob.), and the split between BR-C and BR-S (72 / 0.79).

The COI divergence distribution between the two ridges (Fig. 1.4) revealed that the majority of individuals (40 %) within ridges showed 0-1 % divergence and the majority (82 %) between ridges showed between 4.5 and 6.5 %. However, there was some overlap; 30 % of individuals exhibited higher levels of sequence divergence (4–5 %) within ridges.

14 unique COI haplotypes were identified across both ridges in 47 locations (Fig. 1.5). Workers from the same location (Orel-07 and Orel-29) had identical haplotypes and were excluded from subsequent analysis to avoid inflation of haplotype frequency. In addition, one sequenced individual (B-17) resulted in a spurious haplotype, not coherent with the study and was therefore excluded from analysis. Of the 87 polymorphic sites, 83 were parsimony informative. All haplotypes were joined in a minimum-spanning network with one haplotype (1) comprising 42 % of all individuals. The organization of haplotypes in the network corresponded to the geographic arrangement of sampling locations. LWR-S consisted of 5 haplotypes, each connected to each other by 2-7 mutations. LWR-C contained two unrelated haplotypes, each separated from LWR-S by 22-23 mutations. LWR-N consisted of two haplotypes separated from each other by 12 mutations and from LWR-S by 22 mutations. BR-N was separated from LWR-S by 26 mutations and by 19 mutations from

BR-C. BR-S consisted of three haplotypes and was separated by 15 mutations from BR-C. Haplotype diversity (h) for all samples (47 workers) amounted to 0.802 (SD 0.054) and nucleotide diversity (π) was 0.03. For the BR lineage (12 workers) haplotype diversity (h) amounted to 0.788 (SD 0.09) and nucleotide diversity (π) was 0.02. For the LWR lineage (35 workers) haplotype diversity (h) amounted to 0.664 (SD 0.086) and nucleotide diversity (π) was 0.018. Tajima's D (BR: 1.275, p = > 0.10; LWR: -0.874, p = > 0.10) was not significant but Fu's Fs (BR: 6.171, p = 0.01; LWR: 7.065, p = 0.002) indicated that these populations have undergone a recent expansion.

A linear regression of COI divergence and geographic distance (km) revealed a significant relationship (Fig. 1.6A, r^2= 0.859, p=0). A loess regression curve (span = 0.5, degree = 1) showing an asymptote provided a superior fit to the data (Fig. 1.6B, F = 857.24, df = 3, p = 0.0001).

Morphometrics & Color

Morphometric characters and color values varied among specimens and sites (Table 1.3 and 1.4). A linear discriminant analysis showed different results for quantitative morphometrics and for color (Fig. 1.7). While four clusters corresponding to geographic groupings (BR, LWR-N, LWR-C, LWR-S) were recovered in the color analysis, morphometrics only clearly separated the data into two clusters (LWR-C and rest) with significant overlap of groupings in the second cluster (Fig. 1.7). The first (LD1), second (LD2), and third (LD3) canonical axes explain 100 % of the total data variance for both morphometrics and color. For morphometrics, LD1 explains 74 % of the variation, LD2 explains 14 %, and LD3 12 %. In this case, LD1 discriminates between LWR-C and the rest. The characters that best

discriminate between groups on the first canonical axis are HW and HL. The model matched 80 % of individuals to the correct location. 30 % of specimens from LWR-N were misidentified as BR, and 18 % and 15 % of specimens were misidentified between BR and LWR-S (Table 1.5). For color, LD1 explained 90 % of the variation, LD2 explained 7 %, and LD3 3 %. The most discriminating characters along LD1 are blue and green color on abdomen, antenna and head, and green color on posterior leg. The model matched 100 % of individuals to the correct location (Table 1.5). Values of the canonical discriminant functions are given in Table 1.6.

Discussion

Our genetic results revealed cryptic species-level divergence between *O. relictus* populations on the Lake Wales and Brooksville Ridges. Furthermore, we found considerable genetic structure within ridges that corresponds to geographic distances, with high levels of genetic divergence within ridges. A color analysis revealed significant differences between four geographic groups but other measures of morphology did not track genetic differentiation.

Divergence on islands has been documented in a variety of species and island systems, including between natural islands (Glor et al., 2004; Grant & Grant, 2003; Juan, Oromi, & Hewitt, 1995; Schluter, 1988) and fragment islands like those created by urban development (Harris & Steudel, 2002; Slabbekoorn & den Boer-Visser, 2006). In the present study, we document within a single island system both divergence between "natural" islands (on the species level) and divergence, potentially caused by agriculture and development, within those islands (on the population level). These divergences are accompanied by, at most, modest morphological differences.

Most insect species (which represent the majority of animal species [Zhang, 2013b]) are described based on morphological features. While morphology generally aids in species identification, it also conceals cryptic divergence such as is the case in our study. A preferable approach incorporates natural history, morphology, and molecular techniques (e.g., J.M. Padial & I. De la Riva, 2009; T. Eltz *et al.*, 2011; D. Apolônio Silva De Oliveira *et al.*, 2012). Such integrative methods offer a more comprehensive picture of divergence and diversification and not only result in more strongly supported species hypotheses but also provide a solid base for subsequent research on the causes and consequences of divergence (Dayrat, 2005; Padial, Miralles, De la Riva, & Vences, 2010; Schlick-Steiner et al., 2010; Will, Mishler, & Wheeler, 2005). Unlike studies of morphology alone, this approach offers insights both into taxonomy and evolutionary processes.

Here we found an average sequence divergence in the mitochondrial gene COI between ridges of 4.8 % which is consistent with species level divergence in ants (Fisher & Smith, 2008). *O. relictus* is already considered one of the rarest ants in North America, and it lives in a sparse and threatened habitat (M. Deyrup & Cover, 2004). However, when considering the entire range of this species and its relative abundance on protected lands on the Southern Lake Wales Ridge, one might argue that it is doing relatively well. Our results show that the Brooksville population should be considered as a separate species. This vastly changes the conservation outlook of what we now call *O. relictus*. On the Brooksville Ridge, the species was found in only three areas within a 32 km radius, two within Withlacoochee State Forest and one on unprotected land (BR-N). Such a limited distribution should catapult this species immediately to the top of any threatened species list as has already been suggested for *O. relictus* prior to these findings (M. Deyrup & Cover, 2004).

In addition we found high levels of divergence between some locations within ridges, e.g., 4.7 % between LWR-N and LWR-C, which are approximately 55 km apart, and 3.2 % between BR-N and BR-S, approximately 30 km apart. No such divergence was observed at similar distances in the southern part of the Lake Wales Ridge (e.g., 0.4 % between LWR-S(N) and LWR-S, approx. 45 km apart), where the habitat of these ants is better preserved. We speculate that the relatively greater isolation by distance in the northern parts of the Lake Wales Ridge may be due to anthropogenic change. However, this does not explain why similar divergence was not observed on the Brooksville Ridge where locations that were connected by Withlacoochee State Forest (BR-C and BR-S) were not more closely related than locations that were separated by considerable urban development (the cities of Lecanto, Beverly Hills, and Dunnellon; BR-C and BR-N, see Fig. 1.3).

The extremely high isolation by distance based on COI suggests limited maternal dispersal. Ants exhibit a wide range of mating strategies that are associated with varying dispersal ranges by the sexes (Heinze & Keller, 2000). Wing reduction and complete wing loss have been documented in several genera (Peeters, 2012; Peeters, Keller, & Johnson, 2012; Tinaut & Heinze, 1992) and would be a possibility to explain the genetic pattern observed in this study. During all three summer field seasons, male alates were frequently observed and were numerous in some locations. However, not a single alate queen was observed. Dealate queens were seen and collected in the vicinity of nests. It is unclear whether these queens were the queens of the nearest colony or newly mated queens looking to establish a nest. The dissection of one of these queens (Orel-15) confirmed her mated status by presence of sperm in the spermatheca, however there were no eggs (J. Shik, pers. comm.). The lack of eggs could be consistent with this queen having recently mated,

however, it could also have been a result of malnourishment after keeping it in captivity for several weeks before dissection. A single winged queen specimen (one wing missing) is present in the Archbold Biological Station insect collection, however, winged queens are generally rare. Even when hundreds of alate males are captured in flight traps, there is not a single queen among the samples (M. Deyrup, pers. comm.). It is possible that this species has a mating biology that does not involve mating flights and only disperses over short distances. Permanent wing loss in queens has been documented in another *Odontomachus* species from Madagascar (M. Molet, Peeters, & Fisher, 2007).

While variation in color among individual ants tracked genetic divergence between lineages, this was not the case for other morphological traits, with one exception. One group (LWR-C) was both genetically distinct from the other three (Fig. 1.3) and represented a well-defined morphological group (Fig. 1.7). This location was characterized by an overall larger size compared to all other colonies measured (HW 1.74-1.87, see Table 1.4). And although we only measured a single colony from this location, two additional colonies from this area (W-35, W-36) were noticeably larger in individual worker sizes as well.

Our genetic results suggest that a rare ant species is actually composed of multiple, monophyletic lineages structured by geography, at least two of which meet the criteria to be considered species. Unique lineages have intrinsic and aesthetic values, but in many cases unique features of cryptic species offer applied value. To the extent that some genetic and morphological traits differ among the lineages we discovered, we might hypothesize that chemical and behavioral traits differ as well. The chemistry and behavior of *Odontomachus* ants both have relatively obvious applied uses. *Odontomachus* spp. have been found to harbor alkaloid molecules which have antibacterial, antifungal, and antiviral properties and

have been linked to inhibiting HIV and certain types of cancer (Aniszewski, 2007). *Odontomachus* species have also been studied due to the extraordinary properties of their jaws which close shut at record high speeds and allow the ants to propel themselves backwards into the air (Patek et al., 2006). Recently, a leg-jumping behavior has been described from a species in Borneo which still awaits biomechanical investigation (Sorger, in press). In addition, DMS observed *O. relictus* exhibit a drumming behavior when disturbed. This behavior is entirely new to this subfamily of ants and also remains to be described and studied. Such unstudied behaviors could inspire biomechanical solutions to unsolved problems. For instance, wood wasps (*Sirex noctilio* and *Megarhyssa nortoni nortoni*) served as the inspiration for drill-bits that work in gravity-free environments et al., 2007); mosquitos (Culicidae) inspired pain-free micro needles (Oka et al., 2002); a mantis shrimp (*Odontodactylus spp.*), inspired the development of lighter and tougher body armor (Taylor & Patek, 2010; Weaver et al., 2012).

As ridge endemics, the future of *O. relictus* populations is most immediately threatened by anthropogenic habitat change. Only a small portion of these ridges is currently protected and the majority is under development. Our genetic data suggests that what is now called *O. relictus* represents at least two distinct species, which would be two of America's rarest ants living in a highly threatened habitat. Similar patterns of divergence (and rarity) are likely in other organisms living on these ridges. For instance, significant inter-ridge phylogeograhic genetic structure was found in seven scrub-endemic arthropods (Lamb & Justice, 2005). In addition, a second ridge-endemic ant species, *Dorymyrmex elegans* (Dolichoderinae), has been found to exhibit distinct morphological differences between these same ridges (Hackett & Sorger, unpubl. data). An analysis of the gene COI showed a curious

pattern of genetic divergence of one population cluster, while others were genetically indistinct from a second co-occurring species (*D. bureni*). The next steps in studying these species include a more in-depth phylogenetic study adding nuclear genes and population genetics using microsatellite markers or SNPs. These methods will help shed more light on the evolutionary history and ecology of ridge-endemics. More specifically, these methods will allow us to answer life history questions about breeding structure (e.g. number of queens and males contributing to the gene pool of a single colony), and dispersal (e.g. relatedness within and between colonies, clusters, and ridges). These efforts will lead to understanding why gene flow in these ants is so geographically limited. Given this limited gene flow and the small population sizes of these ants, it will be important to determine how we might best conserve them. Native habitat on these ridges is rare and they are adjacent to a growing Disney World. In addition, they are likely to be strongly influenced by climate change and shifting human demographics.

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Table 1.1. Morphological characters measured from *O. relictus*.

Abbr.	Name	Description
CI	Cephalic index	HW / HL × 100
EL	Eye Length	Maximum eye length in frontal view
HL	Head length	Maximum length of head in full-face view, excluding mandibles, measured from anterior-most point of clypeal margin to posterior-most point of head vertex, parallel to midline
HW	Head width	Maximum width of head in full-face view (including eyes when surpassing head outline)
MdI	Mandible index	MdL / HL × 100
ML	Mandible length	Maximum length of mandible in frontal view of head measured from mandibular insertion to apex
MsL	Mesosoma length	Maximum length of mesosoma, measured in lateral view, diagonal from cervical shield to posterolateral propodeal edge
PnW	Pronotum width	Maximum width of pronotum in dorsal view
PtH	Petiole height	Maximum height of petiole, measured in lateral view (bottom edge of petiole parallel to petiolar apex)
PtL	Petiole length	Measured in lateral view along dorsal outline of petiole from small antero-apical tooth to apex
PtW	Petiole width	Maximum width of petiole in dorsal view
SI	Scape index	SL / HW × 100
SL	Scape length	Maximum length of antennal scape in dorsal view excluding basal constriction

Table 1.2. Percent pairwise sequence divergence in COI among groups.

	BR-N	BR-C	BR-S	LWR-N	LWR-C	LWR-S
BR-N	0.0%					
BR-C	3.0%	0.0%				
BR-S	3.2%	2.7%	0.7%			
LWR-N	4.5%	4.4%	4.9%	1.3%		
LWR-C	4.9%	5.2%	5.9%	4.7%	3.2%	
LWR-S	4.4%	4.7%	5.3%	3.9%	3.9%	0.4%

Table 1.3. Color values for six body parts of *O. relictus* from three color channels per colony.

		Abdomen									Antenna									Anterior Leg								
		Blue			Green			Red			Blue			Green			Red			Blue			Green			Red		
Colony	n	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max
B-08	3	15.82	16.23	16.51	18.91	18.98	19.11	21.24	24.52	28.23	19.60	22.26	25.77	32.14	35.19	40.67	73.33	77.14	83.05	11.42	15.44	20.08	22.60	28.25	34.26	50.92	59.43	70.63
B-17	2	15.10	16.74	18.39	17.78	19.28	20.79	20.47	21.23	21.99	14.01	29.10	44.19	21.37	38.34	55.31	53.24	71.38	89.53	20.74	28.05	35.35	37.13	45.07	53.01	78.73	86.53	94.33
B-18	3	13.87	15.89	17.79	16.01	18.34	20.38	17.32	21.63	27.50	14.93	22.01	29.84	22.15	33.86	42.20	55.15	76.70	93.66	14.27	18.01	22.91	32.54	37.20	43.99	75.26	80.25	89.62
B-25	3	14.52	16.55	17.95	16.78	19.07	20.91	18.78	20.98	23.72	19.98	24.49	29.70	28.71	36.46	40.58	64.04	79.43	93.55	16.53	26.04	44.97	29.64	43.36	67.08	61.59	78.41	103.75
O-06	3	16.35	16.56	16.85	18.78	18.98	19.31	20.80	22.23	23.21	25.56	27.98	30.05	38.53	41.26	43.18	78.05	81.39	86.94	13.48	20.56	33.73	31.17	40.10	56.23	69.52	79.86	96.78
O-07	3	13.84	16.42	18.14	16.86	18.92	20.01	20.57	21.17	21.54	14.71	20.68	30.13	24.19	30.79	42.11	60.60	67.47	78.14	20.57	21.24	22.26	39.93	45.40	49.45	83.75	92.72	102.02
W-06	3	16.52	17.56	18.60	19.29	20.46	21.47	22.58	24.93	26.50	15.02	26.39	36.45	25.07	41.53	53.32	62.14	86.16	99.56	22.21	28.96	42.29	56.74	74.40	92.07	107.26	134.02	151.52
W-10	3	15.10	16.78	17.73	17.68	19.16	20.05	21.67	22.71	23.39	15.60	22.49	31.88	29.90	39.18	53.07	75.76	89.33	104.90	22.73	28.21	36.91	50.46	71.39	90.85	96.10	126.96	150.63
W-16	3	17.60	18.31	19.12	20.92	22.48	24.58	24.07	30.80	41.04	18.62	22.22	25.01	27.62	39.30	47.39	61.94	84.03	99.09	11.90	17.15	24.97	50.25	62.46	85.67	100.65	118.11	147.21
W-30	3	14.99	15.92	16.90	18.07	18.97	19.43	23.65	24.70	25.82	20.27	22.68	27.09	34.46	39.03	47.11	80.36	91.32	105.42	9.42	11.70	15.99	40.91	47.13	57.12	89.90	99.32	109.53
W-36	3	17.14	18.72	20.13	19.50	21.16	22.84	21.81	24.02	25.96	22.05	27.43	35.60	33.32	40.91	48.00	74.58	84.66	92.36	11.91	19.14	29.23	34.68	49.49	64.68	83.00	103.77	124.96

		Head									Posterior Leg									Thorax								
		Blue			Green			Red			Blue			Green			Red			Blue			Green			Red		
Colony	n	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max
B-08	3	13.79	20.72	24.80	20.02	26.62	30.56	44.75	46.63	47.84	11.87	13.27	14.15	24.69	27.00	28.59	49.77	60.83	69.71	11.00	15.23	19.42	14.38	18.20	21.73	26.72	28.82	31.59
B-17	2	15.01	19.13	23.26	20.81	23.93	27.05	40.77	43.33	45.89	12.50	15.63	18.76	29.23	31.26	33.29	70.98	72.50	74.02	12.69	14.82	16.94	15.14	17.46	19.78	22.35	27.90	33.45
B-18	3	16.54	22.46	26.06	24.31	29.63	33.21	51.11	54.05	57.79	9.18	11.13	14.46	21.52	28.72	36.15	50.35	70.60	84.12	13.58	15.70	19.77	15.95	18.20	21.92	27.94	30.80	32.44
B-25	3	15.25	20.61	25.53	22.79	26.61	29.98	44.95	48.65	51.91	10.52	16.57	20.71	27.26	37.56	47.44	72.09	86.04	108.75	13.38	14.43	16.48	15.75	16.97	18.99	23.91	27.02	30.17
O-06	3	13.66	19.85	23.32	20.59	26.41	29.37	49.87	52.80	57.35	13.46	14.44	16.01	32.32	33.88	35.20	75.70	76.45	77.22	9.96	11.68	12.66	12.47	14.72	16.12	25.69	30.56	34.46
O-07	3	20.25	20.86	21.80	24.57	26.26	29.03	41.47	45.99	53.95	8.62	12.86	19.93	21.02	34.06	41.57	56.75	81.06	96.59	10.40	12.08	13.50	13.02	14.88	16.90	25.61	28.50	34.11
W-06	3	19.46	20.89	21.81	27.85	28.66	29.89	53.64	57.96	63.36	10.71	14.68	22.25	45.69	56.77	73.60	97.54	113.99	136.71	11.82	12.85	13.76	15.99	17.54	18.68	37.89	41.63	48.42
W-10	3	22.21	24.25	25.55	28.18	31.21	32.74	49.94	55.52	58.56	11.85	14.19	16.14	51.74	59.82	66.54	103.46	121.14	131.21	12.31	13.29	14.60	15.10	17.09	19.28	28.11	35.42	41.43
W-16	3	13.75	16.02	19.69	23.68	28.20	33.99	54.39	65.65	74.39	12.54	13.93	15.24	54.12	57.69	61.31	100.77	112.52	121.39	11.76	13.25	15.19	16.18	17.69	18.60	34.36	37.12	40.97
W-30	3	14.79	16.73	18.74	26.80	28.31	29.36	63.55	67.68	72.64	13.49	14.20	14.91	41.84	50.84	57.01	86.59	101.95	114.83	10.85	12.29	14.99	15.57	17.58	20.75	38.94	42.77	47.15
W-36	3	16.31	20.36	24.70	24.93	27.86	30.56	57.45	59.93	61.34	14.46	21.78	32.75	32.55	46.24	60.90	79.73	97.79	115.67	13.39	14.33	15.45	16.90	17.61	18.87	34.61	37.88	39.90

Table 1.4. Morphology measurements for ten traits including two indices (see Table 1.1) of *O. relictus* per colony.

			HW			HL			EL			ML			SL			MsL			PtL		
Location	Colony	n	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max
LWR-S	W-25	10	1.43	1.53	1.64	1.76	1.92	2.06	0.91	0.98	1.03	0.28	0.31	0.34	1.73	1.83	1.93	2.20	2.29	2.37	0.57	0.63	0.70
LWR-S	W-26	10	1.52	1.62	1.71	1.86	2.00	2.09	0.95	1.02	1.07	0.32	0.34	0.37	1.77	1.86	1.94	2.24	2.42	2.54	0.61	0.66	0.73
LWR-S	W-29	10	1.61	1.66	1.71	1.96	2.03	2.10	1.00	1.06	1.11	0.32	0.36	0.38	1.86	1.93	1.98	2.26	2.34	2.44	0.65	0.69	0.72
LWR-S	W-01	10	1.48	1.55	1.63	1.84	1.93	1.99	0.94	1.01	1.08	0.32	0.34	0.38	1.76	1.83	1.94	2.19	2.27	2.32	0.60	0.64	0.70
LWR-C	W-34	10	1.74	1.79	1.87	2.14	2.21	2.30	1.05	1.11	1.22	0.36	0.37	0.39	2.04	2.09	2.17	2.51	2.57	2.64	0.73	0.80	0.83
BR	B-19	10	1.52	1.57	1.64	1.89	1.94	2.05	0.96	1.04	1.15	0.30	0.33	0.36	1.78	1.87	1.94	2.25	2.37	2.48	0.64	0.68	0.71
BR	B-20	10	1.38	1.50	1.58	1.70	1.87	1.95	0.90	1.01	1.13	0.28	0.32	0.35	1.69	1.83	1.90	2.14	2.28	2.41	0.59	0.65	0.69
BR	B-22	10	1.55	1.66	1.76	1.93	2.05	2.16	1.00	1.04	1.10	0.32	0.34	0.37	1.83	1.93	1.99	2.25	2.39	2.49	0.66	0.70	0.75
BR	B-24	10	1.57	1.67	1.76	1.95	2.08	2.20	0.94	1.07	1.11	0.30	0.33	0.35	1.88	2.00	2.09	2.34	2.42	2.55	0.65	0.71	0.81
LWR-N	O-03	10	1.51	1.68	1.78	1.84	2.09	2.23	0.96	1.08	1.17	0.29	0.35	0.39	1.77	2.00	2.12	2.23	2.46	2.65	0.63	0.75	0.81
			PtH			PnW			PtW			CI			MdI			SI					
Location	Colony	n	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max			
LWR-S	W-25	10	0.62	0.70	0.75	0.83	0.88	0.95	0.37	0.40	0.43	78.06	79.81	82.23	47.32	51.03	55.03	117.22	119.77	122.72			
LWR-S	W-26	10	0.65	0.76	0.82	0.84	0.93	0.98	0.39	0.42	0.45	78.88	81.08	82.90	46.56	50.87	54.43	108.38	115.14	122.48			
LWR-S	W-29	10	0.76	0.79	0.84	0.89	0.93	0.94	0.39	0.42	0.44	79.06	81.55	83.90	49.19	52.07	56.93	112.85	116.36	119.39			
LWR-S	W-01	10	0.72	0.75	0.79	0.85	0.89	0.94	0.37	0.41	0.45	79.27	80.58	82.25	48.09	52.26	55.03	113.33	117.95	121.67			
LWR-C	W-34	10	0.85	0.89	0.93	0.99	1.02	1.05	0.43	0.49	0.56	79.06	81.14	83.11	47.45	50.27	56.86	113.95	116.34	119.35			
BR	B-19	10	0.71	0.76	0.81	0.84	0.88	0.92	0.37	0.41	0.46	78.64	80.85	82.84	49.79	53.51	60.45	115.75	119.13	124.36			
BR	B-20	10	0.61	0.71	0.81	0.77	0.85	0.90	0.35	0.39	0.42	78.56	80.35	85.40	49.77	53.83	59.27	118.61	121.85	125.23			
BR	B-22	10	0.75	0.79	0.84	0.89	0.94	1.00	0.41	0.44	0.49	79.21	80.85	83.37	49.12	50.80	52.78	112.25	116.81	120.36			
BR	B-24	10	0.74	0.80	0.92	0.88	0.94	1.03	0.38	0.43	0.50	79.43	80.25	81.58	47.65	51.72	55.98	112.59	120.13	125.69			
LWR-N	O-03	10	0.73	0.82	0.89	0.84	0.96	1.04	0.38	0.43	0.49	79.03	80.60	81.99	47.02	51.68	55.44	117.10	118.69	120.18			

Table 1.5. Linear discriminant analysis (LDA) confusion matrices for morphology and color in *O. relictus* between groups.

MORPHOLOGY	BR	LWR-C	LWR-N	LWR-S
BR	78%	10%	30%	15%
LWR-C	3%	90%	0%	0%
LWR-N	3%	0%	60%	0%
LWR-S	18%	0%	10%	85%
COLOR	BR	LWR-C	LWR-N	LWR-S
BR	100%	0%	0%	0%
LWR-C	0%	100%	0%	0%
LWR-N	0%	0%	100%	0%
LWR-S	0%	0%	0%	100%

Table 1.6. Linear discriminant analysis (LDA) group means and coefficients of discriminant functions for color and morphology.

COLOR Group means						COLOR Coefficients of linear discriminants			
	Abd.B	Abd.G	Abd.R	Ant.B	Ant.G		LD1	LD2	LD3
BR	-0.3754339	-0.4236046	-0.3529987	-0.0209836	-0.2213698	Abd.B	5.158676	1.7128768	-1.8587961
LWR-C	1.2172803	0.8748276	0.1003058	0.4604811	0.3388097	Abd.G	-7.1008045	-2.9408142	3.7702736
LWR-N	-0.2649319	-0.3901231	-0.467552	0.0200585	-0.1915549	Abd.R	1.7060944	1.4711869	-1.950808
LWR-S	0.1722937	0.3646589	0.5322817	-0.1059146	0.2139973	Ant.B	-3.5337532	2.5062102	2.6318096
	Ant.R	AntLeg.B	AntLeg.G	AntLeg.R	Head.B	Ant.G	3.5392847	-3.4472578	-3.772947
BR	-0.3186425	0.0233509	-0.6379425	-0.7917092	0.1665656	Ant.R	-0.0705665	1.1391946	1.6270402
LWR-C	0.2500143	-0.2148257	-0.0070501	0.264692	0.0396034	AntLeg.B	0.2217307	0.8231806	-0.7810995
LWR-N	-0.4718003	-0.0226725	-0.3729285	-0.3818309	0.0379535	AntLeg.G	-0.3310739	-0.0808775	1.0722707
LWR-S	0.4654856	0.0436377	0.7730073	0.8504759	-0.1815627	AntLeg.R	2.5616084	-0.4066973	-1.136299
	Head.G	Head.R	PostLeg.B	PostLeg.G	PostLeg.R	Head.B	-5.7321351	-1.913835	2.5568408
BR	-0.21283392	-0.7053363	-0.158867	-0.8048433	-0.7907438	Head.G	3.5322229	1.3664883	-1.1350199
LWR-C	0.03716374	0.5992835	1.4936416	0.2637409	0.2900462	Head.R	-2.4418656	-1.1994658	1.5030751
LWR-N	-0.38272219	-0.6140083	-0.237067	-0.6036422	-0.5231543	PostLeg.B	-2.4086792	-1.6049309	0.1677714
LWR-S	0.37716792	0.8037416	-0.1092488	0.9736589	0.9139142	PostLeg.G	6.2254418	3.0251976	1.0591446
	Tho.B	Tho.G	Tho.R			PostLeg.R	-2.3053801	-1.8434388	-0.7946315
BR	0.6424514	0.2914784	-0.7474456			Tho.B	-0.3933509	-4.1425076	-4.7803022
LWR-C	0.3213448	0.2395059	0.6345677			Tho.G	-1.4607534	4.692501	6.9142196
LWR-N	-0.7489228	-1.0105284	-0.6226271			Tho.R	1.305793	-2.7274984	-2.9150873
LWR-S	-0.2947886	0.1781992	0.8378301						

MORPHOLOGY Group means						MORPHOLOGY Coefficients of linear discriminants			
	HW	HL	EL	ML	SL		LD1	LD2	LD3
BR	-0.2416939	-0.2226653	-0.3413925	-0.0151742	-0.082989	HW	-31.931155	7.3904219	36.52117
LWR-C	1.6998098	1.6667667	1.2621179	1.1421587	1.6247427	HL	14.408643	-2.6122776	-16.041296
LWR-N	0.5795121	0.6270005	0.5711347	0.6010263	0.749275	EL	0.4080786	-0.6275213	0.1710288
LWR-S	-0.3281366	-0.3507764	-0.1169206	-0.4206221	-0.5105154	ML	9.972412	0.2573869	-9.9619746
	MsL	PtL	PtH	PnW	PtW	SL	4.4736906	-2.289396	-7.2435943
BR	-0.1286597	-0.097449	-0.1620479	-0.3029404	-0.1349454	MsL	-0.6733342	0.6352394	-0.2273047
LWR-C	1.6070969	1.704981	1.6738493	1.6322735	1.7564809	PtL	-1.2595496	-0.3763563	-1.7024554
LWR-N	0.6491738	0.961797	0.6556456	0.7157288	0.2098291	PtH	0.0353565	0.0453138	0.710011
LWR-S	-0.435408	-0.5692455	-0.4203258	-0.2840602	-0.3566321	PnW	2.3821795	-3.126203	-1.0221872
	CI	MdI	SI			PtW	-0.9488331	0.7338118	0.7229039
BR	-0.09304164	0.2464591	0.399118			CI	6.8709413	-0.8832006	-7.4814189
LWR-C	0.31054482	-0.573733	-0.592668			MdI	-8.4882722	0.1042632	8.4396636
LWR-N	-0.07494921	-0.0460465	0.1489577			SI	-2.6718656	1.5466871	3.3013209
LWR-S	0.03414273	-0.0915142	-0.2881904						

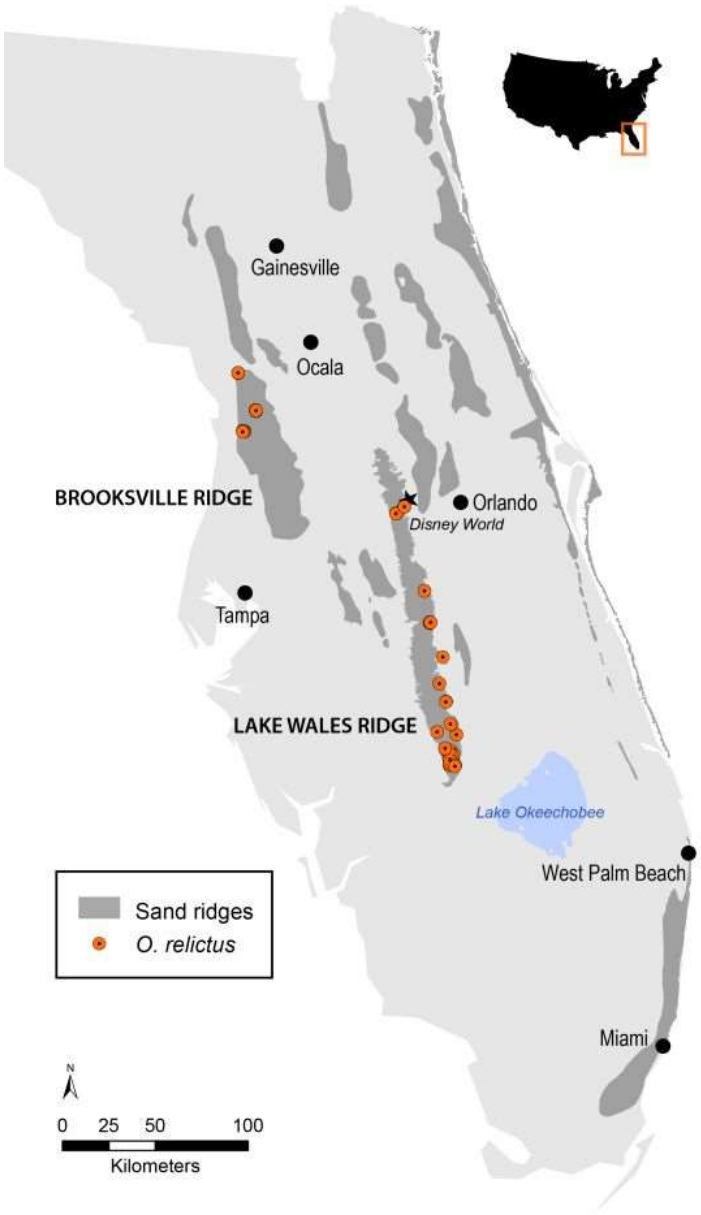

Figure 1.1. Sampling locations for *O. relictus* in Florida.

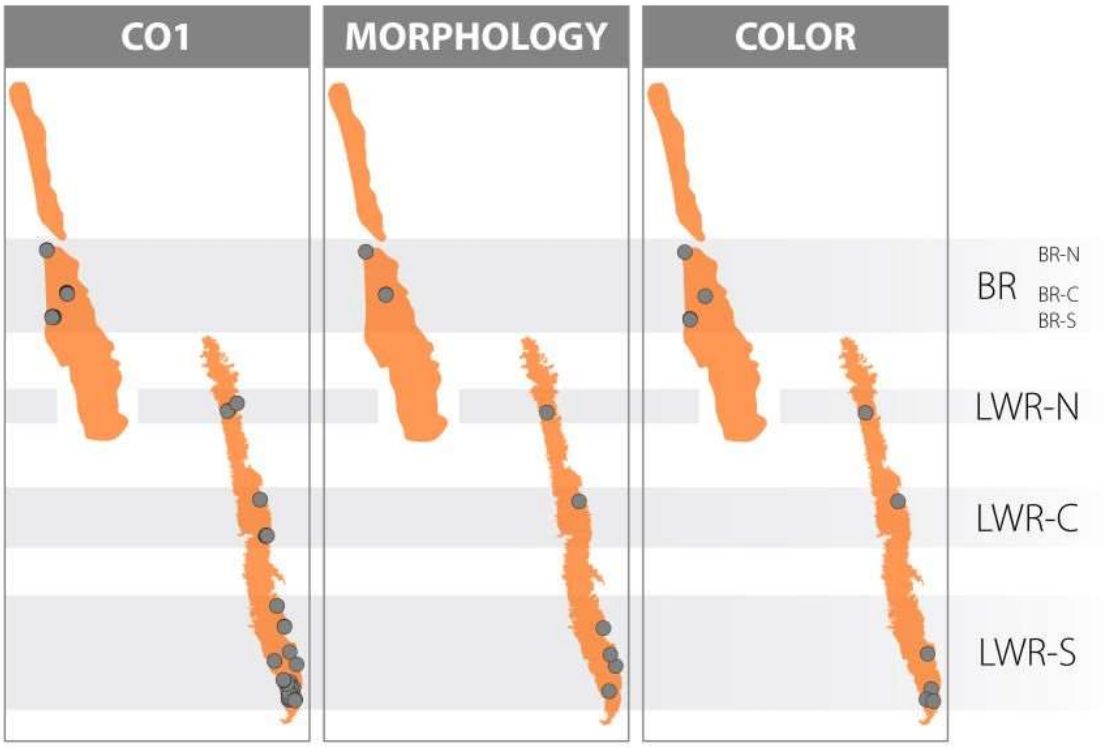

Figure 1.2. Input data (sampling locations) for each analysis type.

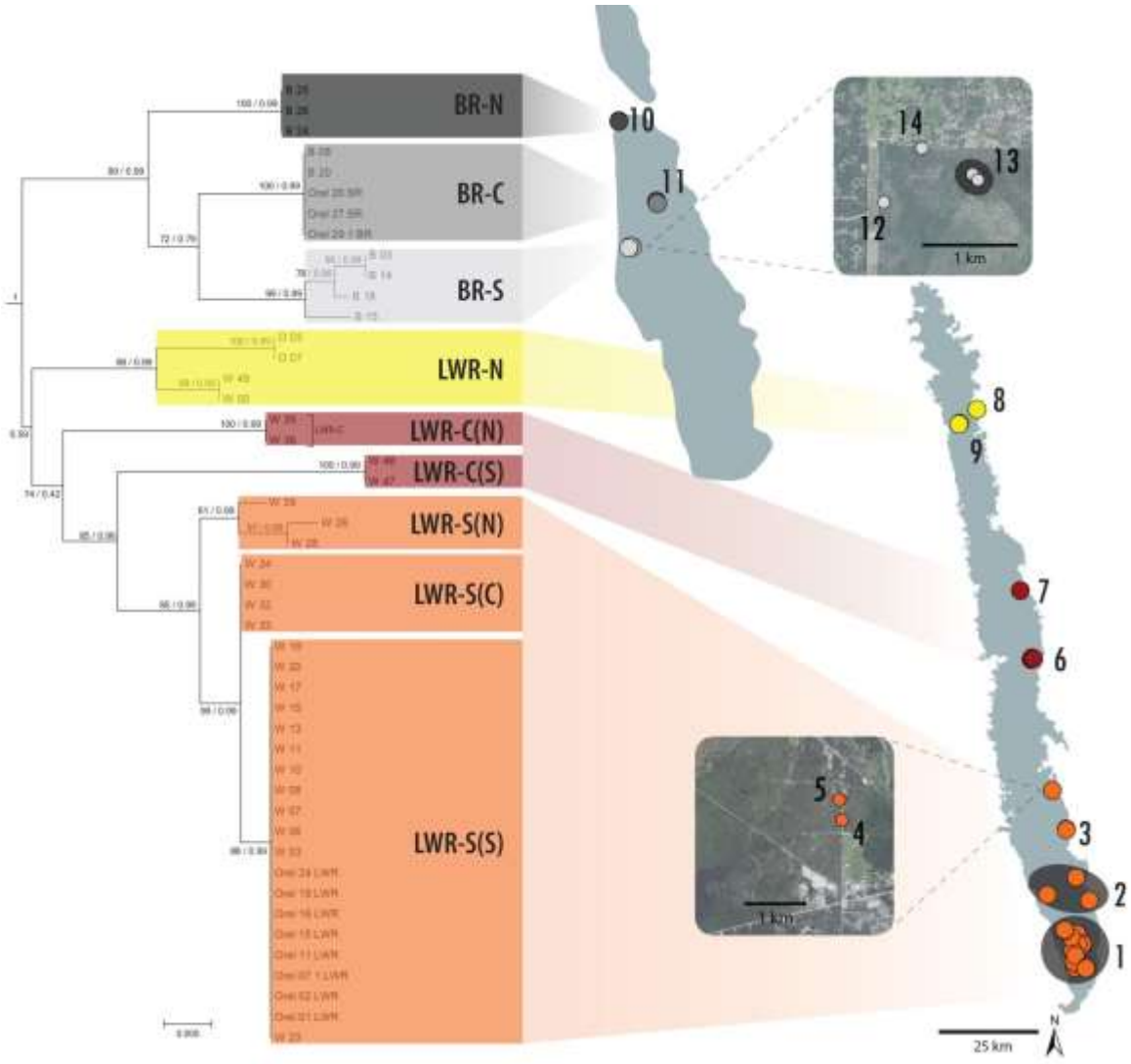

Figure 1.3. Maximum likelihood topology showing bootstrap values (1000 replicates) and posterior probabilities from Bayesian inference including map showing corresponding locations and haplotype numbers.

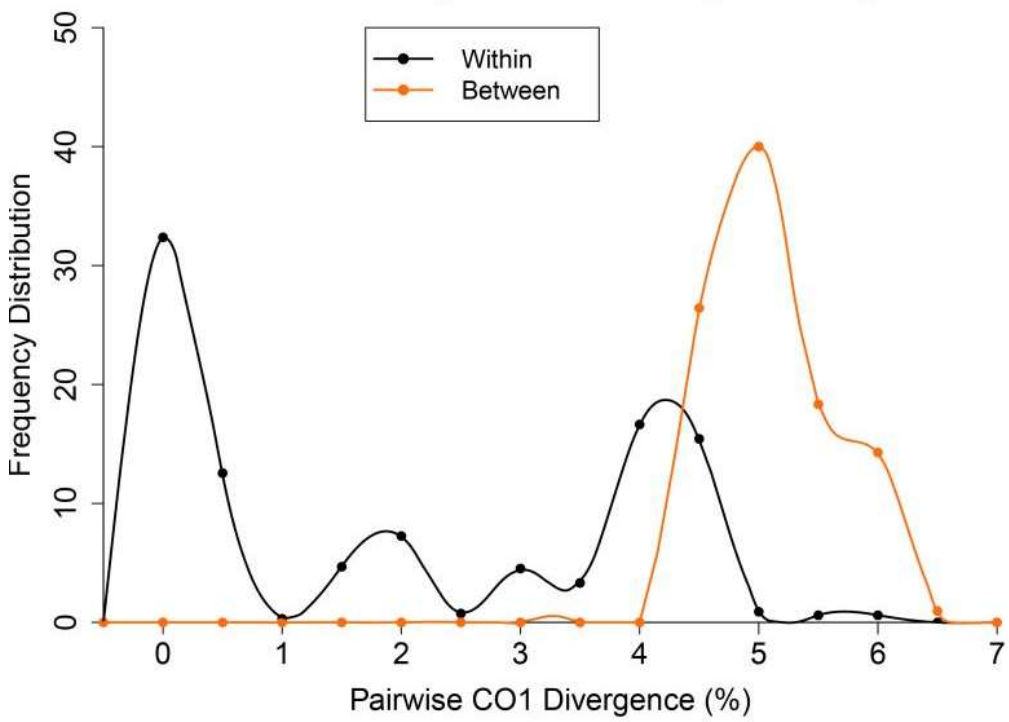

Figure 1.4. Pairwise COI divergence distribution in *O. relictus* between ridges.

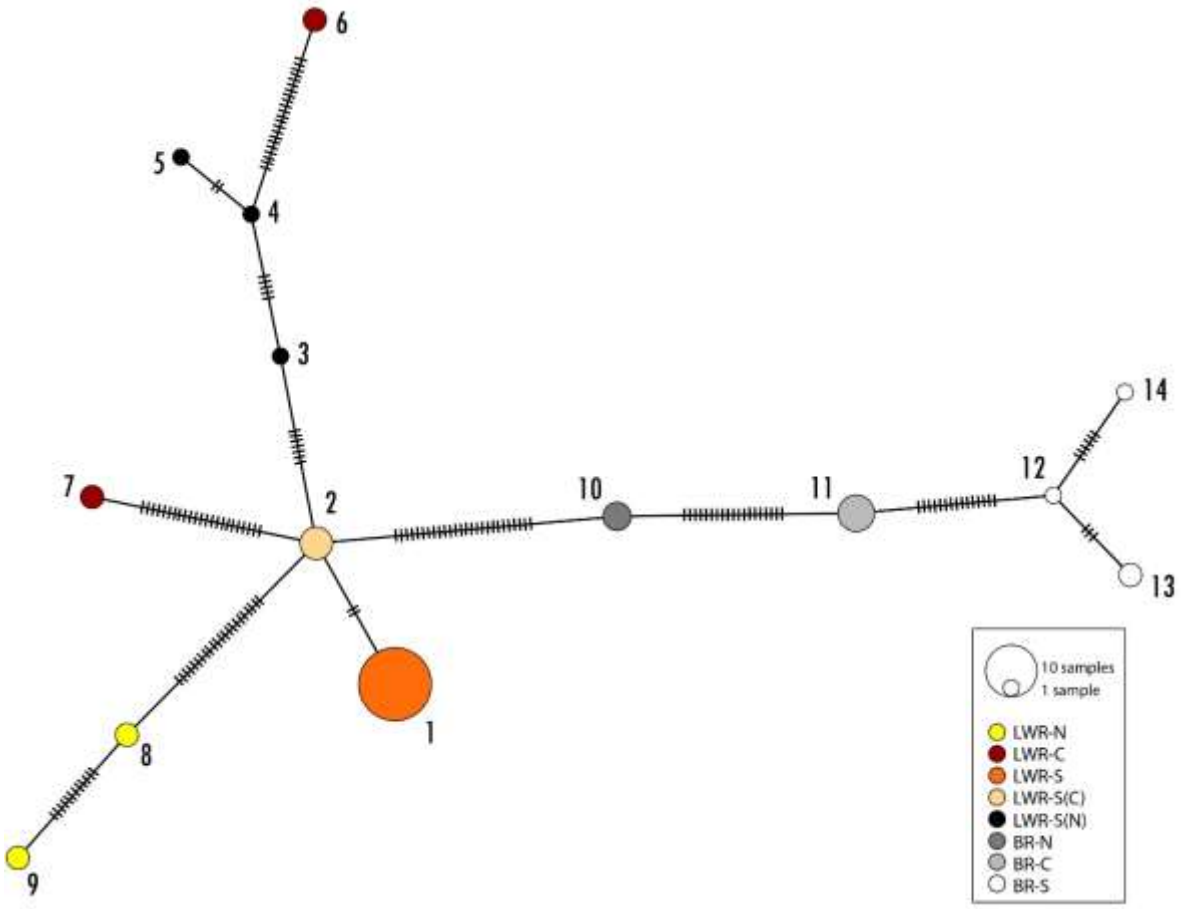

Figure 1.5. Minimum-spanning haplotype network for *O. relictus*. Size of nodes represent number of individuals per haplotype, tick marks between nodes indicate number of mutations between haplotypes.

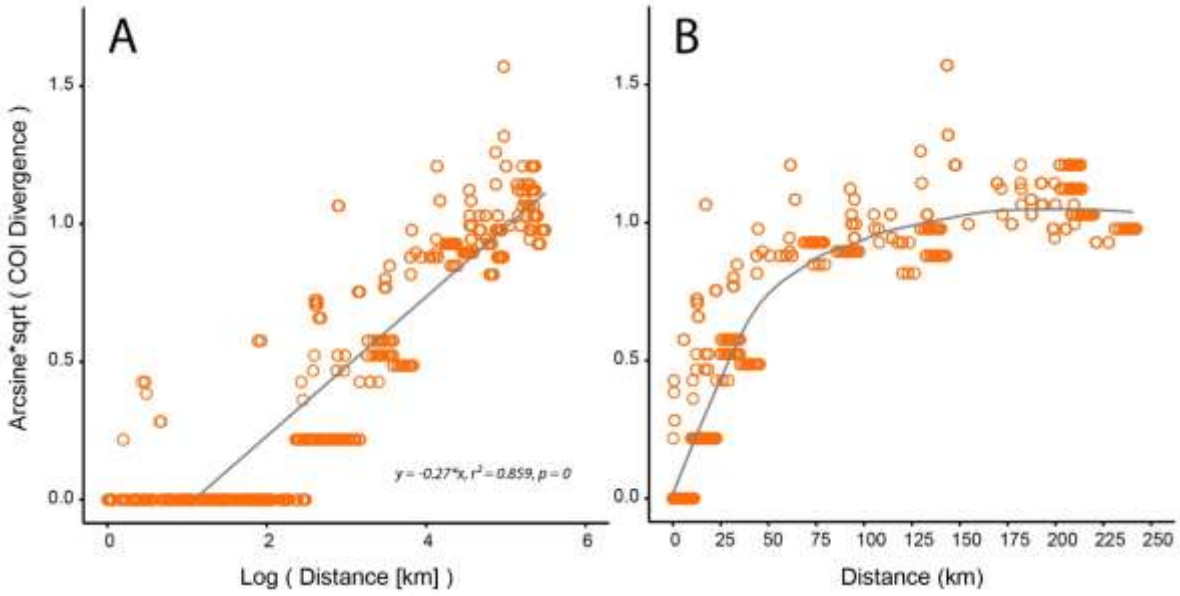

Figure 1.6. Relationship between COI divergence and geographic distance between sampling locations using (A) log transformed distance with linear regression line and (B) non-transformed distance with loess regression curve.

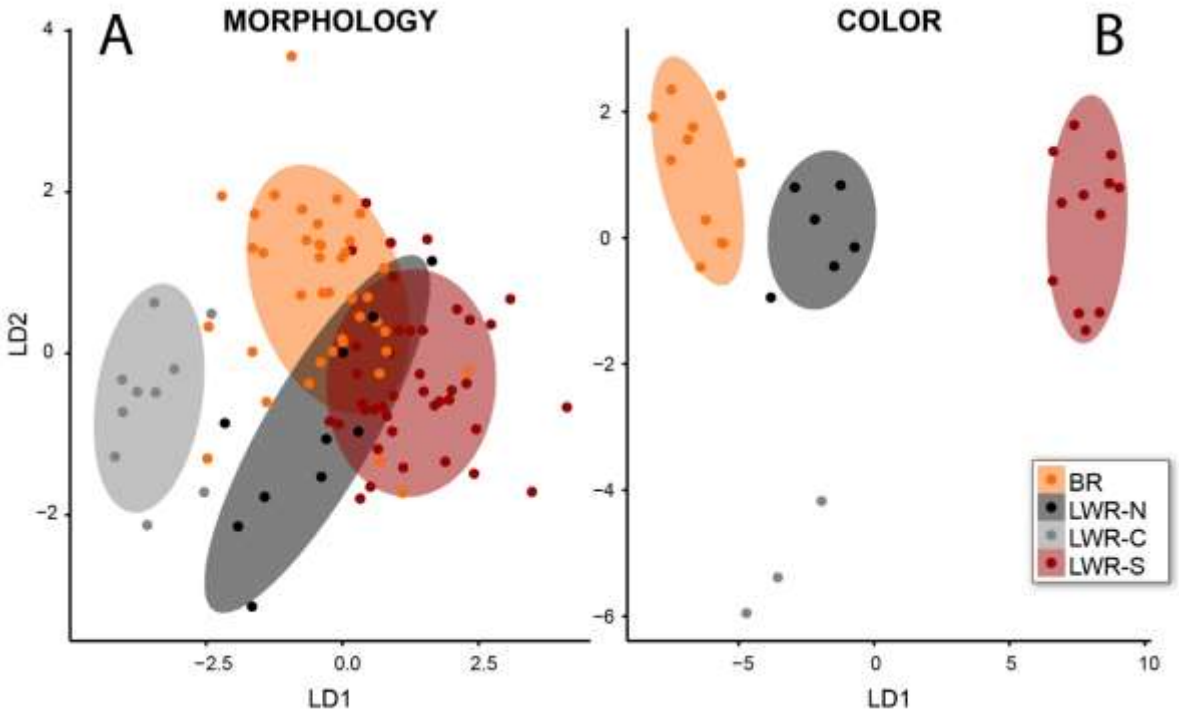

Figure 1.7. Linear discriminant analysis comparing groups based on (A) morphology and (B) color showing first two linear discriminant functions. LWR-C is distinguished from the others groups based on morphology while color differentiates all groups.

CHAPTER 2: Revealing the hidden diversity of trap-jaw ants (Hymenoptera: Formicidae: Ponerinae: *Odontomachus*) along elevational gradients in Borneo using an integrative approach

Prepared for publication

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Abstract

Elevational gradients and the divergence along them have taken an increasingly prominent role in biogeography. Climate varies rapidly and systematically along such gradients resulting in multiple ecological niches along these steep environmental clines. Typically species along elevational gradients are treated as though they are discrete entities with low and high elevation limits. However, most tropical species are described based on morphology which may conceal cryptic species. Here we use genetic (mtDNA and nDNA), morphological, color, and chemical (cuticular hydrocarbon profiles) tools to test the hypothesis of divergence in trap-jaw ant populations (genus *Odontomachus*) along an elevational gradient in Sarawak, Borneo with focus on a widespread species with a broad elevational range. Our results reveal cryptic species diversity within *O. rixosus* along the gradient. We describe two new species, *O. saltans* sp. nov. and *O. dunni* sp. nov., each with a distinct geographic and elevational distribution. A key to *Odontomachus* species in Borneo is included. We discuss these results in the light of the possibility that such genetic divergence patterns may be common along tropical elevation gradients.

Introduction

Since the work of Humboldt, elevational gradients have been recognized as microcosms of the larger world. On a single mountain, one can find environmental conditions as extreme as might be found on an entire continent. As microcosms of the broader world, elevational gradients have informed, disproportionately, our understanding of the drivers of biological diversity (Colwell et al., 2008; Kraft et al., 2011; Sanders et al., 2007; Sundqvist, Sanders, & Wardle, 2013). With anthropogenic climate change, elevational gradients have also become microcosms for the future of biodiversity; "laboratories for ecosystem ecology and global change research" (Malhi et al., 2010). But a major challenge in understanding the diversity of organisms along elevational gradients, particularly in the tropics, is that most tropical species are described only based on their morphology. As a result, a great deal of cryptic variation may exist (Cheviron & Brumfield, 2009; Hodkinson, 2005; McGaughran, Morgan, & Sommer, 2014), cryptic diversity that might either buffer or accentuate the extent of habitat loss and extinction associated with global change.

Here we take a case study approach in which we use molecular tools to consider the morphological and cryptic diversity in a single, relatively well-studied genus of charismatic ants along a comparatively well-studied tropical elevational gradient in Borneo.

Tropical elevational gradients in Southeast Asia are among the most diverse in the world. Two such gradients in Borneo have been studied in the past and are conspicuous: Mt. Kinabalu (4096 m), the highest mountain in Southeast Asia and Mt. Mulu (2376 m), the second highest mountain in Sarawak and fifth highest in Borneo. On Mt. Kinabalu, studies of the diversity of vascular plants (Grytnes & Beaman, 2006), and small mammals (Nor, 2001), found a hump-shaped pattern of diversity. Both moths (I.-Ching Chen et al., 2009; Holloway,

1970), and ants (Brühl, Mohamed, & Linsenmair, 1999; Malsch et al., 2008) on the other hand show monotonous declines in diversity with elevation. On Mt. Mulu the diversity of soil macrofauna including arachnids and several insect groups (Collins, 1980) and dung and carrion-feeding beetle species (Hanski, 1983) also show a steady decline with elevation.

In the studies of elevational gradients, high elevation and middle elevation endemics are common (I.-Ching Chen et al., 2009; Fu et al., 2006; Vetaas & Grytnes, 2002). Because both theory and empirical work suggests that climate change will lead high elevation species to move up in elevation (e.g., J.A. Pounds *et al.*, 1999; R.K. Colwell *et al.*, 2008; I.C. Chen *et al.*, 2009, 2011; W.F. Laurance *et al.*, 2011; K.J. Feeley *et al.*, 2011; G. Forero-Medina *et al.*, 2011; B.G. Freeman & A.M. Class Freeman, 2014), these species are at increased extinction risk if for no other reason than that the area of habitats tends to decline with increases in elevation.

Most metazoan species in the tropics are insects (Zhang, 2013a, 2013b) and most of these species are described based on morphological characters alone. Although morphology is an important factor in facilitating species identification, it may conceal cryptic species diversity (Funk, Caminer, & Ron, 2012; Kadarusman et al., 2012; J. S. Wilson, Clark, Williams, & Pitts, 2012) and as a result integrative approaches (Padial et al., 2010; Schlick-Steiner et al., 2010) incorporating natural history, morphology, and molecular techniques have become the standard (Apolônio Silva De Oliveira et al., 2012; Eltz et al., 2011; Padial & De la Riva, 2009). Morphological studies tend to find relatively few high elevation endemics in tropical insects (I.-Ching Chen et al., 2009; R. D. Wilson, Trueman, Williams, & Yeates, 2007) as is, for example, the case with species of the globally distributed ant genus

Odontomachus (Fig. 2.1). But this pattern may only be true if the morphological species identifications on which such perceptions depend are accurate.

Ants are diverse insects with over 13,000 described species (Agosti & Johnson, 2005) and represent dominant components of most terrestrial ecosystems. This phylogenetically well resolved group (C.S. Moreau & C.D. Bell, 2013) exhibits incredible morphological variation within and among species. Despite numerous studies focusing on intraspecific population structure (Clémencet, Viginier, & Doums, 2005; Doums, Cabrera, & Peeters, 2002; Sanetra & Crozier, 2001), there are only few which consider variation within species along elevational or similar environmental gradients (Purcell, Pellissier, & Chapuisat, 2015). Since alate ant reproductives are capable of flying relatively long distances (Hölldobler & Wilson, 1990; Tschinkel, 2006) this could, in theory, foil local adaptation, particularly in species with large populations and good dispersal (Kirkpatrick & Barton, 1997), if locally adapted populations at high elevations are constantly receiving an influx of gene flow from low elevations.

Within ants, trap-jaw ants in the genus *Odontomachus* have a worldwide distribution and consist of 69 described species with the highest diversity in the tropics. The genus originated about 60 million years ago when ants diversified and assumed ecological dominance (Moreau et al., 2006). It is among the best-studied genera for ants (Brown Jr, 1976; Camargo & Oliveira, 2012b; Cerquera & Tschinkel, 2010; MacGown, Boudinot, Deyrup, & Sorger, 2014; Sorger & Zettel, 2011) and there are currently four recognized *Odontomachus* species present in Borneo: *O. latidens* (*O. rixosus* species group), *O. malignus* (*O. malignus* species group), *O. rixosus* (*O. rixosus* species group) and *O. simillimus* (*O. haematodus* species group) (see Sorger & Zettel [2011] for additional notes on

species groups). These large and charismatic ponerine ants nest and forage in the leaflitter. All species in this genus possess a functionally unique mandible morphology. Their elongated jaws form a hyper fast trap-jaw mechanism to facilitate prey capture of small arthropods and also allows them to jump backwards (Patek et al., 2006). These ants are superficially diverse, and yet this diversity is perhaps modest relative to the age of the lineage. In addition, they occupy broad elevational ranges (Fig. 2.1). Some species of *Odontomachus* have elevational ranges that span up to 2000 m while others have much narrower ones, but in all cases our understanding of these ranges is informed almost exclusively by studies of morphology such that large differences between observed ranges and the actual ranges may exist once cryptic divergence (if present) is accounted for.

Here, we consider diversity and divergence of *Odontomachus* spp. along an elevational gradient in Sarawak, Borneo, one of the most diverse tropical forests in the world. We sampled *Odontomachus* spp. along different elevations throughout Sarawak, then focused molecular, chemical, color and morphological analysis on the most widespread species, *Odontomachus rixosus*, though we examine our results in light of all species. *Odontomachus rixosus* is a common species throughout Borneo and Southeast Asia regarded at low conservation risk due to its widespread distribution. Given the possibility of multiple niches, particularly climate niches, along elevational gradients, we test if populations at high elevations are differentiated from those at low elevations and if there is evidence of cryptic speciation. If the widespread species of *Odontomachus rixosus* currently identified in the region is one good species, then little risk exists for this charismatic ant with regard to climate change. If, however, this species name conceals cryptic diversity, each species will need to be reevaluated concerning its conservation status.

Methods

Sampling – Odontomachus specimens were collected in Sarawak, Borneo, at the localities Bako National Park, Kubah National Park, Niah National Park, Mulu National Park including Mt. Mulu (2,376 m) and Mt. Api (1,750 m), and in the Kuching area between February and March 2012 and during July 2013. Nests were located in the field and ants were collected using an aspirator. All specimens were stored in 99 % ethanol for molecular and morphological analysis. In total, we collected *Odontomachus* specimens from 56 locations in a variety of lowland habitats and different elevations throughout Sarawak. We focused molecular and morphological work on the *O. rixosus* species complex. Additional specimens from Kalimantan (Borneo), Mainland Malaysia, Cambodia, Palau and the Maluku (Moluccas) Islands were included in phylogenetic analyses (see Appendix B for overview of specimens).

Phylogenetic Analysis – To understand the phylogenetic relationships within this group, we considered the barcoding region of the mitochondrial cytochrome oxidase 1 gene (COI) for 1-2 specimens from 45 colonies of *O. rixosus* in Sarawak, Borneo. We also included two workers of *O. malignus*, four workers of *O. rixosus* outside of Sarawak, five workers of *O. latidens*, and four workers of *O. simillimus*. In addition, we analyzed five nuclear genes: Wingless (WG), Long wavelength rhodopsin (LWR), Carbomyl-phosphate synthase II (CAD), Elongation Factor 1-alpha F1 copy (EF1αF1) and the ribosomal gene 28S from a subset of 15 individuals (see Appendix B for overview of specimens). Genomic DNA was extracted from whole ant specimens using the Geneaid Genomic Tissue DNA kit (Taiwan)

and following the manufacturer's protocols. DNA amplifications were carried out using 2 µl of isolated DNA, 1x of PPP Master Mix (Top-Bio, Prague), 0.4 µM of each forward and reverse primers and completed with distilled water to 25 µl. PCR cycling conditions were comprised of an initial denaturation stage of 5 min at 95 °C, followed by 35 cycles each consisting of 30 sec at 94 °C, 50 sec at 50/55 °C, and 90 sec at 72 °C, and a final stage of 5 min at 72 °C. Annealing temperature of primers varied from 50°C in the case of the mitochondrial COI (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) and the nuclear genes wingless, LWR (Philip S. Ward & Downie, 2005)and 28S (Sequeira, Normark, & Farrell, 2000), to 55°C for the nuclear genes CAD (P. S. Ward, Brady, Fisher, & Schultz, 2010) and EF-1αF1 (Schultz & Brady, 2008). Successful PCRs were sequenced at Macrogen (South Korea) using the universal primers *T7promoter* and *T3*. We assembled the chromatograms and aligned and edited the consensus sequences using the program Geneious R7 (Biomatters, 2014). Sequences were aligned using the software modules MUSCLE (Edgar, 2004) and MAFFT (Katoh & Standley, 2013) and AliView (Larsson, 2014). Exonic regions were readily aligned as reading frames were conservative. However, few intronic sections were not clearly aligned after setting different optimization parameters in MUSCLE. We removed from the final analyses those sites that were ambiguous or that we were not certain of their homology. We verified our alignments by checking the congruence of the reading-frame of exonic regions and by eye in the case of introns. We followed the IUPAC nomenclature for heterozygous sites. We used VoSeq (Peña & Malm, 2012) to create all the input files for the follow-up analyses. All nuclear gene sequences are publicly available in GenBank, mitochondrial COI gene sequences in Barcodes of Life database (BOLD, ASPNA project),

and the datasets used for phylogenetic analyses were deposited in TreeBASE (submission ID 17090; http://purl.org/phylo/treebase/phylows/study/TB2:S17090).

Phylogenetic trees were constructed using Bayesian Markov Chain Monte Carlo (MCMC) simulations with BEAST v1.8.2 (Drummond et al., 2012). The program used the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution identified by jModelTest 2 (Darriba et al., 2012) as an appropriate model based on Akaike's Information Criterion (AIC) values. The Bayesian analysis used the default priors on branch lengths, rate parameters, and tree topology with four chains of 50 million generations sampled every 5,000 generations. The first 10 % of trees (4,000) were discarded as burn-in. TaxonDNA software version 1.6.2 (Meier et al., 2006) and MEGA software version 6.0 (Tamura et al., 2013) were used to calculate genetic divergences using uncorrected *p*-distances.

Morphometrics & Color Analysis – All specimens were dry mounted on card triangles. Measurements were taken from 200 individuals (109 *O. rixosus* workers from 13 colonies, mean = 8.4; 81 *O. saltans* sp. nov. workers from 12 colonies, mean = 6.8, 10 *O. dunni* sp. nov. workers from 1 colony) with a Dino-Lite AD-4113T digital microscope at 60x by help of the software Dino-Capture 2.0. Digital photographs for color analysis were taken with a Canon EOS 7D SLR digital camera (Canon EFS 18-55mm lens) in a box lined with white paper at standardized light conditions across all specimens. Color information from three color channels, red (R), green (G), and blue (B), were extracted from six body parts, antennae (Ant), head (Head), thorax (Tho), anterior legs (AntLeg), posterior legs (PostLeg) and abdomen (Abd) from a subset of samples (25 individuals from 9 colonies). Color data was digitalized by help of the program AntColorGrabber developed by Eric Butler (Shaw

University, North Carolina). Images were processed in Adobe Photoshop CS6; body parts were selected by use of the magic wand tool (tolerance: 15) with multiple grabs so that the majority of the body part was colored. Color and morphometrics data were scaled and centered and linear discriminant analyses were conducted in the program R using the packages MASS (Fournier et al., 2012) and Lattice (Sarkar, 2008). LDA visualizations were done with the R package ggplot2 (Wickham, 2009). In addition, boxplots and ANOVAs were constructed in the program R.

Measurements and indices:

CI Cephalic index. HW / HL × 100.

EL Eye Length. Maximum eye length in frontal view.

ELD Eye-Lateral ocellus distance. Distance between eye and lateral ocellus measured in frontal view (only in males).

HL Head length. Maximum length of head in full-face view, excluding mandibles, measured from anterior-most point of clypeal margin to posterior-most point of head vertex, parallel to midline.

HW Head width. Maximum width of head in full-face view (including eyes when surpassing head outline).

ILLD Inner lateral-lateral ocelli distance. Distance between lateral ocelli measured at inner edges of ocelli in frontal view (only in males).

LOCL Lateral ocellus length. Maximum length of lateral ocellus measured in frontal view (only in males).

MdI Mandible index. MdL / HL × 100.

ML Mandible length. Maximum length of mandible in frontal view of head measured from mandibular insertion to apex.

MOCL Medial ocellus length. Maximum length of medial ocellus measured in frontal view (only in males).

MsL Mesosoma length. Maximum length of mesosoma, measured in lateral view, diagonal from cervical shield to posterolateral propodeal edge.

OLLD Outer lateral-lateral ocelli distance. Distance between lateral ocelli measured at outer edges of ocelli in frontal view (only in males).

PnW Pronotum width. Maximum width of pronotum in dorsal view.

PtH Petiole height. Maximum height of petiole, measured in lateral view (bottom edge of petiole parallel to petiolar apex.

PtL Petiole length. Measured in lateral view along dorsal outline of petiole from small antero-apical tooth to apex.

PtW Petiole width. Maximum width of petiole in dorsal view.

SI Scape index. SL / HW × 100.

SL Scape length. Maximum length of antennal scape in dorsal view excluding basal constriction.

Chemical Analysis of Cuticular Hydrocarbons – Cuticular hydrocarbons were extracted on-site. Ants were collected alive in paper-lined containers. Prior to CHC extraction containers were put in the freezer. Ten ants were extracted per colony. Forceps were cleaned with hexane between colonies. One ant and 0.5 ml of hexane were added to an extraction vial and it was manually shaken for 5 min. Then the ant was removed from the vial and the extract

was left to dry until all hexane evaporated. Upon return to the United States, the extract was resuspended in 50 µl hexane and prepared for GC analysis. Samples were analyzed in a 7890A GC system (Agilent) equipped with a DB-5 (20 m x 0.180 mm x 0.40 µm) column (Agilent) and a flame ionization detector (FID). Hydrogen was used as carrier at 56 cm/s average linear velocity. Samples were injected using a 7683B autosampler (Agilent) in pulsed splitless mode at 300 °C and the split valve opened at 2 min. The oven was kept at 60 °C for 2 min, heated to 250 °C at 10°C/min, then to 320 °C at 5 °C/min, and kept at the final temperature for 30 min. The FID was maintained at 320 °C. FID response factors of individual compounds were not determined.

In order to identify the compounds, extracts of single workers were evaporated and re-suspended in 10 µl of hexane. 1 µl of the extract was injected into an Agilent 7890 gas chromatorgraph (Agilent Technologies, Santa Clara, CA), connected to an Agilent 5977 mass selective detector. The GC injection port and the transfer line were set to 315 ºC. The column temperature was held at 60 ºC for 2 min, increased to 220ºC at 40 ºC/min, and then to 315 ºC at 4 ºC/min and held for 20min. Helium was the carrier gas at 1 ml/min, and samples were injected in splitless mode with a purge time of 0.75 min. Electron impact ionization mass spectra were obtained using 70 eV ionizing voltage, with a source temperature of 230 ºC.

Compounds were identified from a combination of their mass spectra, looking specifically for molecular ions or enhanced ions due to fragmentation on either side of methyl branch points, and by comparison of their retention indices to those of straight-chain hydrocarbons (Carlson et al., 1998).

For analysis, GC-FID results for all worker extracts from the 13 colonies surveyed were combined to yield 13 average colony profiles. For each individual worker profile,

relative compound abundances were calculated relative to the cumulative total abundance of all compounds within the extract. The average of these relative compound abundances was calculated for every compound within a colony. Compounds were included in the analysis if they occurred in ≥80% of all average colony samples in one of the two chemotypes. Non-metric multi-dimensional scaling was used to analyze the similarity of profiles within and between chemotypes (Primer 6, PRIMER-E Ltd., Ivybridge, UK). Chord distances were used to calculate the distance matrices. Stress values, representing how well the data are represented in two-dimensions, were also calculated.

Acronyms of repositories:

CSW Coll. D.M. Sorger, Vienna, Austria

NCSUIC North Carolina State University Insect Collection (housed in Department of Entomology), Raleigh, North Carolina, USA

NHMW Natural History Museum, Vienna, Austria

SFDC Sarawak Forest Department Collection, Kuching, Borneo

Results

Molecular Analysis

Our phylogenetic analysis revealed three distinct lineages within the *O. rixosus* clade, two of which are described as new species below (Fig. 2.3, Fig. 2.4). One lineage includes *O. rixosus* specimens from low elevations (below 200 m) from various places throughout Sarawak. A second lineage, *O. saltans* sp. nov. appears to occur predominately at high elevations (> 200 m, < 700 m) on Mt. Mulu. In addition we found a single occurrence of *O.*

saltans sp. nov. in Kalimantan at 390 m. A third lineage, *O. dunni* sp. nov. is only known from a single locality in Kubah National Park near Kuching at 319 m. This third lineage is most closely related to *O. saltans* sp. nov.

The COI divergence distribution between *O. rixosus* and *O. saltans* sp. nov. revealed no overlap in % divergence. The majority of individuals within each lineage showed 0 % divergence and the majority of individuals between lineages showed 7.5 % divergence (Fig. 2.5). Average COI sequence divergence between *O. rixosus* and *O. saltans* sp. nov. amounted to 6.9 %, between *O. rixosus* and *O. dunni* sp. nov. it is 5.5 %, and between *O. saltans* sp. nov. and *O. dunni* sp. nov. 4.2 %.

Distribution of species

Named *Odontomachus* species were distributed non-randomly with respect to elevation (Fig. 2.2). *O. malignus*, a specialist species living in intertidal zones, was found at the lowest elevation while *O. latidens* was found at the highest elevations. Species in the *O. rixosus* species complex (*O. rixosus*, *O. saltans* sp. nov., *O. dunni* sp. nov.) had the broadest elevational distribution (41-676 m). However, *O. latidens* replaced *O. saltans* sp. nov. at higher elevations on Mt. Api and Mt. Mulu at 472 m and 822 m respectively.

Morphometrics & Color

Morphometric characters and color values varied among specimens and sites (Table S2). A key question was whether this variation tracked the molecular divergence of lineages. In short, it did. A linear discriminant analysis of quantitative morphometrics and color shows three clusters (Fig. 2.6). These clusters correspond to the genetic lineages recovered in our

molecular work. The first (LD1) and second (LD2) canonical axes explain 100 % of the total data variance for both morphometrics and color. For morphometrics, LD1 explains 87 % of the variation and LD2 explains 13 %. In this case, LD1 discriminates between *O. rixosus* and *O. saltans* while *O. dunni*, closer to *O. saltans*, differentiates along LD2. The characters that best discriminate between species on the first canonical axis are HW, HL and CI; in addition SL and SI are also informative characters along the second canonical axis. The model matched 97 % of individuals to the correct species. Specimens not correctly identified were mostly *O. rixosus* misidentified as *O. saltans* and vice versa. A single misidentified individual from *O. saltans* as *O. dunni* was a particularly large specimen (Od34_08, MsL 3.628 mm). For color, LD1 explains 75 % of the variation and LD2 explains 25 %. The most discriminating characters along LD1 are green color on head, posterior leg and thorax, blue color on thorax, and red color on posterior leg. Along LD2 green and red color on anterior leg and blue and red color on thorax were the most important characters. The model matched 100 % of individuals to the correct species. Values of the canonical discriminant functions are given in Table 2.3.

Cuticular Hydrocarbons

Analysis of the cuticular extracts clearly revealed the presence of two chemotypes: one consisting of lower elevation *O. rixosus* samples (Od06, 08, 045, 050, 051) and one of higher elevation *O. saltans* sp. nov. samples (Od019, 028, 030, 033, 034, 035, 037, 040). Of the 43 compounds identified from *O. rixosus* and *O. saltans* sp. nov. worker extracts, only 4 compounds were found to be shared between the two chemotypes (Fig. 2.7, Table 2.7). Both chemotypes were rich in unsaturated alkenes and dienes. The lineage two chemotype

displayed many longer chained (C39 and above) compounds that were not present in the lower elevation, *O. rixosus* chemotype. Within chemotype variation is dramatically smaller as compared to between chemotype variation. A two dimensional non-metric multidimensional scaling configuration with an excellent representation (stress value of 0) of the data variation revealed strong clustering within chemotypes and clear separation of both chemotypes (Fig. 2.8).

Taxonomic descriptions

Both new species belong to the *O. rixosus* species group (see Sorger & Zettel [2011] for diagnosis) and, along with *O. rixosus*, are considered part of the *O. rixosus* species complex.

***Odontomachus saltans* Sorger, sp. nov.** (Fig. 2.9)

Type material: Holotype worker (NHMW) and paratypes (16 workers, 1 male CSW, 1worker NCSUIC, 1 worker SFDC): Borneo, Sarawak: Mulu NP, Mt. Mulu, BOR12-047, [Od029], nest between leaves, 658 m, 10.III.2012, (N 4.043356°, E 114.873818°), leg. D.M. Sorger.

Paratypes: Mulu NP, BOR13-006, [Od(13)001], nest in leaflitter, 57 m, 7.VII.2013, N 4.04635°, E 114.81572°, leg. D.M. Sorger (2 workers CSW); Mulu NP, BOR12-041, [Od005], on limestone hill, nest in leaflitter, 76 m, 9.II.2012, N 4.024621°, E 114.822801°, leg. D.M. Sorger (5 workers CSW); Mulu NP, Mt. Api, BOR12-075, [Od044], Kerangas, at base of large tree, 201 m, 15.III.2012, N 4.148511°, E 114.888161°, leg. D.M. Sorger (2 workers CSW); Mulu NP, Mt. Api, Camp 5, BOR12-071, [Od043], at base of fern, 184 m, 14.III.2012, N 4.136618°, E 114.890882°, leg. D.M. Sorger (8 workers, CSW);

Mt. Mulu: BOR12-092, [Od040], at base of tree, 205 m, 11.III.2012, N 4.050511°, E 114.857319°, leg. D.M. Sorger (21 workers CSW); BOR12-061, [Od035], at base of tree, 272 m, 11.III.2012, N 4.049395°, E 114.860032°, leg. D.M. Sorger (11 workers CSW); BOR12-059, [Od036], at base of tree, 274 m, 11.III.2012, N 4.048965°, E 114.860400°, leg. D.M. Sorger (12 workers, 4 males CSW); BOR12-086, [Od039], at base of tree, 285 m, 11.III.2012, N 4.048187°, E 114.861574°, leg. D.M. Sorger (5 worker CSW); BOR12-060, [Od037], at base of tree, 311 m, 11.III.2012, N 4.047825°, E 114.862765°, leg. D.M. Sorger (8 workers CSW); BOR12-015, [Od038], at base of tree, 311 m, 11.III.2012, N 4.047844°, E 114.862669°, leg. D.M. Sorger (18 workers CSW); BOR12-056, [Od034], at base of tree, 360 m, 10.III.2012, N 4.046633°, E 114.864435°, leg. D.M. Sorger (19 workers CSW); BOR12-058, [Od033], at base of tree, 481 m, 10.III.2012, N 4.042326°, E 114.8696°, leg. D.M. Sorger (1 worker CSW); BOR12-057, [Od032], at base of tree, 493 m, 10.III.2012, N 4.042279°, E 114.869933°, leg. D.M. Sorger (5 workers CSW); BOR12-040, [Od019], nest in leaflitter, 543 m, 8.III.2012, N 4.042537°, E 114.871488°, leg. D.M. Sorger (30 workers CSW); BOR12-037, [Od022], at base of small palm in leaf litter, 544 m, 8.III.2012, N 4.042643°, E 114.871353°, leg. D.M. Sorger (11 workers CSW); BOR12-033, [Od024], at base of large tree, 545 m, 8.III.2012, N 4.042471°, E 114.871285°, leg. D.M. Sorger (CSW); BOR12-035, [Od021], nest at base of tree, 545 m, 8.III.2012, N 4.042493°, E 114.871576°, leg. D.M. Sorger (10 workers CSW); BOR12-039, [Od020], nest at base of tree, 554 m, 8.III.2012, N 4.042493°, E 114.871519°, leg. D.M. Sorger (1 workers CSW); BOR12-034, [Od023], at base of large tree, 618 m, 8.III.2012, N 4.042873°, E 114.872704°, leg. D.M. Sorger (CSW); BOR12-052, [Od031], at base of tree, 624 m, 10.III.2012, N 4.042917°, E 114.872863°, leg. D.M. Sorger (2 workers CSW); BOR12-049, [Od030], nest at base of tree,

635 m, 10.III.2012, N 4.042904°, E 114.873055°, leg. D.M. Sorger (CSW); BOR12-028, [Od028], at base of tree, 676 m, 10.III.2012, N 4.043284°, E 114.873871°, leg. D.M. Sorger (CSW).

Description of worker:

Measurements: holotype worker [Od29_09]: CI 71, EL 0.35, HL 2.47, HW 1.76, MdI 60, ML 1.49, MsL 3.43, PnW 1.06, PtH 0.79, PtL 0.79, PtW 0.38, SI 146, SL 2.58; paratype worker with smallest HW [Od40_10]: CI 69, EL 0.31, HL 2.28, HW 1.58, MdI 63, ML 1.44, MsL 3.10, PnW 0.97, PtH 0.77, PtL 0.79, PtW 0.38, SI 156, SL 2.46; paratype worker with largest HW [Od19_06]: CI 74, EL 0.36, HL 2.67, HW 1.96, MdI 59, ML 1.59, MsL 3.59, PnW 1.19, PtH 0.88, PtL 0.93, PtW 0.46, SI 142, SL 2.79.

Structures: Mandibles long, with ca. 6 basal denticles (widely separate from each other) and three apical teeth: proximate tooth truncated, intercalary tooth only slightly shorter than apical. Head rectangular, broadest at level of eyes. Striation on head until ocular ridge, some more striation between ocular and temporal ridge, rest of head smooth and shiny. Microsculpture on head with fine isodiametric reticulum. Mesosoma elongate, slender and low, broadest at level of pronotum. Pronotum rounded, metanotal groove in lateral view present. Coarse rounded sculpture on pronotum (closed circles visible in dorsal view), metanotum and propodeum with coarse transverse sculpture. Petiole short, smooth and shiny, almost conical, with very short petiolar spine, which is rarely absent in small specimens.

Pilosity: Fine loose semi-appressed white pubescence on head, mesosoma and petiole; distance between hairs approximately their length. Head with two standing setae, tergite 1

without setae, tergite 2 with a few setae, number of setae and length increasing towards apex of abdomen.

Colour: Head, mesosoma and gaster dark brown. Legs yellowish.

Measurements (n=4): CI 79-84, EL 0.67-0.756, ELD 0.18-0.19, HL 0.91-1.05, HW 1.15-1.31, ILLD 0.11-0.16, LOCL 0.16-0.20, MOCL 0.18-0.19, MsL 2.51-2.79, OLLD 0.41-0.49, PnW 1.08-1.25, PtH 0.47-0.62, PtL 0.53-0.66, PtW 0.35-0.41.

Colour: Pale yellow.

Habitats: Nests in leaf litter, often found at the base of large tree trunks.

Distribution: Borneo.

Etymology: This species along with *O. rixosus* exhibits a unique leg-jumping behavior (Sorger, in press). The name meaning "jumping" describes this trait.

Notes: *O. saltans* looks very similar to *O. rixosus*. It differs in HW, HL, CI, SI and PtW. For successful identification, a nest series of at least 5-10 workers should be measured.

***Odontomachus dunni* Sorger, sp. nov.** (Fig. 2.10)

Type material: Holotype worker (NHMW) and paratypes (7 workers CSW, 1 worker NCSUIC, 1 worker SFDC) : Borneo, Sarawak: Kubah National Park, BOR12-073, [Od053], at base of tree, 319 m, 29.III.2012 (N 1.608884, E 110.188804), leg. D.M. Sorger.

Description of worker:

Measurements: holotype worker [Od53_10]: CI 72, EL 0.38, HL 2.56, HW 1.85, MdI 61, ML 1.56, MsL 3.59, PnW 1.13, PtH 0.87, PtL 0.85, PtW 0.45, SI 142, SL 2.62; paratype worker with smallest HW [Od53_06]: CI 70, EL 0.34, HL 2.50, HW 1.75, MdI 60, ML 1.51,

MsL 3.40, PnW 1.10, PtH 0.80, PtL 0.82, PtW 0.44, SI 146, SL 2.56; paratype worker with largest HW [Od53_02]: CI 73, EL 0.35, HL 2.63, HW 1.91, MdI 61, ML 1.60, MsL 3.59, PnW 1.16, PtH 0.91, PtL 0.91, PtW 0.45, SI 140, SL 2.68.

Structures: Like *O. rixosus* (see (Sorger & Zettel, 2011) except pronotum smooth and shiny with some striation at anterior base, faint striation may sometimes be present but never as pronounced as striation on rest of mesosoma.

Pilosity: see *O. rixosus* (Sorger & Zettel, 2011)

Colour: Head, mesosoma and gaster dark brown. Legs yellowish.

Habitats: Nest in leaf litter at the base of large tree trunk.

Distribution: Only known from type locality.

Etymology: The species is named in honor of my PhD advisor Dr. Robert R. Dunn who has supported me throughout my graduate studies and played an instrumental part in me becoming the scientist I am today.

Key to *Odontomachus* workers in Borneo

1 Large species (HL > 3.3 mm).....2

– Smaller species (HL < 3.3 mm). 3

2 Head posteriorly with pair of small, but distinct tubercles. Subapical tooth of mandible long and pointed. (***O. malignus*** species group) ..... ***Odontomachus malignus***

– Head posteriorly lacking pair of tubercles. Subapical tooth of mandible short and truncate. (***O. rixosus*** species group) .....***Odontomachus latidens***

3 Subapical tooth and apex of mandible very short. Subapical tooth approximately as long as wide. Temporal prominences striate. Gaster tergite 2 anteriorly with fine microsculpture. (***O. haematodus*** species group) ..... ***Odontomachus simillimus***

– Subapical tooth and apex of mandible elongate. Subapical tooth longer than wide. Temporal prominences smooth. Gaster tergite 2 anteriorly smooth. (***O. rixosus*** species group) 4

4 Pronotum predominately smooth. ..... ***Odontomachus dunni*** sp. nov.

– Pronotum predominately striate. 5

5 Mean of at least 5-10 workers: CI 73-75, HW 1.83-2.18, HL 2.47-2.94, PtW 0.43-0.48, SI 134-141. ..... ***Odontomachus rixosus***

– Mean of at least 5-10 workers: CI 70-73, HW 1.70-1.84, HL 2.38-2.56, PtW 0.38-0.44, SI 144-151. ..... ***Odontomachus saltans*** sp. nov.

Note: *O. monticola*, previously recorded from Borneo (Pfeiffer, Mezger, Hosoishi, Bakhtiar, & Kohout, 2011), is not included in this key. This variable species is not believed to occur south of its type locality in Mainland Southeast Asia (formerly Indochina) (Brown Jr, 1976). Its morphological similarity to the *O. rixosus* species complex appear to have resulted in misidentifications; previous records likely represent *O. rixosus* and *O. saltans* sp. nov.

Discussion

Using an integrative approach of genetic, chemical, morphological and color characters, we were able to reveal two new cryptic species closely related to *Odontomachus rixosus* along an elevational gradient in Borneo. This increases the number of Bornean species in this well-studied genus by a third from what is arguably one of the better-studied tropical gradients. We discovered genetic divergence as well as corresponding morphological, color and chemical differences when investigating variation within *O. rixosus* along this elevation gradient.

Tropical elevation gradients are very steep with temperature dropping up to 6.5° C per 1000 m (Colwell et al., 2008). Ant species richness also declines as one moves uphill (Kaspari, Yuan, & Alonso, 2003; Sanders et al., 2007) and high-elevation endemism is common (Cole, 1940; Glaser, 2006; Sorger, 2011). In the light of climate change, tropical species are more likely to shift their ranges uphill than polewards (e.g., (Chen et al., 2009; Colwell et al., 2008; Freeman & Class Freeman, 2014; Pounds et al., 1999) and as a result the fate of high-elevation endemics is grim (Dirnböck, Essl, & Rabitsch, 2011). But among insects in general, and ant species in particular, species endemic to middle and high elevations are few relative to the great diversity of low elevation taxa (Chen et al., 2009; R. D. Wilson et al., 2007). For example, based on the morphological species concept, of 28 *Odontomachus* species for which we were able to find data, all but seven had elevational ranges that spanned from sea level to approx. 1000 m or more (Fig. 2.1). Only two species seem to be restricted to specific ranges within the gradient. Just like the former species, *O. rixosus* was previously believed to have a broad elevational range of almost 700 m but our results show that *O. rixosus* is in fact restricted to low elevations (< 200 m) while *O. dunni* sp. nov. and *O. saltans* sp. nov. are both considered to be gradient endemics, each occupying distinct elevational ranges (Fig. 2.2).

Cryptic species are frequently discovered when considering molecular markers, especially in the tropics where biodiversity is vast and complex (Funk et al., 2012; Hebert et al., 2004). Elevational gradients are one of the contexts in which cryptic species are most likely to be detected but the traits that allow species to adapt to higher elevations can be subtle (Hodkinson, 2005). As such divergence patterns may be a common occurrence along elevational gradients (Caro et al., 2013; Graves, 1987) and may contribute to an

underestimation of species richness, and as a result an underestimation of species at risk due to climate change. Moreover, in the tropics species discovery is particularly urgent given that habitat change and destruction as well as climate change place a solid conservation deadline on some of these (cryptic) species (Smith et al., 2008). Here we find that what was considered one named species is indeed three, each with a distinct elevational range and likely also distinct life history traits.

One might reasonably ask the question why anyone should care about the conservation of cryptic species of ants too subtly different to be noticed by scientists. Here, the biology of *Odontomachus* suggests features of these new species that might be uniquely valuable. For instance, alkaloid molecules which have been found in *Odontomachus* among a handful of other ant genera display antibacterial, antifungal and antiviral properties and have been linked to inhibiting HIV and certain cancer cells (Aniszewski, 2007). It is likely that the alkaloids of the new and heretofore unnoticed species are unique and scientifically novel. Moreover, *Odontomachus* spp. are charismatic ants with peculiar biomechanics. The ants are capable of shutting their mandibles at rapid speeds (60 m/s). This mechanism propels them into the air when deployed against a hard surface and elucidates interesting insights into the biomechanics of ballistics and propulsion (Patek et al., 2006). A recent discovery revealed that *O. rixosus* and *O. saltans* sp. nov. as the only members of this genus also jump using their legs (Sorger, in press), the biomechanics of which still remain to be studied.

Our genetic data revealed clear divergence within the *O. rixosus* species complex resulting in two new species: *O. dunni* sp. nov. and *O. saltans* sp. nov. which represent the sister clade to *O. rixosus*. The barcoding region of the mitochondrial gene COI showed an average divergence between these species ranging from 4.2 – 6.9 %. These divergences are

consistent with species-level differences (Fisher & Smith, 2008). In addition, we recovered these same monophyletic species clades after adding a set of nuclear markers providing further support for this hypothesis. We also document subtle differences in morphology and color between all three species. While morphology does not always track genetic divergence (Funk et al., 2012), in this case we found subtle but quantitatively clear separation between the three lineages.

While we use morphology and color to differentiate between species, the ants themselves primarily use cuticular hydrocarbons to identify each other. These chemical profiles are not only a means of recognizing nestmates and individual fertility status but are also physiologically important for desiccation resistance (Blomquist & Bagnères, 2010). Here we found significant differences in cuticular hydrocarbon profiles between *O. rixosus* and *O. saltans* sp. nov. The two chemotypes are largely distinguished by qualitative differences, in that the types only share 4 of the 43 identified compounds. However, the chemotypes show little evidence of their compound differences being due to the shifting of homologous series of compounds to a longer or shorter chain length. For instance, almost all of the methyl- and dimethyl-branch alkanes that are present in the *O. rixosus* chemotype are absent in the *O. saltans* sp. nov. chemotype. In summary, the chemical evidence adds weight to our conclusion that the two chemotypes are divergent beyond within-species population-level variation even in ways the ants themselves are likely to recognize.

The documented elevational range of the genus *Odontomachus* in Borneo covers sea level (*O. malignus*) to 1122 m (*O. latidens*). While most species are restricted to either low or high elevations on a single mountain, *O. saltans* sp. nov. covers a relatively wide elevational range (619 m). The species seems to be able to withstand colder climates at higher elevations

while also surviving at warmer conditions at low elevations. What allows *O. saltans* sp. nov. to persist at low elevations is unclear. The species may occupy a different (cooler) microhabitat. For instance, nesting deeper in the soil could offset the temperature difference at higher elevations (Strathdee & Bale, 1998). This notion coincides with the first author's observations of this species in the field. Whenever DMS found *Odontomachus* workers, it was usually easy to find the nest (indicated by brood) which was often located between leaves in close vicinity to foraging workers. However, at two *O. saltans* sp. nov. locations at low elevations, DMS did not find nests and workers seemed to quickly disappear deeper into the ground where the nest might have been.

Here we considered a single species with a broad elevational range but we found that it really represents three separate species restricted to specific elevational ranges. In light of climate warming, some of these species may be at risk of extinction. While most species may be able to move uphill to track climate change, *O. latidens* which is restricted to higher elevations may have nowhere to go. For instance, Mt. Kinabalu has warmed 0.7 ° C over 42 years (Chen et al., 2009). Recognizing these cryptic species is important for modeling responses to climate change. If we considered the *O. rixosus* species complex as a single species, results may be misleading. *O. rixosus*, previously believed to be a widespread species with a broad elevational range, is actually restricted to low elevations (< 200 m) and may be able to move uphill. *O. saltans* sp. nov. and *O. dunni* sp. nov., on the other hand, are possibly more sensitive to warming andmay be more limited in their ability to move up further as temperatures rise.

Our focus here has been on a single species with a broad elevational range in Borneo which really consists of three cryptic species but in reality there are many species with broad

elevational ranges (e.g., Fig. 2.1). If *gradient endemics* such as demonstrated in this study are common, then this pattern of genetic structure could be representative of other species as well (Longino & Colwell, 2011; M. A. Smith, Hallwachs, & Janzen, 2014). Relative to other ant genera *Odontomachus* ants are well studied and compared to insects more generally, ants are among the best studied groups. Therefore, we speculate that many species with widespread elevational ranges, in particular on tropical elevation gradients around the world, are composed of multiple cryptic species, some restricted to high elevations which automatically puts them at high risk with climate change. The discovery of such hidden species may not only reveal unique benefits to humans, but will inform our conservation regime and, as a result, we might have to revise our current estimate of the number of species threatened by climate change.

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Table 2.1. Morphology measurements for ten traits including two indices (see Table 1.1) of *O. rixosus*, *O. saltans* sp. nov., *and O. dunni* sp. nov. per colony.

			HW			HL			EL			ML			SL			MsL			PtL			PtH			PnW			PtW			Cl			MdI			SI		
Morph	Colony	n	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max	min	mn	max
rixosus	Od01-45F	10	1.79	1.95	2.07	2.48	2.67	2.82	0.30	0.33	0.35	1.53	1.59	1.64	2.62	2.74	2.87	3.32	3.51	3.69	0.86	0.92	0.98	0.86	0.89	1.01	1.07	1.14	1.22	0.42	0.45	0.51	72	73	74	57	60	63	136	141	146
rixosus	Od08-64M	10	1.89	1.98	2.03	2.57	2.66	2.73	0.39	0.36	0.39	1.52	1.58	1.69	2.55	2.72	2.82	3.35	3.48	3.60	0.82	0.88	0.92	0.85	0.89	0.95	1.10	1.14	1.17	0.44	0.46	0.48	72	75	78	56	59	65	135	138	145
rixosus	Od08-64M	10	1.89	1.98	2.03	2.57	2.66	2.73	0.39	0.36	0.39	1.52	1.58	1.69	2.55	2.72	2.82	3.35	3.48	3.60	0.82	0.88	0.92	0.85	0.89	0.95	1.10	1.14	1.17	0.44	0.46	0.48	72	75	78	56	59	65	135	138	145
rixosus	Od12-45N	10	1.88	2.00	2.13	2.55	2.70	2.84	0.32	0.35	0.40	1.50	1.56	1.66	2.64	2.72	2.83	3.39	3.50	3.62	0.83	0.90	0.99	0.86	0.91	0.99	1.11	1.18	1.25	0.42	0.45	0.48	72	74	76	56	58	60	132	136	141
rixosus	Od16-34N	10	1.69	1.86	1.91	2.32	2.52	2.60	0.27	0.30	0.34	1.38	1.49	1.56	2.45	2.59	2.68	3.15	3.41	3.53	0.78	0.86	0.91	0.79	0.88	0.94	0.99	1.08	1.13	0.44	0.46	0.48	71	73	75	56	59	60	136	140	145
rixosus	Od42-138A	10	1.92	2.01	2.12	2.61	2.67	2.75	0.33	0.35	0.38	1.49	1.55	1.60	2.59	2.70	2.79	3.32	3.46	3.58	0.83	0.86	0.88	0.77	0.86	0.89	1.13	1.17	1.20	0.43	0.45	0.49	73	75	79	57	58	60	129	134	138
rixosus	Od45-158A	10	1.73	1.90	2.00	2.32	2.54	2.68	0.30	0.33	0.36	1.44	1.51	1.59	2.38	2.61	2.76	3.15	3.41	3.58	0.74	0.83	0.89	0.79	0.86	0.93	1.14	1.09	0.99	0.37	0.43	0.46	74	75	77	56	60	63	134	137	141
rixosus	Od48-143A	10	1.82	1.97	2.08	2.53	2.67	2.78	0.32	0.35	0.38	1.47	1.53	1.62	2.56	2.70	2.84	3.22	3.45	3.69	0.84	0.88	0.94	0.77	0.87	0.96	1.06	1.14	1.22	0.42	0.45	0.49	72	74	75	55	57	59	135	137	140
rixosus	Od49-57M	10	1.66	1.83	2.00	2.25	2.47	2.64	0.28	0.32	0.36	1.40	1.49	1.57	2.35	2.55	2.67	3.06	3.32	3.53	0.74	0.80	0.85	0.75	0.81	0.87	0.98	1.08	1.18	0.39	0.45	0.51	72	74	77	58	60	64	133	140	145
rixosus	Od51-41M	10	1.82	1.92	2.08	2.50	2.61	2.76	0.30	0.33	0.36	1.43	1.50	1.55	2.51	2.61	2.72	3.21	3.41	3.65	0.71	0.82	0.93	0.74	0.83	0.94	1.04	1.10	1.16	0.40	0.44	0.51	72	73	75	55	58	61	128	137	140
rixosus	Od52-31S	10	1.85	1.93	2.01	2.47	2.59	2.68	0.32	0.34	0.36	1.50	1.56	1.60	2.56	2.67	2.74	3.23	3.43	3.51	0.86	0.90	0.93	0.90	0.92	0.96	1.09	1.13	1.19	0.44	0.47	0.50	73	74	76	58	60	63	135	139	142
rixosus	Orix01-45M	2	2.16	2.18	2.20	2.91	2.94	2.97	0.40	0.40	0.40	1.62	1.63	1.64	2.92	2.93	2.94	3.73	3.73	3.73	0.93	0.95	0.96	0.94	0.95	0.97	1.24	1.26	1.28	0.48	0.48	0.49	74	74	74	55	55	56	133	134	135
rixosus	Orix03-51M	2	1.95	2.00	2.04	2.62	2.68	2.74	0.35	0.35	0.35	1.54	1.55	1.56	2.63	2.71	2.79	3.44	3.47	3.50	0.85	0.90	0.95	0.87	0.90	0.93	1.09	1.13	1.18	0.47	0.48	0.49	74	75	75	57	58	59	135	136	137
saltans	Od(13)01-59M	2	1.66	1.71	1.75	2.37	2.38	2.40	0.30	0.30	0.30	1.50	1.53	1.55	2.54	2.55	2.56	3.28	3.33	3.38	0.87	0.87	0.88	0.80	0.83	0.86	1.04	1.04	1.05	0.40	0.40	0.41	69	72	74	63	64	66	146	150	153
saltans	Od05-50M	5	1.63	1.70	1.76	2.39	2.45	2.49	0.30	0.31	0.32	1.41	1.46	1.56	2.49	2.58	2.62	3.22	3.32	3.44	0.81	0.83	0.85	0.79	0.80	0.81	0.99	1.01	1.05	0.36	0.38	0.40	68	70	71	57	60	63	149	151	152
saltans	Od19-553	10	1.64	1.78	1.96	2.31	2.46	2.67	0.31	0.33	0.36	1.41	1.50	1.59	2.41	2.59	2.79	3.17	3.36	3.59	0.77	0.83	0.93	0.76	0.83	0.92	1.01	1.09	1.19	0.38	0.42	0.46	71	73	75	59	61	63	141	145	148
saltans	Od29-658	10	1.74	1.81	1.94	2.39	2.53	2.68	0.30	0.33	0.38	1.45	1.52	1.59	2.58	2.65	2.77	3.30	3.43	3.55	0.79	0.85	0.93	0.78	0.85	0.96	1.06	1.10	1.16	0.37	0.41	0.45	69	72	74	57	60	64	141	146	152
saltans	Od31-624	2	1.75	1.80	1.86	2.53	2.56	2.60	0.29	0.31	0.34	1.39	1.44	1.48	2.62	2.64	2.67	3.37	3.42	3.48	0.84	0.85	0.86	0.79	0.84	0.88	1.08	1.10	1.12	0.39	0.39	0.39	69	70	71	55	56	57	144	147	150
saltans	Od32-498	5	1.63	1.74	1.82	2.28	2.42	2.51	0.30	0.32	0.33	1.43	1.48	1.57	2.45	2.51	2.58	3.11	3.28	3.43	0.74	0.79	0.83	0.74	0.79	0.85	0.99	1.04	1.08	0.38	0.40	0.42	70	72	73	60	61	63	141	144	151
saltans	Od34-360	10	1.69	1.82	1.89	2.38	2.54	2.65	0.29	0.32	0.35	1.45	1.54	1.61	2.52	2.64	2.74	3.30	3.48	3.64	0.76	0.82	0.89	0.77	0.84	0.92	1.04	1.08	1.13	0.37	0.41	0.44	70	71	75	59	61	65	141	146	151
saltans	Od36-274	10	1.67	1.74	1.81	2.35	2.46	2.59	0.28	0.30	0.32	1.39	1.50	1.58	2.41	2.50	2.60	3.13	3.30	3.42	0.79	0.81	0.83	0.77	0.80	0.85	0.99	1.05	1.09	0.36	0.40	0.44	69	71	73	56	61	63	137	144	149
saltans	Od38-311	10	1.74	1.84	1.92	2.47	2.54	2.68	0.31	0.33	0.37	1.46	1.53	1.59	2.59	2.65	2.77	3.37	3.46	3.57	0.82	0.86	0.92	0.80	0.86	0.93	1.06	1.10	1.15	0.42	0.44	0.46	71	72	73	58	60	63	143	145	149
saltans	Od39-285	5	1.59	1.70	1.75	2.28	2.42	2.49	0.31	0.33	0.35	1.42	1.47	1.52	2.42	2.51	2.60	3.16	3.30	3.42	0.73	0.79	0.82	0.73	0.77	0.82	0.99	1.04	1.05	0.39	0.40	0.43	70	70	72	59	61	63	144	148	153
saltans	Od40-205	10	1.58	1.74	1.83	2.28	2.47	2.54	0.31	0.32	0.34	1.41	1.47	1.55	2.46	2.60	2.66	3.10	3.32	3.50	0.78	0.83	0.90	0.75	0.81	0.87	0.97	1.05	1.08	0.38	0.41	0.44	69	70	72	57	60	63	142	150	156
saltans	Od44-158AK	2	1.68	1.73	1.78	2.43	2.47	2.51	0.29	0.32	0.34	1.39	1.43	1.48	2.57	2.61	2.65	3.29	3.38	3.46	0.83	0.84	0.85	0.79	0.83	0.86	0.99	1.03	1.08	0.39	0.41	0.43	69	70	71	57	58	59	148	151	153
dunni	Od53-319K	10	1.75	1.85	1.91	2.50	2.61	2.74	0.33	0.35	0.38	1.51	1.58	1.63	2.54	2.67	2.78	3.40	3.55	3.62	0.82	0.88	0.92	0.80	0.88	0.92	1.10	1.14	1.18	0.42	0.45	0.48	69	71	73	56	61	63	137	144	150

Table 2.2. Color values for six body parts of *O. rixosus, O. saltans* sp. nov., *and O. dunni* sp. nov. from three color channels per colony.

			Abdomen									Antenna									Anterior Leg								
			Blue			Green			Red			Blue			Green			Red			Blue			Green			Red		
Species	Colony	n	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max
rixosus	Od08-64M	3	13.54	14.08	14.97	17.02	18.18	19.51	28.68	33.07	36.47	27.46	33.00	40.90	40.18	45.77	56.06	79.19	86.51	94.19	15.83	31.04	44.67	48.18	65.79	91.26	94.46	112.15	146.85
rixosus	Od45-158A	3	13.11	14.60	15.48	18.43	19.38	20.10	27.41	32.43	36.59	29.96	33.95	38.05	43.67	48.93	52.76	83.57	90.73	95.70	27.92	34.74	40.02	64.21	71.48	76.13	102.98	109.93	114.67
rixosus	Od51-41M	3	11.48	13.22	14.43	15.84	17.85	19.19	28.16	31.98	34.15	28.27	41.94	64.26	39.60	53.82	75.14	76.74	90.60	106.78	30.04	32.71	34.85	61.65	65.49	69.19	92.75	101.95	109.35
rixosus	Orix01-45M	2	11.27	11.98	12.69	15.69	16.11	16.53	27.74	28.52	29.29	34.96	35.25	35.54	39.16	42.26	45.35	55.84	71.10	86.37	15.29	22.97	30.65	37.57	51.03	64.48	70.07	86.37	102.67
rixosus	Orix03-51M	2	11.37	11.78	12.20	15.18	15.75	16.33	28.00	28.51	29.02	21.42	27.72	34.01	36.27	40.24	44.20	82.91	85.90	88.89	31.05	37.71	44.37	67.69	78.67	89.65	110.96	123.14	135.32
saltans	Od(13)01-59M	2	13.81	13.84	13.88	16.65	16.78	16.91	24.74	25.38	26.01	26.86	43.24	59.62	36.56	53.07	69.57	73.84	86.32	98.81	17.45	30.81	44.18	41.49	68.05	94.60	77.29	107.60	137.90
saltans	Od19-553	3	13.04	14.21	15.54	16.98	17.85	18.80	27.67	29.81	33.04	27.47	30.63	32.47	42.68	48.12	54.36	90.05	98.64	107.93	38.28	43.23	49.12	82.55	92.42	103.48	129.43	139.55	152.04
saltans	Od34-360	2	14.17	14.56	14.96	18.37	18.71	19.05	32.38	32.95	33.53	30.04	33.71	37.39	44.13	46.65	49.18	84.15	85.68	87.20	21.41	23.86	26.30	58.95	65.53	72.11	101.13	108.55	115.96
saltans	Od40-205	3	14.79	15.20	15.41	19.32	19.40	19.46	31.59	32.69	34.25	23.75	27.07	31.86	32.41	39.32	45.57	63.43	80.61	92.74	41.93	43.57	45.80	82.93	86.43	89.65	118.37	127.81	134.39
dunni	Od53-319K	2	14.83	15.42	16.01	16.56	17.27	17.99	25.88	27.10	28.32	11.79	11.80	11.81	26.19	26.45	26.71	77.48	77.51	77.53	29.86	34.37	38.89	79.53	89.66	99.79	126.10	139.57	153.03

			Head									Posterior Leg									Thorax								
			Blue			Green			Red			Blue			Green			Red			Blue			Green			Red		
Species	Colony	n	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max
rixosus	Od08-64M	3	19.39	21.40	24.70	27.39	29.39	32.15	53.31	56.39	59.93	20.80	23.04	24.89	50.85	62.04	70.21	98.75	113.80	127.94	14.90	16.71	18.03	17.79	19.36	20.78	27.99	29.56	30.65
rixosus	Od45-158A	3	21.26	23.39	26.76	28.58	29.85	31.69	49.09	50.41	52.29	23.92	27.21	31.87	53.67	59.88	69.39	91.08	97.87	107.89	14.44	15.01	15.32	17.46	17.75	18.00	25.69	27.25	29.34
rixosus	Od51-41M	3	21.70	22.88	23.71	31.42	32.63	34.46	55.25	59.40	65.14	27.39	31.00	38.00	70.03	76.00	86.01	114.51	122.14	131.17	15.01	16.11	17.12	17.88	19.07	20.60	29.28	31.36	35.30
rixosus	Orix01-45M	2	17.97	18.17	18.37	26.16	27.72	29.28	50.65	54.90	59.15	23.63	25.53	27.43	54.79	58.90	63.00	92.79	98.05	103.32	13.88	15.15	16.42	16.79	18.24	19.69	26.07	28.46	30.85
rixosus	Orix03-51M	2	18.65	23.10	27.56	27.28	29.82	32.35	51.01	52.89	54.77	26.15	32.38	38.60	63.64	75.19	86.73	107.59	122.14	136.68	14.01	15.30	16.58	16.87	18.40	19.94	27.98	30.38	32.77
saltans	Od(13)01-59M	2	17.50	18.76	20.02	22.92	23.83	24.73	38.47	38.57	38.68	26.34	27.54	28.74	62.49	65.71	68.92	103.99	108.54	113.08	10.96	13.05	15.14	12.99	15.16	17.34	16.01	18.33	20.65
saltans	Od19-553	3	18.85	19.38	20.21	24.20	24.54	24.81	42.63	46.17	48.97	20.68	25.19	33.94	60.08	69.25	84.29	108.66	119.95	136.65	16.69	18.08	18.82	18.93	20.37	21.13	24.70	25.97	27.19
saltans	Od34-360	2	20.78	24.07	27.35	31.47	31.98	32.50	54.35	59.75	65.15	23.87	28.49	33.11	63.30	73.62	83.94	106.02	118.92	131.82	14.83	17.55	20.28	17.47	20.15	22.82	26.57	28.95	31.32
saltans	Od40-205	3	14.58	21.07	26.26	29.13	30.28	31.39	49.72	56.89	67.19	31.85	33.48	34.52	83.69	86.71	89.33	130.76	133.24	136.98	14.00	15.81	17.02	16.36	18.34	19.04	22.06	25.65	27.74
dunni	Od53-319K	2	15.91	16.30	16.69	17.65	18.14	18.62	28.64	27.75	26.85	21.31	22.45	23.59	52.72	56.95	61.18	89.57	96.92	104.26	13.04	14.06	15.08	14.88	15.81	16.73	22.32	22.59	22.86

Table 2.3. Linear discriminant analysis (LDA) group means and coefficients of discriminant functions for color and morphology in *O. rixosus*, *O. saltans* sp. nov., and *O. dunni* sp. nov..

COLOR Group means						COLOR Coefficients of linear discriminants		
	Abd.B	**Abd.G**	**Abd.R**	**Ant.B**	**Ant.G**		**LD1**	**LD2**
rixosus	-0.4663721	-0.1413937	0.19740151	0.23616677	0.1760444	**Abd.B**	5.5046363	-4.048151
saltus	0.3937803	0.26996	-0.05197303	0.05029991	0.1049351	**Abd.G**	-8.2841881	5.620534
dunni	1.0625171	-0.4307408	-1.0232447	-1.7865835	-1.6689641	**Abd.R**	7.3524635	-3.324124
						Ant.B	10.4187257	9.926856
	Ant.R	**AntLeg.B**	**AntLeg.G**	**AntLeg.R**	**Head.B**	**Ant.G**	-13.3767311	-9.457549
rixosus	-0.01746238	-0.22183778	-0.4147521	-0.4170789	0.26866963	**Ant.R**	5.1227904	2.133284
saltus	0.16897053	0.2850479	0.3611035	0.3296377	-0.08600224	**AntLeg.B**	-0.8912993	6.441344
dunni	-0.73134719	0.01670608	0.8903716	1.0628247	-1.31634144	**AntLeg.G**	-2.3835666	-17.63658
						AntLeg.R	2.739435	12.397908
	Head.G	**Head.R**	**PostLeg.B**	**PostLeg.G**	**PostLeg.R**	**Head.B**	0.3570458	4.042179
rixosus	0.4483698	0.3896058	-0.00804351	-0.2134447	-0.2014907	**Head.G**	-4.9929705	-8.638906
saltus	-0.1191977	-0.04356752	0.205616862	0.4741634	0.4842079	**Head.R**	-1.7170312	3.735463
dunni	-2.3184153	-2.31460009	-0.97580148	-0.9834262	-1.1113497	**PostLeg.B**	-2.5663843	-2.237194
						PostLeg.G	20.026747	8.268639
	Tho.B	**Tho.G**	**Tho.R**			**PostLeg.R**	-16.2810477	-7.831708
rixosus	-0.04593907	0.09147848	0.562482			**Tho.B**	19.2716501	24.185742
saltus	0.23421688	0.12471997	-0.5148376			**Tho.G**	-20.5503887	-32.696044
dunni	-0.87248048	-1.21821	-1.0819446			**Tho.R**	3.1806499	12.048608

MORPHOLOGY Group means						MORPHOLOGY Coefficients of linear discriminants		
	HW	**HL**	**EL**	**ML**	**SL**		**LD1**	**LD2**
rixosus	0.599194	0.4377817	0.2548466	0.2306774	0.2979648	**HW**	-9.88698992	-36.94927316
saltus	-0.7899131	-0.6316712	-0.4202372	-0.4191242	-0.4408394	**HL**	9.40930879	32.93702144
dunni	-0.1329182	0.3447165	0.626094	0.8805223	0.3229825	**EL**	0.42737777	0.31105626
						ML	1.26709487	3.3719842
	MsL	**PtL**	**PtH**	**PnW**	**PtW**	**SL**	-4.42585623	-8.15318767
rixosus	0.1907316	0.2908164	0.3667348	0.4134736	0.5323655	**MsL**	1.18028096	0.21361636
saltus	-0.3768911	-0.4582651	-0.5560846	-0.6271958	-0.7803119	**PtL**	-0.14207096	0.14758913
dunni	0.9738439	0.5420489	0.5068758	0.5734239	0.5177418	**PtH**	-0.08688541	-0.07336243
						PnW	1.34775688	0.76435783
	CI	**MdI**	**SI**			**PtW**	-0.28851408	0.75981971
rixosus	0.6518052	-0.3382122	-0.6966744			**CI**	5.09382755	19.57992379
saltus	-0.7654706	0.4000297	0.8759195			**MdI**	-0.6816318	-2.81370319
dunni	-0.9043652	0.4462731	0.4988032			**SI**	4.54523672	7.32147244

Table 2.4. Compound identifications corresponding to number labels of Figure 7. Relative compound abundances given in average (minimum, maximum). Kovat's retention index and diagnostic ion listed for each compound, when available. Sample sizes: 8 colonies *O. saltans* sp. nov. chemotype, 5 colonies *O. rixosus* chemotype. Colony samples consist of the average relative compound abundances compiled from 10 worker profiles per colony, extracted and analyzed separately.

Compound Identification	*O. rixosus* average (min, max)	*O. saltans* average (min, max)	Retention index	Diagnostic Ions
x-Tritriacontene	0	0.16 (0.03, 0.33)	32.73	462 (M^+), 83, 97, 111
unknown unsaturated C34	0	1.44 (0.98, 2.21)	33.47	476 (M^+), 83, 97, 111, 446
3-Methyltritriacontane	0	2.35 (1.75, 2.73)	33.74	463 (M^+-15), 57/449
x,y-Pentatriacontadiene	0	0.89 (0.81, 0.95)	34.49	488 (M^+), 82, 96, 110, 124, 138
x,y-Pentatriacontadiene	0	1.47 (1.24, 1.64)	34.54	488 (M^+), 82, 96, 110, 124, 138
x,y-Pentatriacontadiene	0	3.52 (2.96, 3.85)	34.6	488 (M^+), 82, 96, 110, 124, 138
x,y-Pentatriacontadiene	0	1.72 (0.12, 4.9)	34.68	488 (M^+), 82, 96, 110, 124, 138
x-Pentatriacontene	0	6.74 (1.85, 8.08)	34.76	490 (M^+), 83, 97, 111
x-Pentatriacontene	0	2.16 (1.08, 3.14)	34.81	490 (M^+), 83, 97, 111
17-;15-;13-Methylpentatriacontane	1.21 (0.78, 2.13)	0.58 (0.28, 0.75)	35.28	491 (M^+-15), 253/281; 225/309; 197/337
13, 17-Dimethylpentatriacontane	0.69 (0.06, 1.33)	0	35.52	505 (M^+-15), 197/351, 267/281
unknown unsaturated C36	0	27.89 (24.05, 29.96)	35.54	504 (M^+), 83, 97, 111, 474
3-Methylpentatriacontane	0	3.2 (2.99, 3.36)	35.75	491 (M^+-15), 57/477
16-;14-Methylhexatriacontane	0.64 (0.23, 1.18)	0	36.27	505 (M^+-15), 239/309; 211/337
x,y-Heptatriacontadiene	0	3.76 (0.7, 7.53)	36.53	516 (M+), 82, 96, 110, 124, 138
x,y-Heptatriacontadiene	0	26.01 (21.8, 30.27)	36.6	516 (M+), 82, 96, 110, 124, 138
x,y-Heptatriacontadiene	0	5.14 (4.57, 6.12)	36.64	516 (M+), 82, 96, 110, 124, 138
x-Heptatriacontene	2.53 (1.81, 4.23)	2.51 (2.2, 2.82)	36.76	518 (M^+), 83, 97, 111
unkown	0	1.58 (1.17, 1.9)	37.08	
19-;17-;15-;13-Methylheptatriacontane	10.06 (8.09, 12.41)	0	37.32	519 (M^+-15), 281/281; 253/309; 225/337; 197/365
13,17-Dimethylheptatriacontane	0.87 (0.55, 1.62)	0	37.51	533 (M^+-15), 197/379, 267/309
unknown unsaturated C38	0	3.47 (0.18, 4.63)	37.49	532 (M+), 83, 97, 111, 502
11,17-Dimethylheptatriacontane	0.86 (0.21, 1.93)	0	37.56	533 (M^+-15), 169/407, 267/309
x-Octatriacontene	1.85 (1.02, 2.52)	0	37.69	532 (M^+), 83, 97, 111
18-;16-;14-Methyloctatriacontane	1.73 (1.41, 2.99)	0	38.29	533 (M^+-15), 267/309; 239/337; 211/365
x,y-Nonatriacontadiene	3.77 (0.41, 5.95)	1.22 (0.34, 1.75)	38.47	544 (M^+), 82, 96, 110, 124, 138
x,y-Nonatriacontadiene	5.23 (3.67, 8.31)	1.3 (0.96, 1.68)	38.51	544 (M^+), 82, 96, 110, 124, 138
x-Nonatriacontene	10.03 (8.65, 11.44)	0	38.73	546 (M^+), 83, 97, 111
x-Nonatriacontene	1.68 (0.2, 2.88)	0	38.8	547 (M^+), 83, 97, 111
19-;17-;15-;13-Methylnonatriacontane	5.76 (3.75, 7.19)	0	39.3	547 (M^+-15), 281/309; 253/337; 225/365; 197/393
x,y-Tetracontadiene	1.92 (1.15, 2.29)	0	39.44	558 (M+), 82, 96, 110, 124, 138
13,17-Dimethylnonacontane	2.13 (1.66, 2.57)	0	39.54	561 (M^+-15), 197/407, 267/337
x-Tetracontene	0.62 (0.19, 1.66)	0	39.68	(no M^+), 83, 97, 111
unknown	3.3 (1.26, 8.11)	0	40.35	
x,y-Hentetracontadiene	8.78 (0.84, 16.11)	0	40.52	(no M^+), 82, 97, 110, 124, 138
x,y-Hentetracontadiene	6.82 (5.63, 8.09)	0	40.6	(no M^+), 82, 97, 110, 124, 138
x-Hentetracontene	1.45 (0.31, 2.62)	0	40.71	(no M^+), 83, 97, 111
17-;15-;13-Methylhentetracontane	1.29 (0.31, 1.75)	0	41.3	(no M^+), 253/351; 225/379; 197/407
x,y-Dotetracontadiene	1.39 (0.74, 2.25)	0	41.45	(no M^+), 82, 97, 110, 124, 138
x,y-Dotetracontadiene	0.45 (0.01, 1.09)	0	41.55	(no M^+), 82, 97, 110, 124, 138
unknown	3.41 (3.05, 4.14)	0	42.3	
x,y-Tritetracontadiene	3.47 (1.19, 5.29)	0	42.45	(no M+), 82, 97, 110, 124, 138
x,y-Tritetracontadiene	3.86 (2.22, 6.34)	0	42.51	(no M+), 82, 97, 110, 124, 138

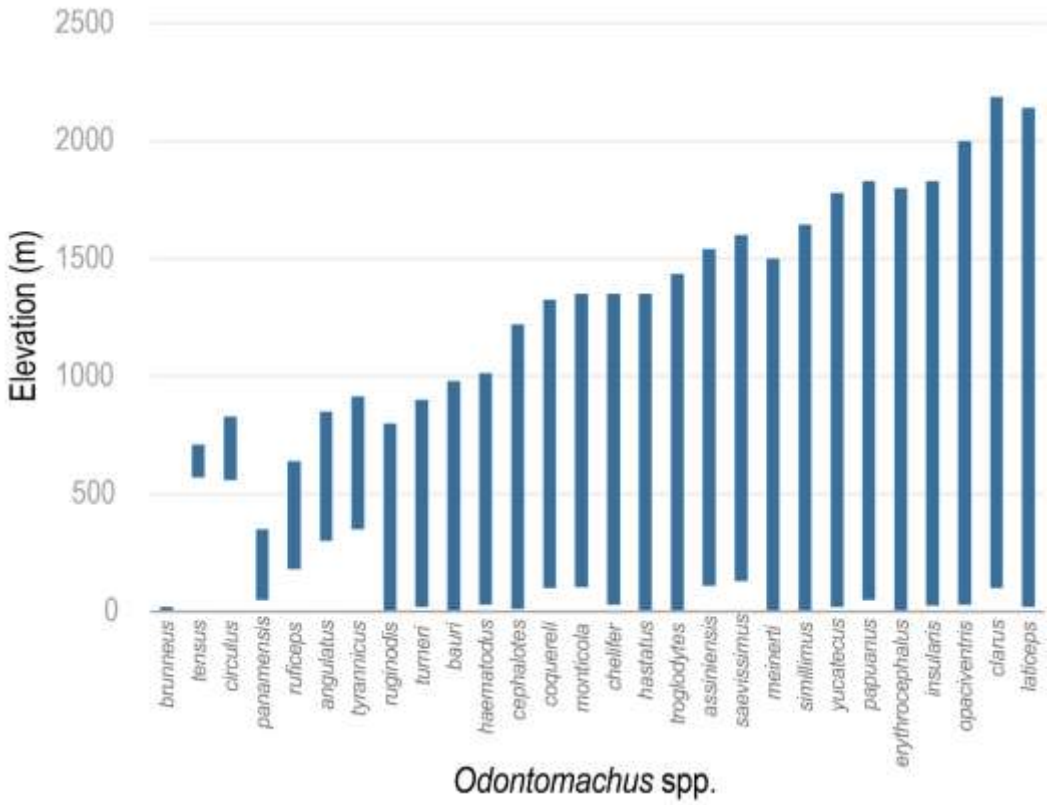

Figure 2.1. Elevational ranges of *Odontomachus* spp. Data from Antweb.org (only species with >5 elevational records were included), additional records from newguineaants.org.

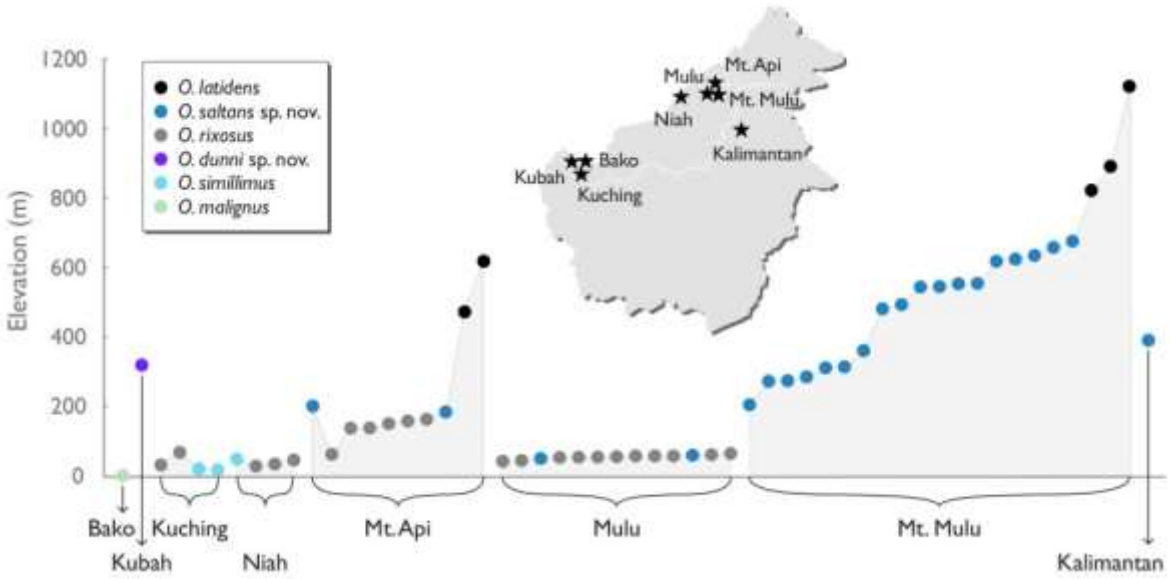

Figure 2.2. *Odontomachus* sampling locations showing elevation and map (Borneo).

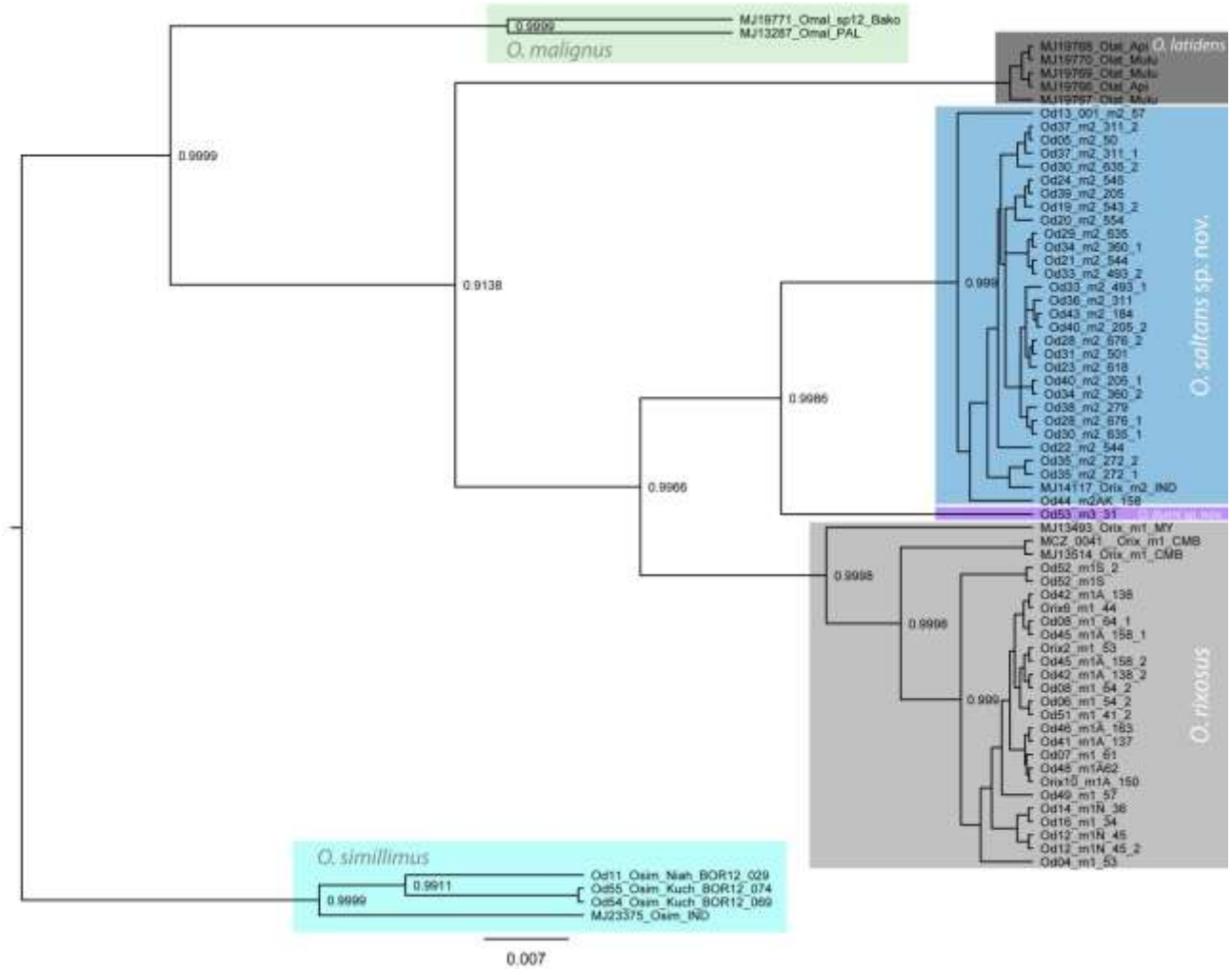

Figure 2.3. COI Bayesian inference topology (BEAST), branch nodes show posterior probabilities, branch length indicates sequence divergence. Colors indicate species.

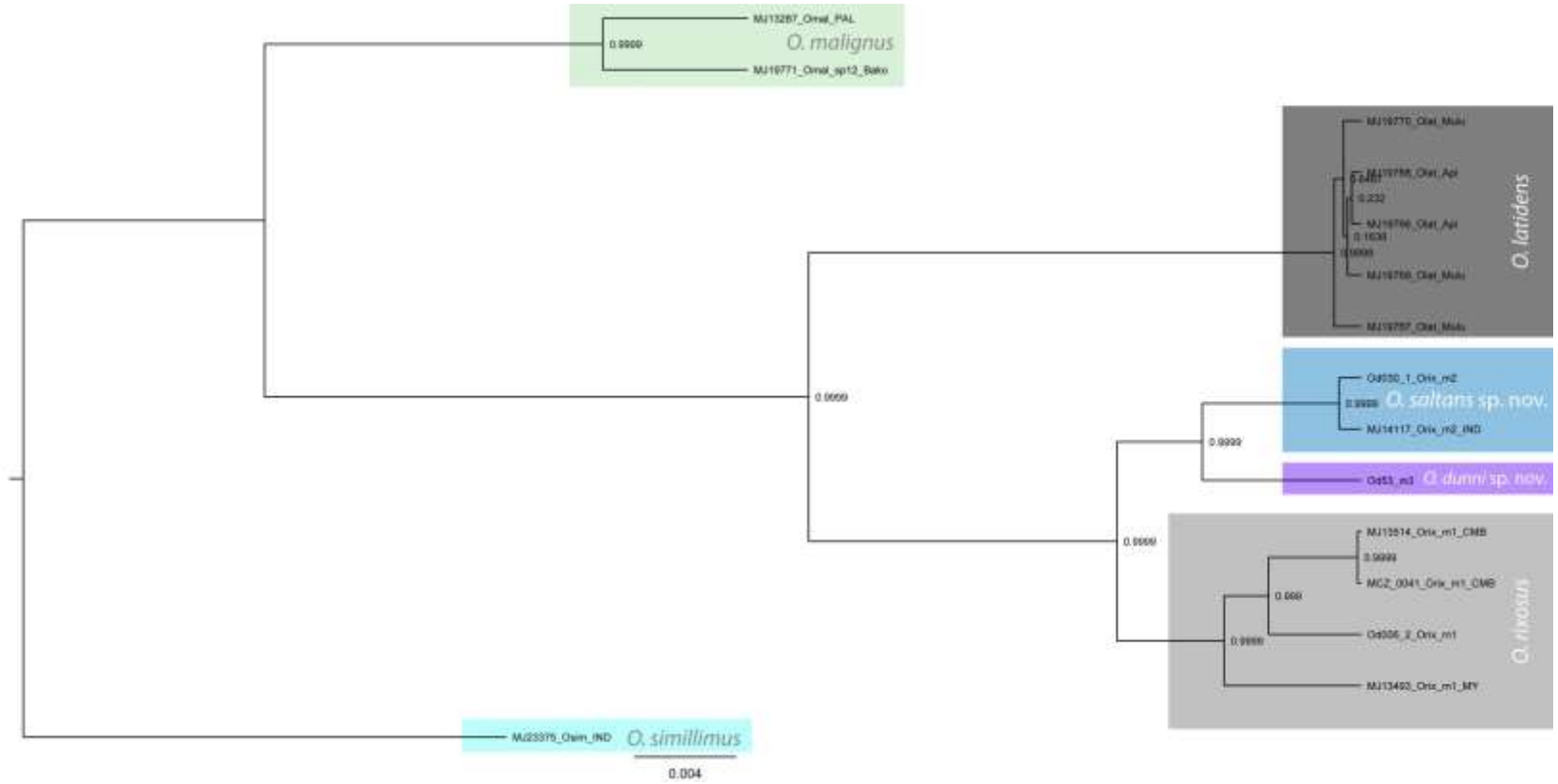

Figure 2.4. Bayesian inference topology (BEAST) based on nuclear and mitochondrial DNA, branch nodes show posterior probabilities, branch lengths indicate sequence divergence. Colors indicate species.

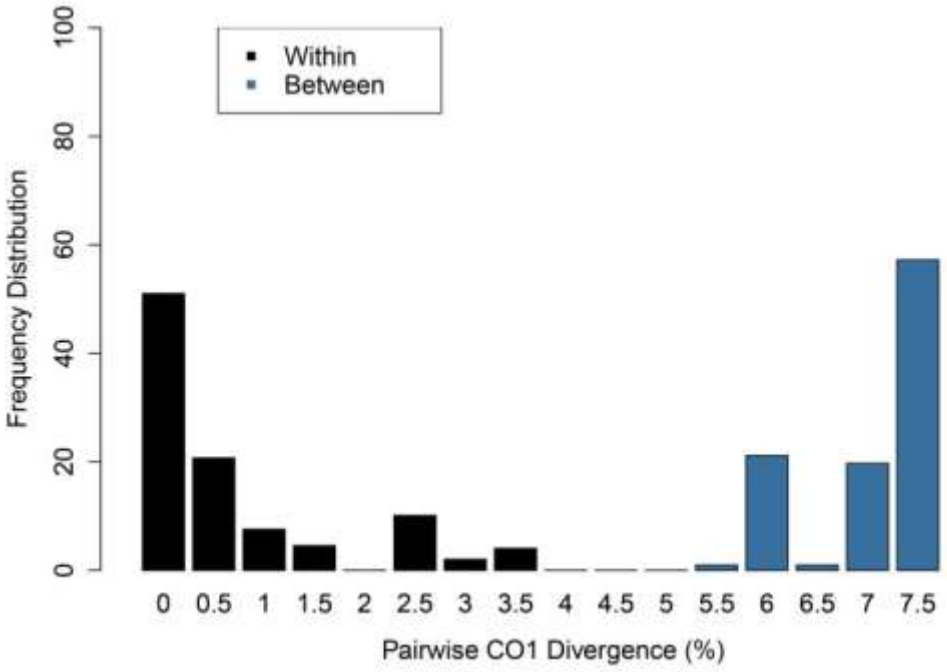

Figure 2.5. Histogram showing pairwise divergence distribution COI within the *O. rixosus* species complex (considering *O. rixosus* and *O. saltans* sp. nov.) and between *O. rixosus* and *O. saltans* sp. nov..

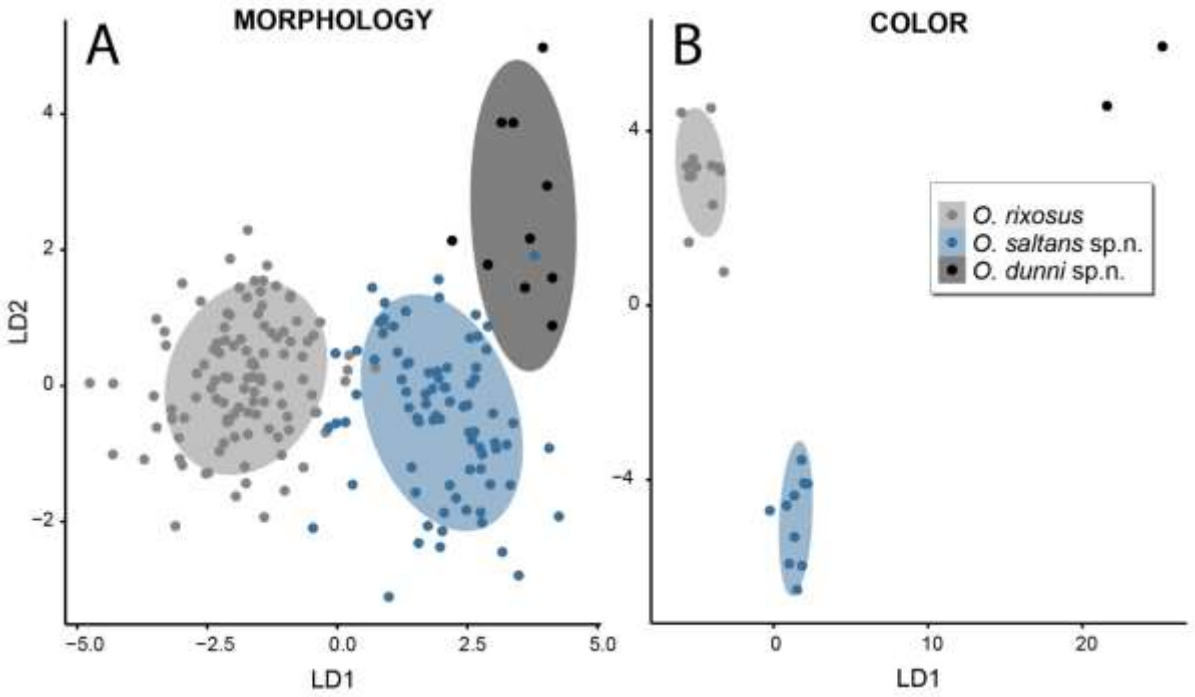

Figure 2.6. Linear discriminant analysis comparing groups based on (A) morphology and (B) color showing first two linear discriminant functions. Both morphology and color differentiate between species.

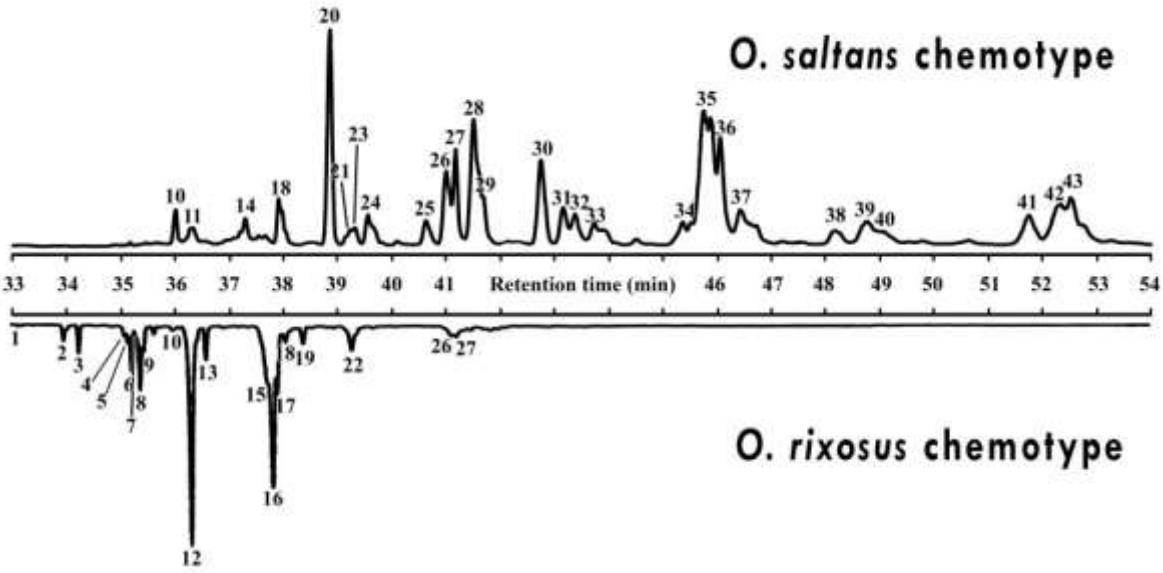

Figure 2.7. Representative chromatograms of worker cuticular hydrocarbon profiles from the two observed chemotypes. Labeled peeks correspond to compound identifications in Table 2.7.

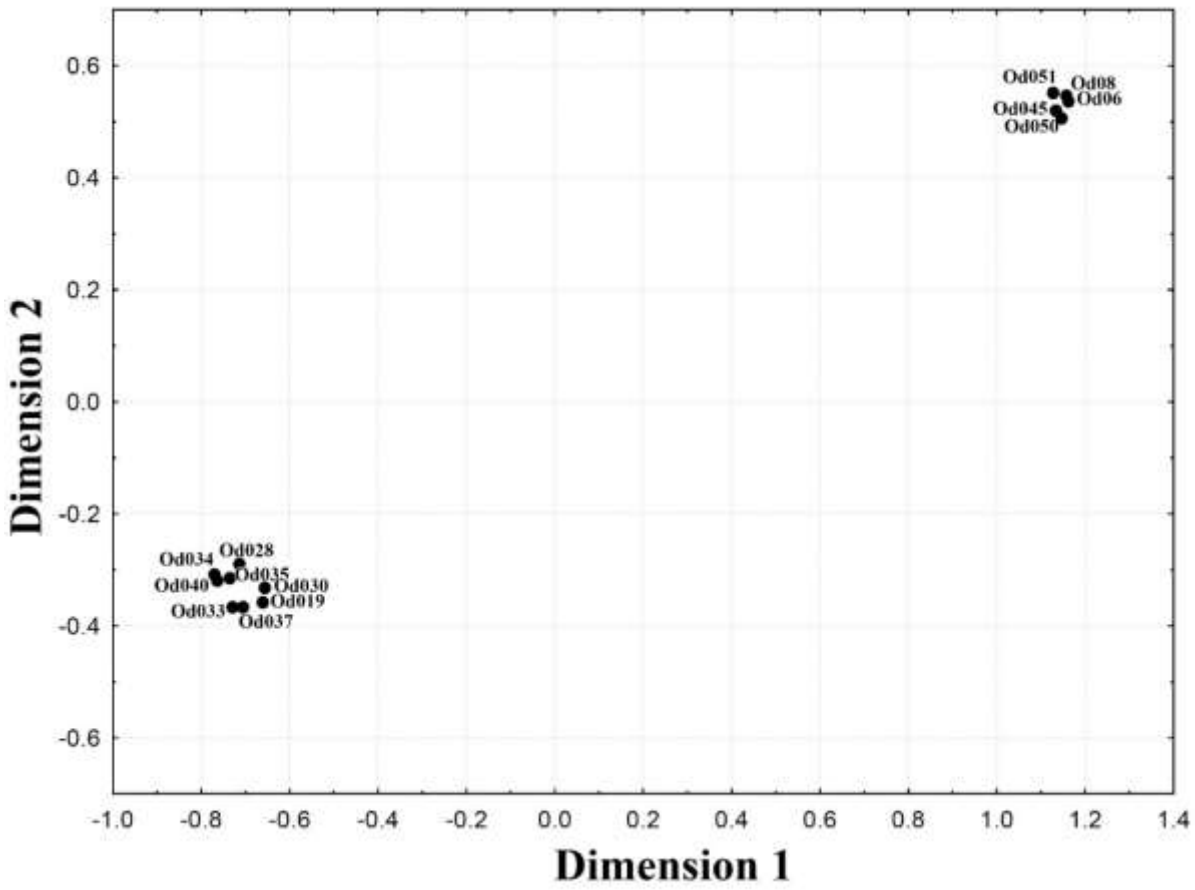

Figure 2.8. Two-dimensional configuration of non-metric, multidimensional scaling of differences within and between *O. rixosus* and *O. saltans* sp. nov. chemotypes.

Figure 2.9. *Odontomachus saltans* worker and male. (1) Worker head, (2) worker dorsal view, (3) worker lateral view, (4) male head, (5) male lateral view. Scale bars represent 1 mm. Photo credit: Matthew Bertone.

Figure 2.10. *Odontomachus dunni* worker. (1) head, (2) dorsal view, (3) lateral view. Scale bars represent 1mm. Photo credit: Matthew Bertone.

CHAPTER 3: The geography of gene flow: genetic (and morphological) structure within a species of trap-jaw ant (Hymentopera: Formicidae: *Odontomachus*) along a tropical elevational gradient

Prepared for publication

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Abstract

A variety of breeding systems exist in social insects including monogyny, polygyny, monoandry, polyandry, parthenogenesis and a combination of these. Many of these systems can be present even within a single species. At least in theory, and a few empirical examples, this variation within species may deterministically track climate and more generally environmental conditions. Environmental variables change systematically (and sometimes rapidly) along elevational gradients and for species distributed along such gradients this represents a highly heterogeneous habitat. Here, we investigate genetic structure, breeding system and morphological differences along an elevational gradient in Borneo in the trap-jaw ant *Odontomachus saltans* (50 – 676 m in elevation) and compare our results to *O. rixosus*, its sister taxon, found on the lower portion of the same gradient (< 200 m). Our results show no genetic structure and no changes in breeding systems along the gradient but indicate a morphological change consistent with macroecological theory, i.e. larger size at higher elevations. We also present results from sequencing what appears to be a founding chamber of *O. saltans*. This represents the first genetic study of the colony breeding structure of a species in the genus *Odontomachus*.

Introduction

Social insects exhibit a variety of breeding systems. In ants, these vary from colonies headed by a single single-mated queen to multiple multiply-mated queens and even parthenogenetic systems have been documented (Kronauer, Tsuji, Pierce, & Keller, 2013; Schilder, Heinze, & Hölldobler, 1999; Timmermans, Hefetz, Fournier, & Aron, 2008). Breeding structure is usually associated with colony reproductive strategies (Hölldobler & Wilson, 1977). For instance, the presence of a single queen (monogyny) is often linked to sexuals engaging in mating flights, extensive queen dispersal, and independent colony founding (Ross & Keller, 1995). Polygyny (multiple queens), on the other hand, is often associated with loss of mating flights, limited dispersal (e.g., colony budding), and dependent colony founding (Ross & Keller, 1995). Variation in breeding structure may be phenotypically plastic and respond to local conditions within the lifetime of a queen (Keller & Reeve, 1994; Ross, 2001; Ross & Keller, 1995); it may also be adaptive and respond to local selection (Gage, 1995), within species, along gradients.

In general, polygyny is predicted to be favored in areas where independent colony founding is difficult, both in the societies of insects (Ross & Keller, 1995), and humans (Ember, Ember, & Low, 2007; Quinlan, 2007). Factors that make independent founding difficult and hence may promote polygynous colonies include areas with higher population densities and limited availability of nest sites (e.g., Herbers 1986; Molet *et al.* 2008). In variable environmental conditions, a genetically more diverse colony can be favorable. Comparative studies suggest that polyandry (a female mating with multiple males) may counteract the effects of genetically incompatible matings, and improve division of labor as

well as resistance to pathogens and parasites (Boomsma & Ratnieks, 1996; Crozier & Fjerdingstad, 2001; Oldroyd & Fewell, 2007).

Elevational gradients offer ideal contexts for understanding the geography of mating systems in societies. Environmental variables such as temperature and precipitation change as one moves up in elevation. For instance, along tropical elevation gradients temperature changes up to 6.5° C per 1,000 m (Colwell et al., 2008). For a species with a wide elevational distribution this means facing very different conditions at the top and bottom of a mountain.

Insects make up 65 % of all animals species (Zhang, 2013a, 2013b). Ants are among the most diverse and well-studied insects with 13,000 described species (Agosti & Johnson, 2005) and a well resolved phylogeny (Moreau & Bell, 2013). The group is a dominant component of most terrestrial ecosystems where it plays a considerable role in ecosystem functioning (e.g., Folgarait 1998; Wardle *et al.* 2011). Ant reproductives are winged and some are capable of flying long distances (Hölldobler & Wilson, 1990; Tschinkel, 2006). However, it is unclear if this also allows them to maintain a steady gene flow within an environmentally heterogenous habitat like an elevational gradient.

The genus *Odontomachus* is among the best studied ant genera (Brown Jr, 1976; Camargo & Oliveira, 2012b; Cerquera & Tschinkel, 2010; MacGown et al., 2014; Sorger & Zettel, 2011). It has a worldwide distribution and consists of 69 extant species with its highest diversity in the tropics. The mating biology and colony breeding structure of *Odontomachus* have not been studied genetically but observations indicate that nests of some tropical species are large (more than 1000 workers) and that monogyny and polygyny are both common among species and sometimes vary within species (Camargo & Oliveira, 2012b; De la Mora, Pérez-Lachaud, & Lachaud, 2008; Ehmer & Hölldobler, 1995).

Odontomachus rixosus is a common species throughout Southeast Asia. This species has highly polygynous colonies (up to 82 queens) that consist of anywhere from 40 to as many as 1000 workers (Bickel, Brühl, Gadau, Hölldobler, & Linsenmair, 2006; Ito, Yusoff, & Idris, 1996). A recent phylogeographic study with focus on the elevational distribution of this species revealed that *O. rixosus* in Borneo consists of a cryptic species complex of three species, each with distinct elevational ranges (see CHAPTER 2). For instance, while the distribution of *O. rixosus* does not exceed 200 m, *O. saltans*' distribution covers over 600 m in elevation, and the third species, *O. dunni*, has only been found at 319 m.

Here, we focus on the genetic structure of the trap-jaw ant *O. saltans* along a 626 m elevational gradient in Borneo. Additionally, we consider phenotypic morphological variation in as much as morphological and genetic structure might track each other. We compare our results to those for its close relative *O. rixosus* which does not occur higher than 200 m in elevation. First we investigate if there is genetic structure within *O. saltans* associated with elevation. We consider metrics such as relatedness and heterozygosity among both, sterile workers and reproductives. Previous research on genetic structure along elevational gradients in insects has produced mixed results (Gilles, Litrico, Tillard, & Duvallet, 2007; Sabando, Vila, Peñaloza, & Véliz, 2011) but provided some indication that a panmictic population structure may be more common in unstable habitat conditions (i.e. conditions fluctuate, e.g., due to flooding or temperature changes) while isolation-by-distance patterns may be more common in stable conditions. For instance, studies of two mayfly species (*Atalophlebia* spp.) produced variable results with one species associated with habitat stability showed no genetic structure while the other, living in less stable habitats, exhibited isolation-by-distance (Wright, 1943).

Secondly, we want to know whether there is variation in social structure that coincides with elevation. There is evidence that monogyne colonies are more common at higher elevations (Purcell et al., 2015) and that inbreeding increases in colder climates (Vargo et al., 2013). Ant queens at higher elevations i.e. in colder, unpredictable and patchier habitats may be larger and better at establishing colonies independently while the more stable lowland conditions favor large, fast-growing polygynous colonies (Purcell et al., 2015). Similarly, inbreeding in termites is associated with cool, moist habitats, in particular mean annual temperature and soil moisture (Kaspari & Vargo, 1995). The authors hypothesize that larger colonies at colder temperature (Kaspari & Vargo, 1995) may require an increased egg production that can only be supported by daughters mating with their father and thereby increasing inbreeding levels (Vargo et al., 2013). Lastly, we also investigate elevation-associated changes in a suite of morphological characters. Generally, body size should increase in colder temperatures due to better food storage by larger organisms (Arnett & Gotelli, 1999; Bergmann, 1847; Shelomi, 2012). Support for this prediction in insects has been variable but, generally, it should be investigated within species rather than among species (Shelomi, 2012).

Methods

Sample collection

Odontomachus saltans and O. rixosus specimens were collected in Sarawak at Niah National Park, in the Kuching area and at Mulu National Park including Mt. Mulu (2376 m) and Mt. Api (1750 m) in February and March 2012 and July 2013. Nests were located in the field and ants were collected using an aspirator. All specimens were stored in high percentage ethanol

for molecular and morphological analysis. In total, we collected *O. saltans* specimens from 23 locations (50 m – 676 m elev.) and *O. rixosus* specimens from 22 locations (31 m – 163 m elev.) (Fig. 3.1). Three additional *O. rixosus* specimens from Cambodia and mainland Malaysia as well as a specimen of *O. saltans* from Kalimantan, Indonesia were included in COI data analysis (Appendix C).

Genotyping

DNA data processing

DNA was extracted from whole specimens or from three legs using the Genomic DNA Mini Kit (Tissue) (GeneAid). Universal microsatellite primers for ants were used to generate the data for population level analyses (Butler, Siletti, Oxley, & Kronauer, 2014). We tested 23 loci in our focal species for consistent amplification, polymorphism and clearly scorable allelic pattern. Out of 23 loci tested, eight were polymorphic for *O. saltans* and seven for *O. rixosus* (although four of these only weakly so). These loci were amplified in two multiplex sets using fluorescently labeled primers (Applied Biosystems) and Multiplex PCR Master Mix (QIAGEN), following the manufacturer´s instructions for PCR parameters and composition with annealing temperature 54°C and total volume of each reaction 10 µl. Allelic patterns were scored manually using the software GeneMapper 3.7 (Applied Biosystems).

For *O. saltans* we genotyped 9-28 (mean = 15) workers of 20 colonies. We also genotyped a total of 10 queens from 7 colonies. For *O. rixosus* we genotyped 12-28 individuals from 18 colonies. In addition we genotyped what appeared to be a founding

colony of *O. saltans* consisting of four queens, three workers and one larva contained within a small chamber in the leaf litter.

Mitochondrial sequence data were obtained from 26 *O. rixosus* colonies (2 workers per colony from 5 colonies) and 29 *O. saltans* colonies (2 workers per colony from 6 colonies) (see Appendix C). From each specimen, a 568 bp fragment of the mitochondrial Cytochrome Oxidase subunit I (COI) gene was amplified by PCR. DNA amplifications were carried out using 2 µl of isolated DNA, 1x of PPP Master Mix (Top-Bio, Prague), 0.4 µM of each forward and reverse primers and completed with distilled water to 25 µl. PCR protocols consisted on 95°C for 5 minutes, 35 cycles of: 94°C for 30 seconds, 50/55°C for 50 seconds and 72°C for 90 seconds, finalizing the program with 72°C for 5 minutes. Annealing temperature of primers was 50°C (Folmer et al., 1994). Successful PCRs were sequenced at Macrogen (South Korea) using the universal primers *T7promoter* and *T3*. DNA sequences were assembled, edited and aligned in Geneious R7. (Biomatters, 2014). Sequence aligment was performed with MAFFT v. 7 (K. Katoh & D.M. Standley, 2013) and coding regions were checked for consistency with a reading frame.

Genetic Data Analysis

Basic population genetic parameters such as number of alleles per locus and observed and expected heterozygosities were calculated using the software GenAlEx 6.5 (Peakall & Smouse, 2012) and FSTAT (Goudet, 2001). Diversity parameters, including nucleotide diversity (π) and haplotype diversity (h), were computed with DnaSP 5 (Librado & Rozas, 2009). Genetic distances between haplotypes were reconstructed using a minimum-spanning network algorithm implemented in PopART 1.7 (epsilon = 0) (Bandelt et al., 1999). Trends

in the demographic history of both species were investigated using Tajima's D and Fu's Fs statistics. A significant negative value in these two tests indicates a population expansion event.

We assigned parentage using the program Colony vers. 2.0.5.9 (Wang, 2004) to confirm that all nests sampled along the gradient represent distinct colonies. We then analyzed worker and queen genotypes to assess genetic structure along the gradient. For nests with no sampled queens, we reconstructed putative queen genotypes based on worker genotypes (mean n = 15) in Colony using the full likelihood method with estimated allele frequencies. Since the mating system is unknown for this group, we ran the data set with both all combinations of monogyny/monoandry and polygyny/polyandry of males and females. Genotyping error rates were derived from a re-genotyped subsample (approx. 10 %) of the dataset. When inferring genotypes, we averaged the output across four independent runs with different seed numbers. If more than one possible parental genotype was generated, we only considered genotypes with a likelihood of 0.75 or higher. For colonies with genotyped queens (see Appendix C), we used a consensus between Colony reconstructions and sequenced queen genotypes for analysis.

To identify monogynous and polygynous colonies, we analyzed parentage output from Colony. Results were visualized in the program Pedigree Viewer vers. 6.5b (Kinghorn & Kinghorn, 2010). In addition, we calculated pairwise relatedness (r) values among individuals within each colony using the algorithm of Queller and Goodnight (1989) in GenAlEx 6.5 (Peakall & Smouse, 2012).

An Isolation by distance analysis using geographic distance was done using the Isolation By Distance Web Service Version 3.23 (Jensen, Bohonak, & Kelley, 2005) with

both queen and worker genotypes using F_{ST} and relatedness (r) values. For isolation by distance analyses, we included only colonies on Mt. Mulu (*O. saltans:* 16 colonies) and from Mulu (*O. rixosus*: 8 colonies) to control for geographic distance. In addition, we conducted linear regressions using relatedness (r), F_{ST}, heterozygosity (H_e, H_o), allelic richness with elevation as the independent variable. All values were considered as colony averages.

Morphometrics

200 specimens from 25 colonies (2-10 workers per colony, mean = 8) were used for morphometric analysis. All specimens were dry mounted on card triangles. Measurements were taken with a Dino-Lite AD-4113T digital microscope at 60x by help of the software Dino-Capture 2.0. We measured ten standard morphometric characters and three indices (see Table 3.1 and Appendix C) and conducted linear regressions in the software R (R Development Core Team, 2011). Visualizations were done using R packages ggplot2 (Wickham, 2009), reshape2 (Wickham, 2007) and plyr (Wickham, 2011).

Results

Descriptive statistics of microsatellite variability

We analyzed eight polymorphic loci in *O. saltans* and detected a mean of 11.6 alleles (range 4–19) per locus. Allelic richness averaged 18.8 alleles (range 15–28.8) per locus and mean gene diversity was 3.6 (range 2.5–5). We analyzed the same eight loci in *O. rixosus* and detected a mean of 7.7 alleles (range 2–19) per locus. However, overall, these loci were less polymorphic in *O. rixosus* (see Table 3.2), e.g. loc40 was only polymorphic in one colony (Od01-67F). Allelic richness averaged 13.6 alleles (range 9–17.3) per locus and mean gene

diversity was 2 (range 0.8–3.1). There was no overlap in alleles at loc41, loc40, loc6, loc5 and loc21 between the two species.

Descriptive statistics of haplotype data

O. saltans had 7 haplotypes throughout the sampled region. Haplotype diversity (h) amounted to 0.522 (SD 0.124), nucleotide diversity (π) was 0.002. Tajima's D (-1.638, p = > 0.10) and Fu's Fs (-2.488, p = 0.053) indicate that the population has not undergone a recent expansion. In comparison, for *O. rixosus* seven haplotypes were identified throughout the sampling region. Haplotype diversity (h) amounted to 0.684 (SD 0.097), nucleotide diversity (π) was 0.01. Tajima's D (-1.114, p = > 0.10) and Fu's Fs (2.283, p = 0.113) also indicate that the population has not undergone a recent expansion.

Is there genetic structure associated with elevation?

No iteration of the isolation by distance analysis using microsatellite data rendered significant results with the exception of *O. rixosus* workers in Mulu using F_{ST} values (Fig. 3.2). However, this result is probably due to a single outlier colony that was geographically further away than the rest. We also did not recover a significant relationship between relatedness (r), F_{ST}, heterozygosity (H_e, H_o), and allelic richness when regressed against elevation (see Fig. 3.3 for r and H_o).

All haplotypes for each species were joined in a minimum-spanning network (Fig. 3.4). In *O. saltans*, one haplotype (1) comprised 73 % of all individuals and a total of four haplotypes were present on Mt. Mulu, each separated by a single mutation (Fig. 3.4A). In *O.*

rixosus, haplotype 1 comprised 55% of all individuals and a total of four haplotypes were present in Mulu separated by 2-7 mutations (Fig. 3.4B).

Does social structure vary with elevation?

In a colony headed by one single-mated queen, relatedness between workers is expected to be approx. 0.75 as ant males are haploid and females are diploid (e.g., Queller & Strassmann 1998). In a polygynous colony, relatedness is expected to be lower due to the mixing of more alleles from mother/s and father/s. Therefore, we used relatedness as a proxy for mating system and investigated a possible correlation with elevation. A linear regression between relatedness and elevation did not reveal a significant relationship in either species. Relatedness values were greater than 0.69 in 16 out of 20 colonies (80 %) in *O. saltans* and 9 out of 18 colonies (50 %) in *O. rixosus* (Fig. 3.5). Colony consistently reconstructed 11 colonies as single queen/single male across all settings in *O. saltans* and 5 for *O. rixosus*. Colony outputs including relatedness values for both species are summarized in Table 3.3. COI sequences were identical within colonies.

Does morphology change with elevation?

Five morphological characters (PnW: $r^2 = 0.21$, p = 2.07e-05; EL: $r^2 = 0.12$, p = 0.002; CI: $r^2 = 0.12$, p = 0.002; HW: $r^2 = 0.11$, p = 0.003; PtH: $r^2 = 0.05$, p = 0.046) in *O. saltans* showed a positive relationship with elevation and one character showed a negative relationship (SI: $r^2 = 0.10$, p = 0.004) (Fig. 3.6). Three additional characters (MsL, HL, SL) showed a positive trend but were not significant.

Results from sequencing possible founding chamber

All four queens shared one haplotype at each locus and had a maximum of three alleles in all but one locus where there were four alleles. Workers and the larva had no more than three alleles at each locus and shared at least one allele at each locus with one of the queens. This suggests that all individuals were related as indicated by shared alleles.

Discussion

Our results revealed no patterns in genetic or social structure associated with elevation in *O. saltans* (elevational range 50 – 676 m), nor were genetic and social structure of *O. saltans* different from those of *O. rixosus* which does not occur at elevations exceeding 200 m. However, we did find changes in six morphological features that were correlated with elevation and gained insights into the possible colony founding mechanism of *O. saltans*.

The lack of genetic structure within *O. saltans* suggests that reproductives are capable of flying up and down the mountain to mate. Previous studies considering the influence of elevation have suggested (and in some cases shown) that panmictic population structures are common where habitats are unstable (and hence local adaptation is foiled by that instability). On the other hand, isolation-by-distance patterns are more common in stable environments (Baggiano, Schmidt, Sheldon, & Hughes, 2011; Purcell et al., 2015). While the elevational gradient on Mt. Mulu covered by *O. saltans* is heterogeneous in regards to temperature with an estimated difference of 3 °C between the top and bottom of the 600-m gradient (Collins, 1980), the species occurs only within one, relatively stable, forest type (high mixed dipterocarp forest, 150 – 800 m; Collins 1980). Yet, we did not find isolation by distance in *O. saltans*, suggesting that these ants are indeed able to maintain a steady gene flow within a

climatically heterogeneous elevational gradient. These results are consistent with results documented for flies (Gilles et al., 2007), rodents (Gonçalves, Marinho, & Freitas, 2009), and mayflies (Sabando et al., 2011).

The breeding structure of *O. saltans* indicates that both monogyny and polygyny occur in this species but monogyny seems to be prevalent (80 %). Likewise in *O. rixosus* both monogyny and polygyny occur, however, contrary to *O. saltans,* polygyny and monogyny appear to be equally common. However, it is important to note that there are limitations to our results for *O. rixosus*. Only three out of seven microsatellite loci were sufficiently polymorphic, resulting in a limited genetic sample size. Consequently, relatedness values may be inflated and colony reconstructions may be incorrect. For instance, colony reconstructions in *O. rixosus* assigned the same queen to colonies several hundreds of meters apart in Mulu. This could either mean that these colonies are indeed very large or it could mean that there was simply not sufficient information to infer these relationships.

There is evidence from an ant species in the Swiss Alps, *Formica selysi*, that monogyne colonies are more common at higher elevations (Purcell et al., 2015). Contrary to the notion that a diverse gene pool is advantageous in unstable conditions, the authors hypothesize that in this case stable conditions favor polygynous colonies due to the greater availability of resources. The higher proportion of monogynous colonies at higher elevations may be due to a number of reasons including faster development time of brood which may be advantageous in temporally unpredictable environments. It is important to note that the authors consider conditions at higher elevations to be more unstable (colder, unpredictable and patchy environment) than conditions at low elevations (continuous habitat). This may be the opposite for the tropical elevational gradient we are considering in this study. At 700 m

we still find the same habitat, high mixed dipterocarp forest, that we do at 150 m, which is approximately where we start to see *O. saltans* on Mt. Mulu. Other forest types featuring *Odontomachus* are found at low elevations and include frequently flooded habitats. As a result conditions within *O. saltans*' elevational range on Mt. Mulu might be more stable compared to conditions at low elevations in Mulu. *Odontomachus* nests were usually found at the bases of trees, tree trunks or other elevated structures onto which the colony could evacuate in case of flooding. And colonies were indeed observed moving their nests repeatedly within only a few days. Perhaps, for ants, the relevant scale for considering the harshness and instability of environments is far smaller than elevational bands but instead the scale of habitat patches.

While we did not find a significant correlation between relatedness and elevation in *O. saltans*, we did find a positive relationship between morphological character sizes and elevation. Individual body size relates to ecological, physiological and life-history traits. Generally, body size should increase at colder temperatures due to better food storage by larger organisms, but in reality support for this prediction is variable (Chown & Gaston, 2010; Geraghty, Dunn, & Sanders, 2007; Kaspari & Vargo, 1995). We found a positive correlation in six morphological features including one index. Although the variation explained by the linear model is a modest 20 %, the pattern is clear. However, if we consider variation in morphology on the species level between *O. rixosus* and *O. saltans*, using the average elevational range of each species, we did not find support for larger size (using HW as a proxy for body size (Weiser & Kaspari, 2006)) at higher elevations. *O. saltan*s is smaller than *O. rixosus* (see Fig. 3.7). Although, these results are limited by a small sample size both within and between species, they suggest that considering such macroecological patterns on

the population level within species renders different results than on the species level (also see Shelomi 2012).

The results from sequencing a possible founding chamber indicate pleometrosis (multiple queens start a colony) of related queens. Observations from other *Odontomachus* species put these observations into context. For instance, in *O. assiniensis*, isolated groups of dealate females have been observed in natural conditions (Ledoux 1952 cited in Brandao 1983) and observations in *O. troglodytes* suggest that there are no nuptial flights as queens are mated within the maternal nest and then leave in small groups (Brandao, 1983; P. Colombel, 1971; Pierre Colombel, 1970). However, we can only speculate about whether this is the principal founding mechanism in this species and if it results in secondary monogyny, or if multi-queen colonies are founded this way (i.e. primary polygyny).

Based on colony reconstructions, the most likely breeding system for *O. saltans* is female monoandry paired with either male monogamy or polygamy. All other Colony settings reconstructed fewer queens than were collected and genotyped for some colonies, which seems improbable assuming that all queens collected within a nest were also reproductively active within the colony. Multiple sequenced queens matched reconstructed genotypes and were consistent with workers being their daughters, which supports the hypothesis that these queens were indeed reproductively active within the colony.

We did not find genetic structure along a tropical elevational gradient in *O. saltans*, suggesting that these ants maintain a panmictic population structure along a heterogeneous (variable in regards to temperature) environmental gradient. While our results within *O. saltans* were clear, future studies shall seek to address these questions on a broader spatial scale of sampling. Also, here we used universal microsatellite markers which were

sufficiently variable for *O. saltans*, but did not appear to be sensitive enough for *O. rixosus* despite these two species being closely related. Species-specific microsatellite markers or SNPs discovered by methods for non-model organisms like ddRADSeq (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) may prove to be superior alternatives to resolve the population structure of this species.

Acknowledgements

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Table 3.1. Morphological characters measured from *O. rixosus* and *O. saltans*.

Abbr.	Name	Description
CI	Cephalic index	HW / HL × 100
EL	Eye Length	Maximum eye length in frontal view
HL	Head length	Maximum length of head in full-face view, excluding mandibles, measured from anterior-most point of clypeal margin to posterior-most point of head vertex, parallel to midline
HW	Head width	Maximum width of head in full-face view (including eyes when surpassing head outline)
MdI	Mandible index	MdL / HL × 100
ML	Mandible length	Maximum length of mandible in frontal view of head measured from mandibular insertion to apex
MsL	Mesosoma length	Maximum length of mesosoma, measured in lateral view, diagonal from cervical shield to posterolateral propodeal edge
PnW	Pronotum width	Maximum width of pronotum in dorsal view
PtH	Petiole height	Maximum height of petiole, measured in lateral view (bottom edge of petiole parallel to petiolar apex)
PtL	Petiole length	Measured in lateral view along dorsal outline of petiole from small antero-apical tooth to apex
PtW	Petiole width	Maximum width of petiole in dorsal view
SI	Scape index	SL / HW × 100
SL	Scape length	Maximum length of antennal scape in dorsal view excluding basal constriction

Table 3.2. Microsatellite information for *O. saltans* and *O. rixosus*.

Locus	Gene diversity		Number of alleles		Allelic Richness	
	saltans	*rixosus*	*saltans*	*rixosus*	*saltans*	*rixosus*
loc41	4.542	8.134	4	10	3.017	5.919
loc7	7.316	3.217	4	6	3.273	2.451
loc18	10.82	8.17	13	10	7.944	5.547
loc6	9.673	1.465	18	3	8.142	1.798
loc5	10.892	N/A	19	N/A	9.811	N/A
loc40	8.786	0.485	10	2	5.791	1.217
loc20	10.827	10.297	19	19	9.48	8.025
loc21	9.759	3.71	6	4	4.69	2.849

Table 3.3. Predicted number of queens in each colony and males they mated with determined by reconstructions from COLONY considering four breeding structures for each species: MM = male monogamy, MP = male polygamy, FM = female monoandry, FP = female polyandry. Q represents number of queens, M represents number of males. Within-colony relatedness (r) values of workers are included.

				MMFM		MMFP		MPFM		MPFP	
Colony	Species	Elevation (m)	Relatedness (r)	Q	M	Q	M	Q	M	Q	M
Od05-50M	*saltans*	50	0.856	1	1	1	1	1	1	1	1
Od(13)01-59M	*saltans*	59	0.839	1	1	1	2	1	1	1	2
Od43-184A	*saltans*	184	0.878	2	1	1	3	2	2	1	3
Od44-201AK	*saltans*	201	0.782	1	1	1	1	1	1	1	1
Od40-205	*saltans*	205	0.414	3	3	2	3	5	4	2	4
Od35-272	*saltans*	272	0.76	1	1	1	1	1	1	1	1
Od36-274	*saltans*	274	0.493	3	3	1	4	3	3	1	4
Od39-285	*saltans*	285	0.527	4	4	2	7	9	4	4	5
Od38-311	*saltans*	311	0.793	1	1	1	1	1	1	1	1
Od37-314	*saltans*	314	0.718	1	1	1	1	1	1	1	1
Od34-360	*saltans*	360	0.74	1	1	1	2	2	1	2	1
Od33-481	*saltans*	481	0.754	2	2	1	2	2	2	1	2
Od22-544	*saltans*	544	0.829	1	1	1	1	1	1	1	1
Od19-553	*saltans*	553	0.374	2	2	2	3	2	2	2	3
Od20-554	*saltans*	554	0.751	1	1	1	1	1	1	1	1
Od23-618	*saltans*	618	0.804	1	1	1	1	1	1	1	1
Od31-624	*saltans*	624	0.698	1	1	1	1	2	1	2	1
Od30-635	*saltans*	635	0.786	1	1	1	1	1	1	1	1
Od29-658	*saltans*	658	0.866	1	1	1	1	1	1	1	1
Od28-676	*saltans*	676	0.799	1	1	1	1	1	1	1	1
Od52-31S	*rixosus*	31	0.618	3	3	1	3	3	2	1	3
Od16-34N	*rixosus*	34	0.835	1	1	1	1	1	1	1	1
Od07-36M	*rixosus*	36	0.591	3	3	3	6	3	3	3	6
Od51-41M	*rixosus*	41	0.424	4	4	4	4	6	4	4	4
Orix6-44M	*rixosus*	44	0.414	5	5	4	7	8	6	6	8
Od12-45N	*rixosus*	45	0.729	1	1	1	1	1	1	1	1
Od04-53M	*rixosus*	53	0.578	1	1	1	1	1	1	1	1
Orix2-53M	*rixosus*	53	0.899	1	1	1	4	1	1	1	4
Od06-54M	*rixosus*	54	0.798	1	1	1	2	5	3	4	2
Orix03-55M	*rixosus*	55	0.716	3	3	3	3	3	3	5	5
Od49-57M	*rixosus*	57	0.828	3	3	2	4	4	3	3	3
Od08-64M	*rixosus*	64	0.454	3	3	3	4	4	4	4	8
Od01-67F	*rixosus*	67	0.546	3	3	1	4	3	3	2	4
Od41-137A	*rixosus*	137	0.784	1	1	1	1	1	1	1	1
Od42-138A	*rixosus*	138	0.767	2	2	4	5	4	3	4	3
Od48-143A	*rixosus*	143	0.852	1	1	1	1	1	1	1	1
Od45_12-158A	*rixosus*	158	0.462	4	4	2	6	7	4	2	6
Od46_01-163A	*rixosus*	163	0.643	4	4	2	6	5	3	4	4

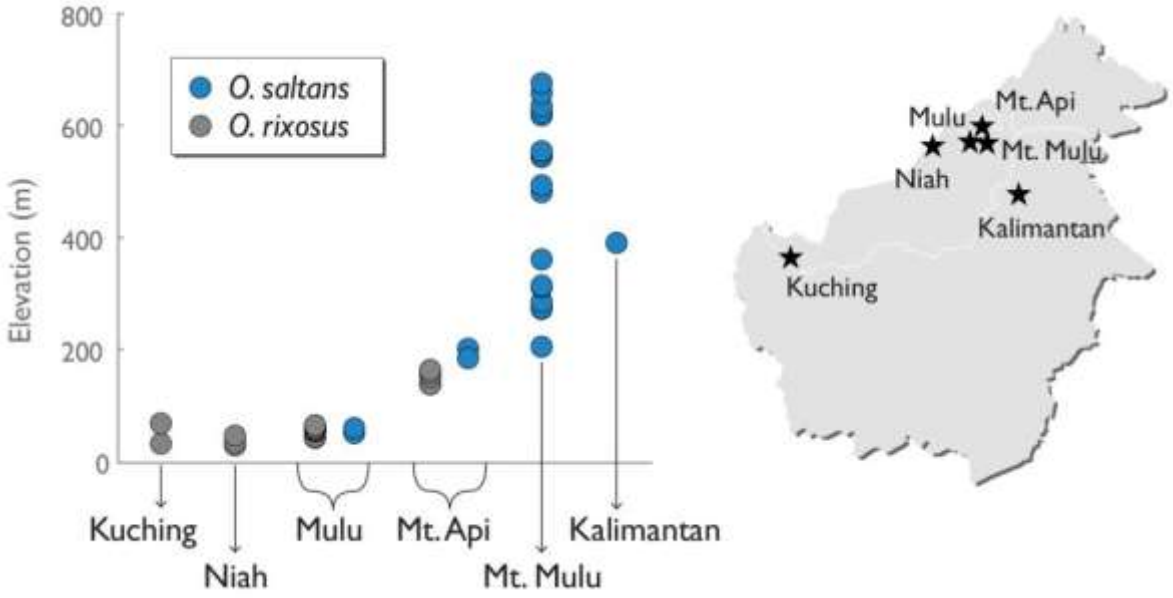

Figure 3.1. *O. saltans* and *O. rixosus* sampling map showing locations and elevational ranges.

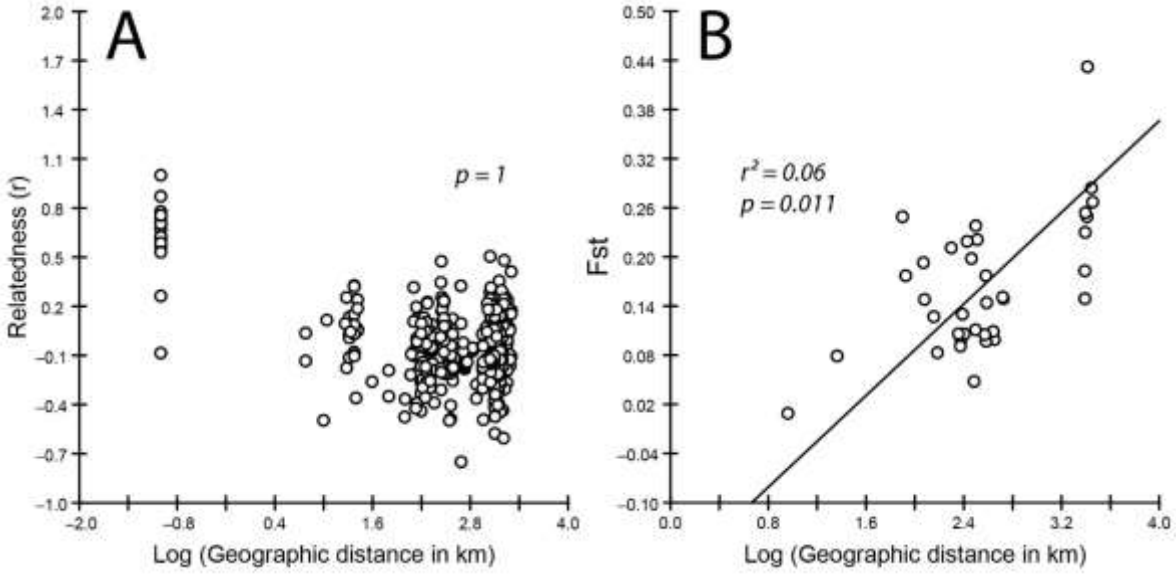

Figure 3.2. Isolation by distance analysis: (A) the relationship between relatedness in *O. saltans* queens and (B) F_{ST} in *O. rixosus* workers with geographic distance. Relationship in (B) driven by one colony that is geographically further away from rest.

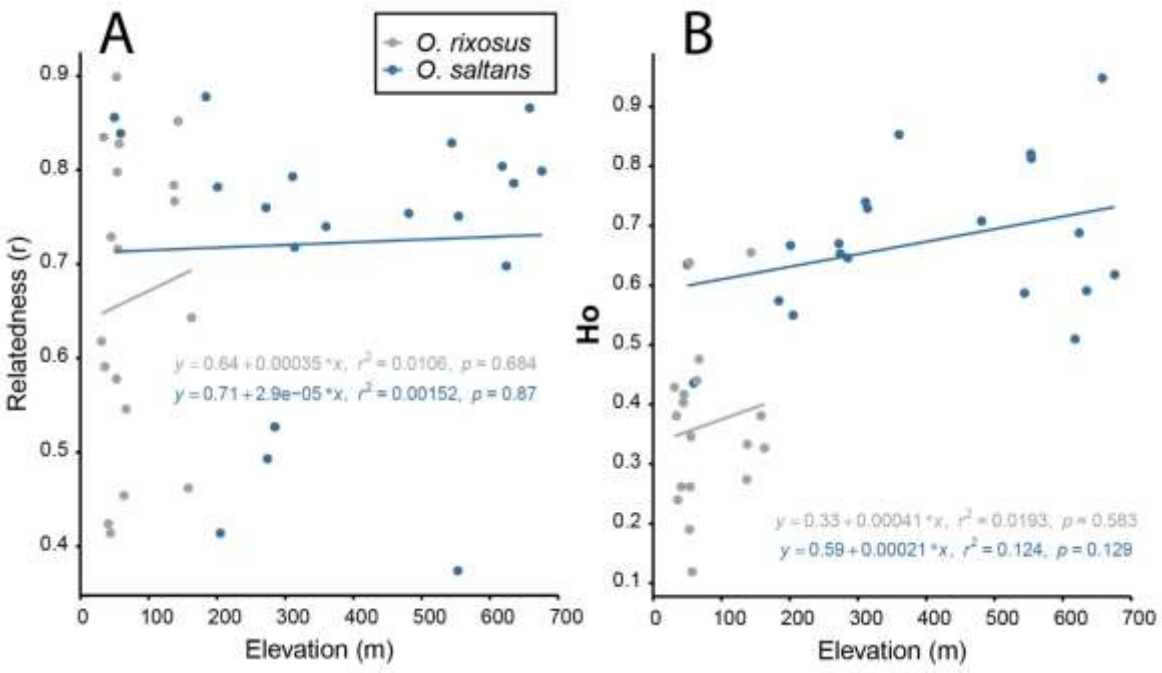

Figure 3.3. Linear regression of (A) relatedness & (B) heterozygosity by elevation (workers) in *O. saltans* and *O. rixosus*.

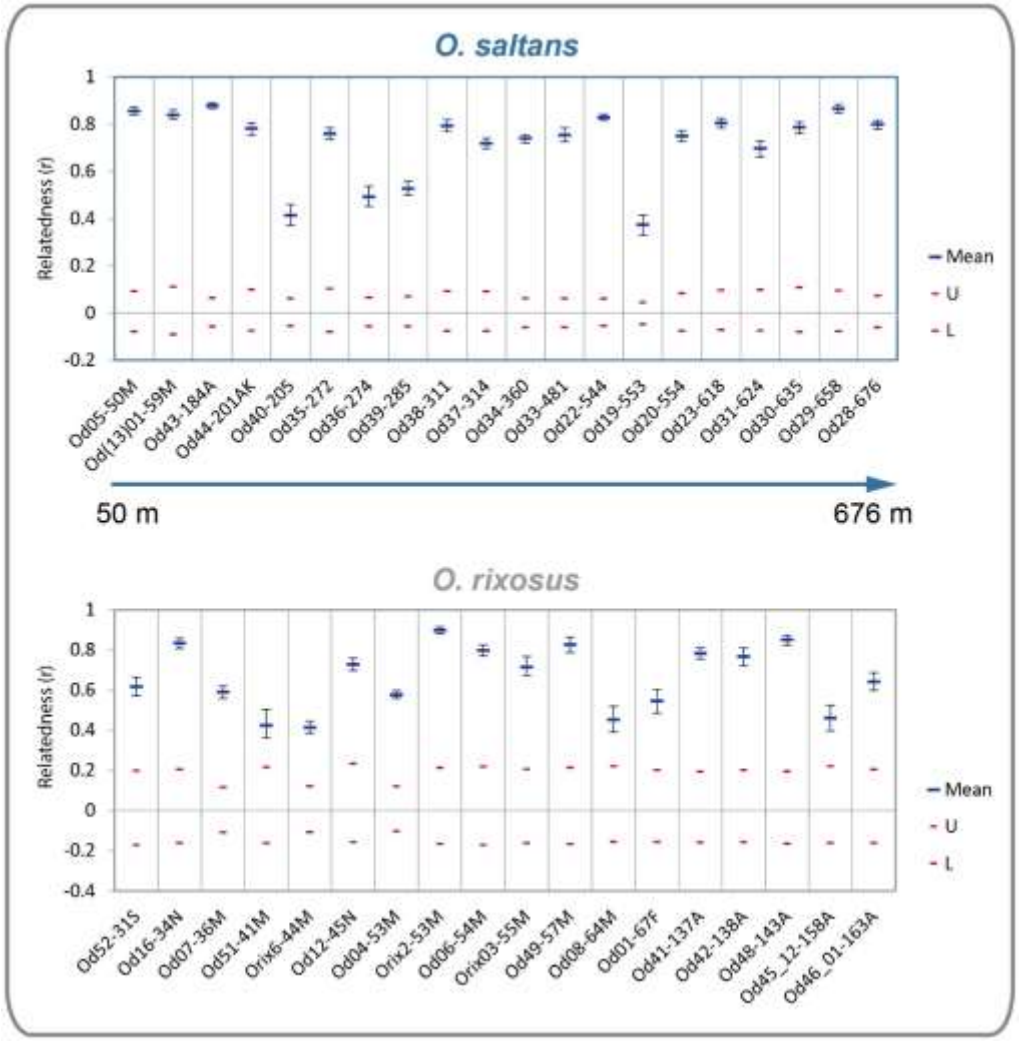

Figure 3.4. Mean within colony pairwise relatedness (workers) in (A) *O. saltans* and (B) *O. rixosus*. Upper and lower error bars bound the 95% confidence interval about the mean values as determined by bootstrap resampling. Upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of 'No Difference' across the populations as determined by permutation. Elevation gradient indicated for *O. saltans* (50 – 676 m).

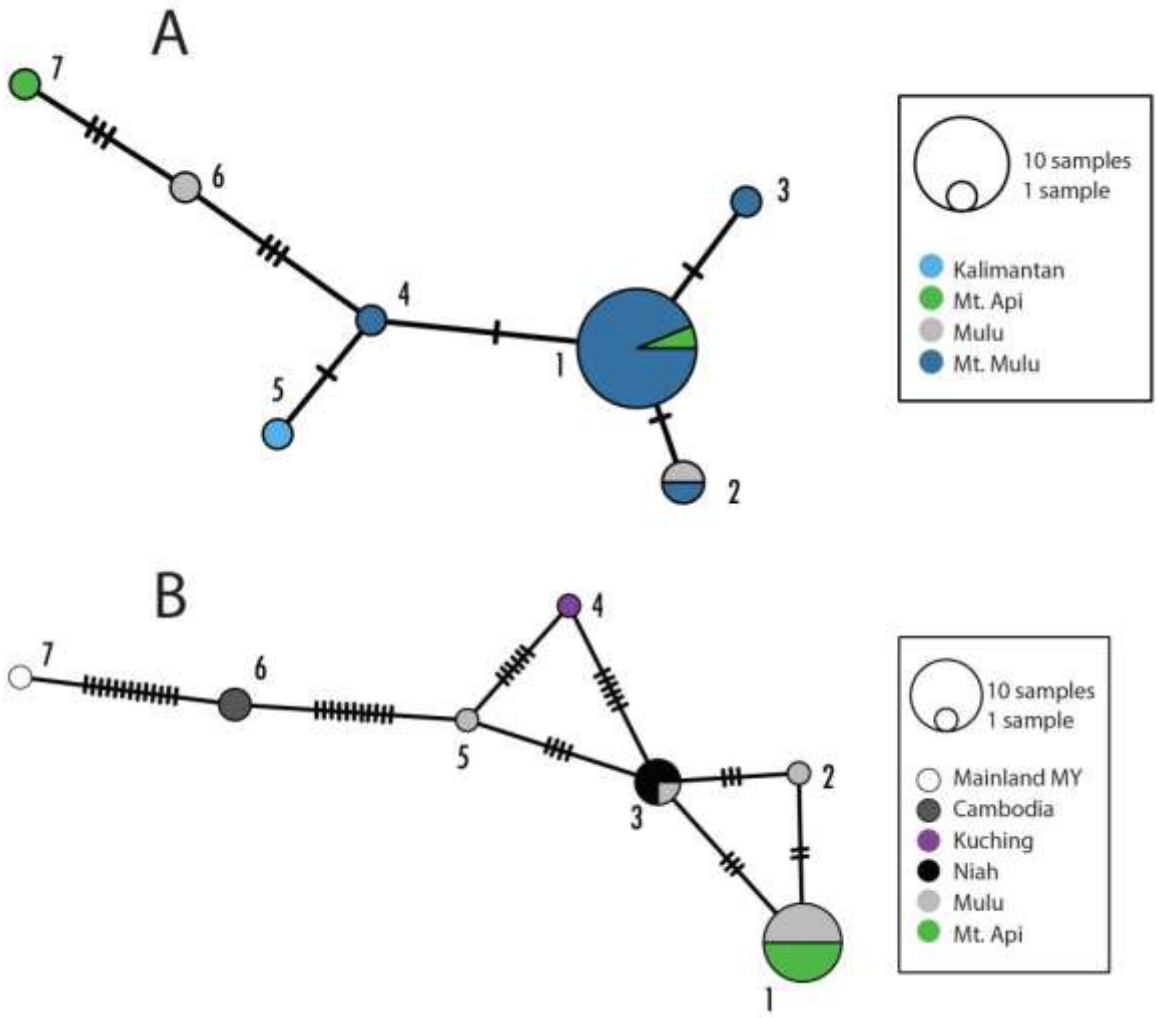

Figure 3.5. Minimum-spanning haplotype network for (A) *O. saltans* and (B) *O. rixosus*. Size of nodes represent number of individuals per haplotype, tick marks between nodes indicate number of mutations between haplotypes.

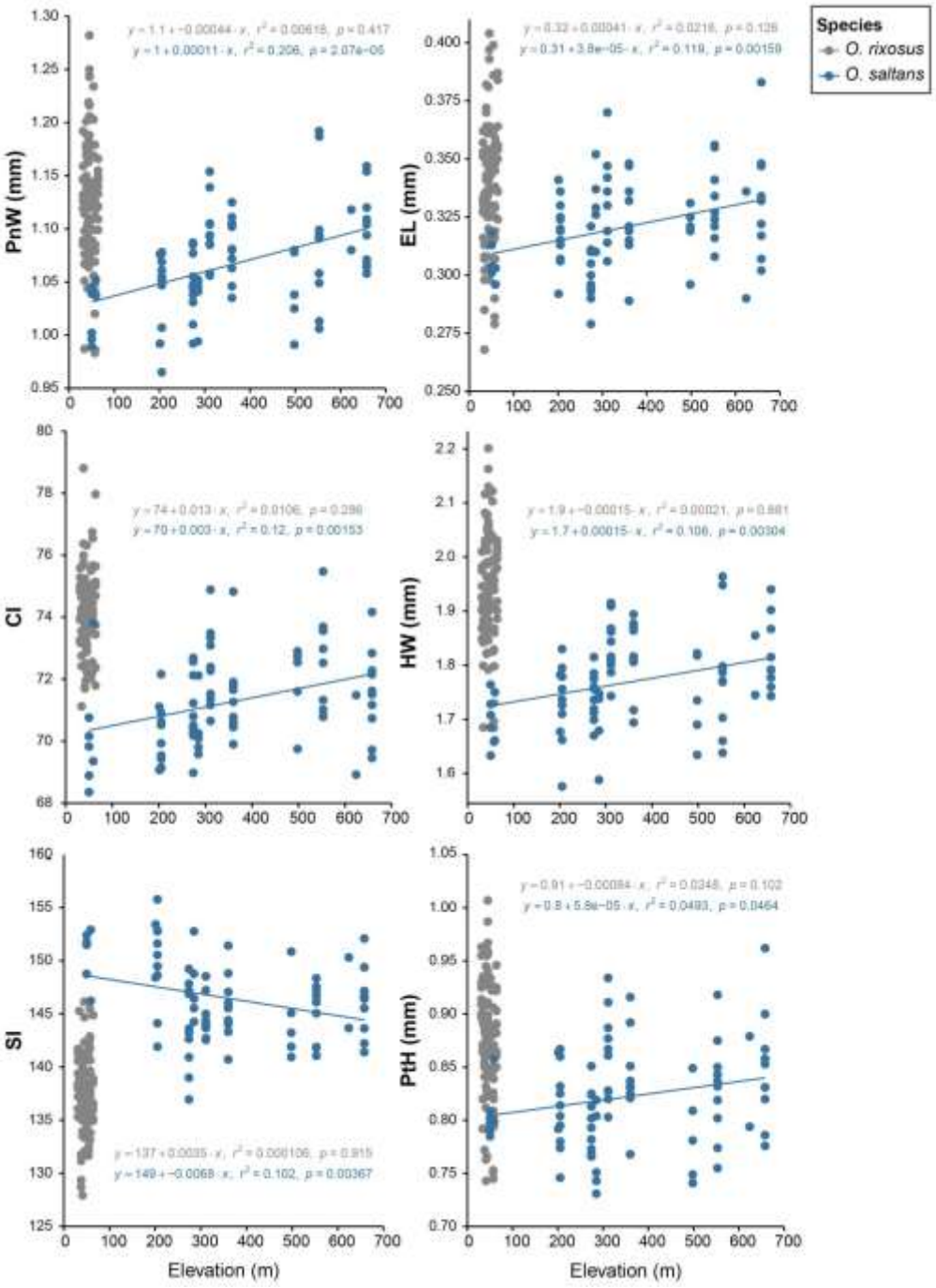

Figure 3.6. Linear regressions of six morphological traits by elevation. See Table 3.1 for key to abbreviations. In these six traits there is an increase in body size with elevation. SI is an index, a negative slope indicates increase in both SL and HL.

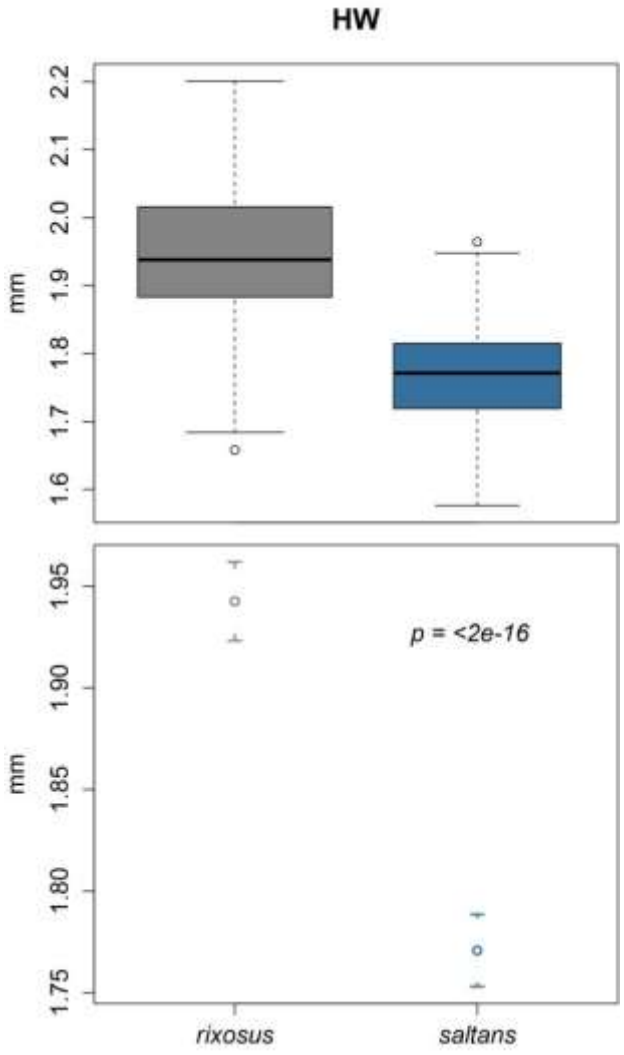

Figure 3.7. Top graph showing box plot (median, 25-75 %, range) and bottom graph showing mean ± SE of head width (HW) of *O. saltans* and *O. rixosus*. Head width was larger in *O. rixosus* than *O. saltans* (F = 81.09, df = 2, p = <2e-16).

CHAPTER 4: Trap-jaw ants in Borneo jump in two ways – with their jaws and with their legs

Paper accepted in *Frontiers in Ecology and the Environment*

D.M. Sorger

A wide variety of animals jump – kangaroos, frogs, grasshoppers, and even humans – but one rarely sees this behavior in ants. Only three out of 326 ant genera (Bolton, 2014) are known to jump using their legs: *Gigantiops* (Formicinae) in tropical South America, *Harpegnathos* (Ponerinae) in Southeast Asia, and *Myrmecia* (Myrmeciinae) in Australia, New Zealand, and New Caledonia. However, other ants have evolved the ability to jump by using their jaws.

These so-called trap-jaw ants snap their jaws – specialized elongate mandibles also used to catch prey – closed onto a hard surface to propel themselves backwards and escape threats (Patek et al., 2006; Wheeler, 1922). This behavior has been best-studied in *Odontomachus* and *Anochetus* (Ponerinae), two closely related genera, but there are a few references (Biró, 1897; Creighton, 1937; Mayr, 1887) from a third unrelated genus, *Strumigenys* (Myrmicinae). These tiny ants can jump as far as 47 cm, over 100 times their body length (Biró, 1897). This curious behavior has not been mentioned in the literature since 1937 and may be rare, or else seen but not reported.

In 2011, I received a grant to study trap-jaw ants along elevational gradients in Borneo. I focused on a common species in Southeast Asia, *Odontomachus rixosus* (Fig. 4.1). These ants are relatively large (1.3 cm) and live on the complex forest floor. As part of the project I was mapping nests and collecting individuals. One of my first field sites in Borneo was at Niah National Park, located in the heart of Sarawak. Upon discovering an *O rixosus* nest near the river banks in a recently flooded lowland rainforest, my local friend and field

assistant Syria Lejau and I crouched down to collect some ants. But then we both froze – these ants were doing something I had never seen before. They were jumping. *Forward*. I subsequently observed this behavior many times at various locations throughout Sarawak; whenever I disturbed *O rixosus* nests, in addition to backwards-oriented mandible-jumps, ants would jump from leaf to leaf on the low vegetation and litter surrounding the nest entrance. These leg-powered jumps, spanning several inches, were forward-oriented (Fig. 4.2A), and resembled the leaps of a jumping spider. Video recordings of this behavior are available on YouTube (https://www.youtube.com/watch?v=lOQgvlAakh4). I could not find any record of leg-powered jumps in the literature.

In 2013, I returned to Borneo to document this newly discovered jumping behavior through a series of field and laboratory observations and experiments. *Odontomachus*' trap-jaw mechanism is a particularly well developed, hyper-fast motion, reaching speeds of over 60 m s^{-1} (J. C. Spagna et al., 2008). The ants have two distinct backwards-oriented, mandible-triggered jumps: a "bouncer defense jump" (Carlin & Gladstein, 1989) and an "escape jump" (Patek et al., 2006). For the bouncer defense jump, the ants approach a large intruding object and then snap their jaws against it, propelling themselves backwards away from it. For the escape jump, they try to avoid an intruder by shutting their mandibles against the substrate, which propels them vertically into the air. The trap-jaw mechanism almost certainly evolved for prey capture but over time the ants started using it for jumping as well (Joseph C. Spagna, Schelkopf, Carrillo, & Suarez, 2009). Trap-jaw-triggered, backwards-oriented jumps generally appear erratic; the ants do not seem to direct their trajectories toward a target, but rather try to move away from a threat quickly. As a result, they land haphazardly, often on their backs (Fig. 4.2B).

My research revealed that the previously undocumented leg-powered jumps in *O rixosus* always occurred as a result of disturbance, rather than general locomotion, and were directed at clear targets. I observed the behavior of workers after brushing leaf litter over nests with a wooden stick, and compared the results to undisturbed control nests; I never saw the ants jump unprovoked. In the field laboratory at Mulu National Park, I was able to induce leg-powered jumping only through targeted disturbance (ie touching the ant's legs). I also investigated whether ants relied on visual cues to orient their leg-powered jumps by presenting 12 ants from nine nests with a high-contrast and a low-contrast target. I disturbed the ants for 10 minutes and logged each jump. In total, I recorded 2825 jumps, 96% of which were forward-oriented leg-jumps, while the remainder were backwards-oriented, mandible-triggered jumps. On average, when performing leg-powered jumps the ants showed a slight preference to jump onto a dark rather than a white surface (ca 60%).

The evolution of two distinct jumping behaviors in *O rixosus* is surprising. Why has a second jumping behavior evolved in this species? In other ants where leg-jumping has evolved, these species share several characteristics: (1) they are diurnal, solitary hunters that catch live prey and forage in the complex leaf litter (*Gigantiops* and *Myrmecia* also forage in the canopy; see (Beugnon, Chagné, & Dejean, 2001; Jayatilaka, Raderschall, Narendra, & Zeil, 2014); (2) they possess relatively large eyes to track arthropod prey; and (3) they jump primarily to escape and for prey capture, although *Gigantiops* and *Harpegnathos* also use it for general locomotion (Beugnon et al., 2001; Urbani, Boyan, Blarer, Billen, & Ali, 1994).

Odontomachus can use mandible-jumping as an escape mechanism when startled, but this gives them little or no control over the direction or distance of their trajectory. When they use leg-jumping instead, there may be an advantage to using a directed motion where

individuals land on their feet and are therefore able to make swift headway in a specific direction. Mandible-jumping results in a chaotic landing and is not suitable for this purpose (Fig. 4.2B). Leg-jumping may therefore have evolved as a more efficient escape mechanism to increase fitness or to better traverse the complex leaf litter environment.

In addition to using directed leg-powered jumping to flee from threats, *O rixosus* may also use it to capture prey. I did not observe *O rixosus* jumping during prey capture, but such observations would be problematic to document in the field, even if common, because of the difficulty of maintaining sight of individuals in the leaf litter. In addition, prey-capture-related jumps, if they occur, may be inconspicuous, as they are in *Harpegnathos* where the ants leap forward only short distances (usually about 2 cm) or just progress by means of short forward "jerks" (Musthak Ali, Baroni Urbani, & Billen, 1992). So I cannot rule out the possibility that *O rixosus*' leg-jumping plays a role in prey capture. Successfully capturing live prey also requires strong visual abilities. All other leg-jumping ant species have extremely large eyes and while the eyes of *Myrmecia* are smaller than those of *Gigantiops* and *Harpegnathos*, the eyes of *Odontomachus* are smaller still (Fig. 4.3). The eyes of *O rixosus* are no larger than those of other non-jumping congenerics. In the case of *Odontomachus*, jumping to catch prey may be less important because of their elaborate trap-jaw mechanism, including trigger hairs specifically designed to catch prey items at close range (Gronenberg, 1995), which other leg-jumping species lack. However, vision must still play an important role in jumping; otherwise directed jumps like those from leaf to leaf would not be possible.

Finally, it is entirely possible that species other than *O rixosus*, also use leg-propelled jumps. Indeed, several colleagues have since shared anecdotal observations of jumping ants

in other genera (outside of the known “jumping ants”). Sometimes it is hard to distinguish between an intentional leg-triggered jump and the directed falls exhibited by many ants. Nonetheless, leg-jumping is probably underreported and may be present in more ant species. If it is, I predict that they will tend to be species living in structurally complex environments such as the forest canopy or a leaf-litter-covered forest floor and that they have excellent vison, like other jumping ants.

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This project received funding from the Lewis and Clark Fund for Exploration and Field Research (2011), The Explorers Club Exploration Fund (2013), the Southeast Climate Science Center, and NSF-CAREER (09533390). Acknowledgements are given to Mulu National Park staff, Sarawak Forestry, CA Penick, MW Moffett, AV Suarez, DR Tarpy, AL Traud, and RR Dunn.

Figure 4.1. *Odontomachus rixosus* worker.

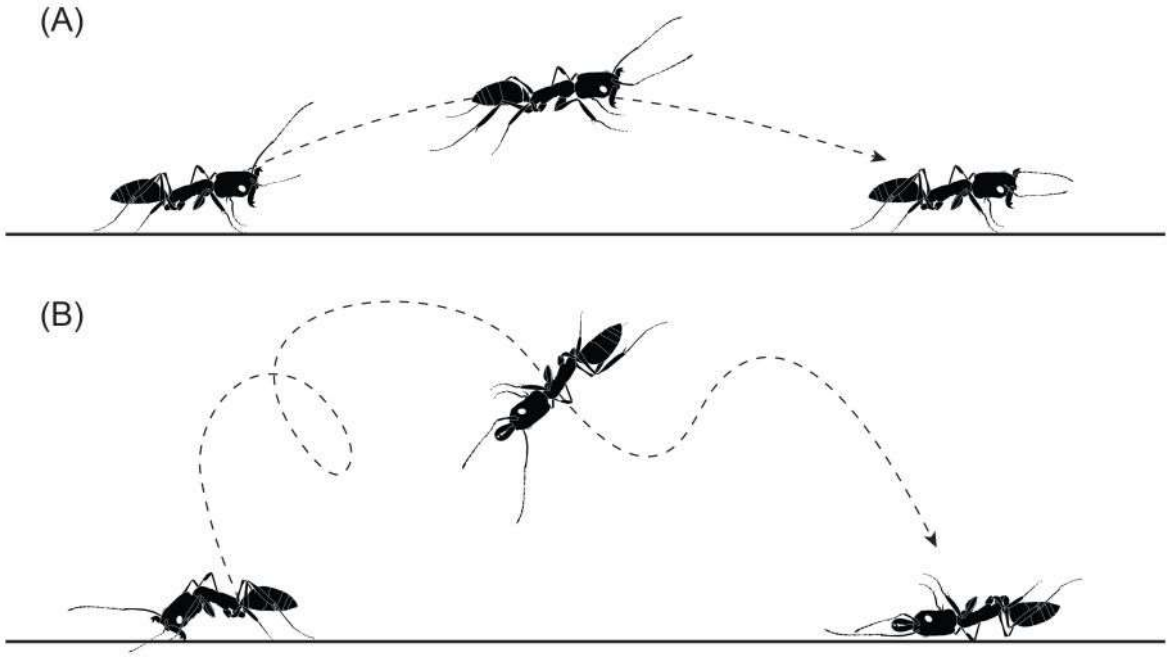

Figure 4.2. Jumping trajectory of *O rixosus* in (A) a leg-powered jump and (B) a mandible-powered jump. The leg-powered jump moves forward in a controlled motion while the mandible-powered jump causes the ant to flip and land haphazardly.

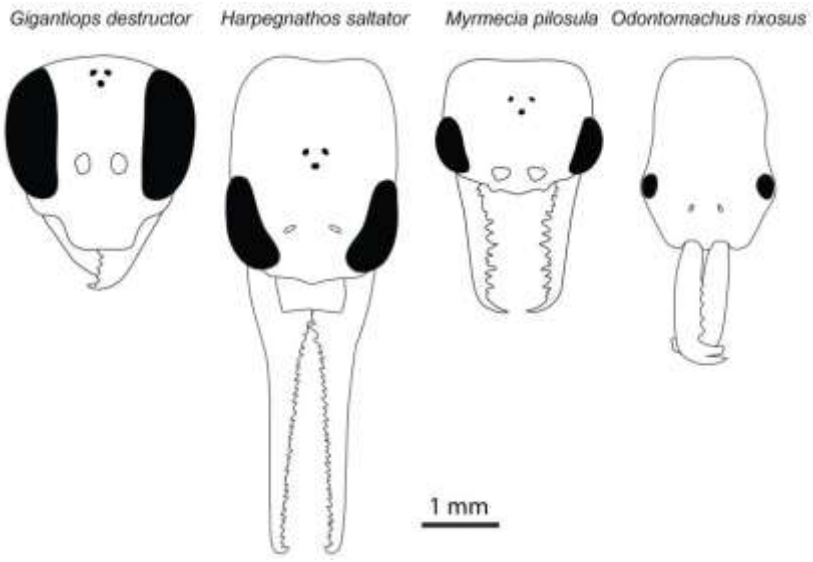

Figure 4.3. Head illustration showing variation in eye sizes of jumping ants.

CHAPTER 5: Is the ant a tramp? A *Lepisiota* (Hymenoptera: Formicidae) species from Ethiopia with genetically diverse supercolonies and invasive ant traits

Prepared for publication

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Abstract

An undescribed species of *Lepisiota* in Ethiopia, with what appear to be supercolonies spanning at least several kilometers, occurs at high densities where there is sufficient moisture and/or herbaceous cover, and dominates the local ant community. These traits would suggest that it is an invasive species within this area. Unlike many invasives however, the Ethiopian supercolonies contain substantial genetic diversity, indicating they have not gone through a population bottleneck usually characteristic of species invasions. The absence of any discernable genetic structure geographically suggests that this may result from multiple independent introduction events. We therefore propose that this ant may in fact be a *local invasive*, having recently expanded its range into this area as a result of habitat changes and the human-mediated dispersal opportunities afforded by the construction of local infrastructure. This *Lepisiota* species nevertheless exhibits the *invasive ant syndrome* and therefore could have the makings of a potential invasive species at an international scale, given the many general similarities in its basic biology to other invasive ant species.

Introduction

Many species of ants have been described as invasive (Porter & Savignano, 1990; Ross, Vargo, & Keller, 1996; Suarez et al, 1999; Tschinkel, 1998; Wetterer, 2005). Indeed, five

species of ant feature in the top 100 of the world's worst invasive alien species (Lowe et al., 2000), with the most significant of these having severe environmentally disruptive effects (Cremer et al., 2006; Drescher, Feldhaar, & Blüthgen, 2011). Given that the total diversity of ants is unlikely to be much greater than 20,000 species (Hölldobler & Wilson, 1990) and that upwards of eight million species are thought to exist (Mora et al., 2011), ants are 20 times more likely to be serious invaders than expected by chance.

Invasive ant species, such as the red imported fire ant (*Solenopsis invicta*), the yellow crazy ant (*Anoplolepis gracilipes*), and the Argentine ant (*Linepithema humile*), share multiple characteristics that appear to promote establishment in foreign environments. Though outliers exist (e.g., the original monodomous and monogynous form of *S. invicta* that first colonized the southeastern United States [(Tschinkel, 2006)]), these traits, which make up the *invasive ant syndrome* (Cremer et al., 2008), include: a) indiscriminant nesting, b) generalist diet, c) polygyny (with successive generations of queens staying within their natal colony), d) ecological dominance, e) polydomous colonies, and f) colony expansion through budding, by which means the ants may span large areas but belong to the same colony (Holway et al., 2003). As in ants generally (Hölldobler & Wilson, 1990; Tsutsui, 2004), polydomous ants identify their colony by specific cuticular hydrocarbon profiles. This identification is retained following budding (Torres, Brandt, & Tsutsui, 2007) such that, when the environment permits, individuals may move freely among nests, intermixing and thereafter cooperating indiscriminately with any other portion of that colony (in some instances low level aggression may occur, at least temporarily [Suarez et al., 1999]). Furthermore, in species like the Argentine ant, colony growth seems to continue as long as suitable unoccupied space is available (Buczkowski & Bennett, 2008; Moffett, 2012; Van

Wilgenburg, Torres, & Tsutsui, 2010). Therefore a colony can take over huge expanses but nevertheless have a clear membership and distinct boundaries, as maintained through distinct hydrocarbon profiles ((Torres et al., 2007). The capacity of a colony to expand its range without constraints is the strongest basis for describing such ants as having *supercolonies* (Moffett, 2012).

Nesting and dietary generalism allow a supercolony to quickly invade and spread across land previously unoccupied by the species, with workers and queens that retain the same colony identity moving into any suitable nest site that is in close proximity to a potential source of sustenance (Holway et al., 2002). In supercolonial species, multiple queens within a colony (polygyny) permit its exponential growth rate and spatial expansion to occur not only through budding, but also through jump-dispersal. Here, ants are carried often as stowaways in human cargo and potentially over long distances (Holway et al., 2002). Over the last century, jump-dispersal has expanded the natural range of numerous species of invertebrate (e.g., Ascunce et al., 2011; Booth et al., 2011; Gotzek et al., 2012; Saenz et al., 2012), and in the ants has led to supercolonies that extend between continents (Sunamura et al., 2009; Vogel, Pedersen, d'Ettorre, Lehmann, & Keller, 2009; Vogel, Pedersen, Giraud, Krieger, & Keller, 2010), even when propagule pressure was small. For example, it is believed that an estimated 6-13 mated Argentine ant queens founded the supercolony that now extends across much of western Europe and harbors billions of individuals (Giraud, Pedersen, & Keller, 2002).

The frequent association of the above traits has led many myrmecologists to develop an intuitive sense of which species might be invasive based on their behavior in the field. Care must be taken however as many of the traits outlined above (a –f) are often exhibited in

species within their native ranges. This syndrome may predict and promote the success of a given species both in non-native habitats, and also in non-analogue habitats (i.e., human-modified [disturbed] habitats for which nothing directly comparable exists today under natural conditions [(Menke et al., 2010)]) within their native range. For example, the odorous house ant, *Tapinoma sessile*, a North America native, is reported to become dominant in urban areas, where disturbance results in the creation of supercolonies (Barbani, 2003; Buczkowski & Bennett, 2008; Menke et al., 2010; M. R. Smith, 1928). In undisturbed areas, however, this species is described as subdominant, occupying small, single nests (Fellers, 1987; Milford, 1999). Supercolony formation has also been observed within the native range of some ant species exhibiting the invasive ant syndrome. Some populations of the narrow-headed ant, *Formica exsecta*, exhibit both monogyny and multicoloniality, whereas others are polygynous and polydomous (i.e., are composed of supercolonies: Pamilo & Rosengren, 1984; Seppä et al., 2012). Similarly, Argentine ants in their native range may form supercolonies several hundred meters wide, overlapping with the size of the smaller supercolonies of this species in its invaded range (Suarez, Holway, & Tsutsui, 2008; Vogel et al., 2010). As such, as an alternative hypothesis to a species being an invader, it may be a native species with a high potential to turn invasive if it manages to disperse from its native range, in that it shows traits typical of successful invasive ants.

While we are still lacking a general understanding of how some introduced populations become established (Bock et al., 2015), we can potentially address the contrasting hypotheses of invader vs. invasive syndrome through the use of population genetic analyses. Theory predicts that due to the process of population foundation, genetic diversity will be reduced in the colonized range relative to the native range. This is due to the

likelihood that a propagule may contain only a small fraction of the genetic diversity from the native population, and that an even smaller fraction of that transported propagule is likely to become established (i.e. founder effects and bottlenecks [Nei, Maruyama, & Chakraborty, 1975]). Nei et al. (1975) suggest that in order to observe genetic erosion between the native and invading populations, such a bottleneck would have to be severe with fewer than 10 individuals being released into the new area. However, social insect colonies tend not to be representative of the genetic diversity of the entire population, but instead that of a smaller subpopulation. Thus, the movement of a propagule consisting of individuals from a single colony could therefore have a severe effect on genetic diversity resulting in a significant reduction in the invaded range (e.g., Tsutsui et al., 2000). As such, we might predict both limited genetic diversity and, as a result, limited genetic differentiation among colonies across the invaded range (e.g., Suarez et al., 1999; Tsutsui & Case, 2001; Corin et al., 2007; Thomas, 2010). Also, the supercolonies of an introduced ant species are likely to be much larger than in its native population because colony expansion is no longer constrained by the presence of many hostile neighboring supercolonies (Vogel et al., 2009).

In order for genetic diversity to become elevated in the invaded range, two processes may contribute: mutation from the pre-existing standing genetic variation, and/or multiple introductions from genetically diverse source populations (Dlugosch & Parker, 2008). The former process may be slowed due to the lengthy process by which new mutations arise and spread within a population. However, following establishment, subsequent population demographic expansion may result in the accumulation of mutations that generate a *starburst* haplotype network, given the likelihood that single, not multiple, mutations will arise per mutational event. Multiple introductions, in contrast, are limited by a number of factors,

including transport vector type, transport pathway type and frequency of use, propagule pressure, genetic diversity within the native range, the area over which propagules may originate, and the likelihood of establishment in the introduced range (Lockwood, Hoopes, & Marchetti, 2013; Wares, Hughes, & Grosberg, 2005). Multiple introductions into non-native areas have been recorded for a number of ant species following genetic analyses (e.g., Abbott et al., 2007; Ascunce et al., 2011; Gotzek et al., 2012), but are likely to be rare if dispersal distance is far (e.g., transcontinental). In the absence of having range-wide collections, which might enable the source of target populations to be assessed if indeed invasive, assessing genetic structure and diversity may therefore offer clues as to the origin(s) of populations within a given area.

The genus *Lepisiota* is likely to have originated in Africa (Peter Hawkes, pers. comm.), however recent evidence of translocations and subsequent establishment have been reported in Australia (Jonathan Majer and Marc Widmer, pers. comm.) and South Africa (Sithole, Smit, & Parr, 2010), suggesting that members of this genus may have the potential to become a global nuisance. While studying patches of forest scattered across the Ethiopian highlands, DMS and MWM noticed that a presently undescribed (Peter Hawkes, pers. comm.) species of *Lepisiota* exhibited invasive traits, e.g., appearing to forage and nest over wide areas at high densities, and, being a small and innocuous-looking monomorphic ant, it thus at least superficially resembles the Argentine ant. These were initially found in the so-called *church forests* which surround Orthodox churches, some of which are over 1,500 years old. The forests range in size from only a few hectares to more than 400 ha and are relictual oases within largely barren land and agricultural fields. As part of a bio survey in which three church forests were sampled, *Lepisiota* was found only within one, Zhara Church Forest (8

ha). This site appeared to be the most disturbed of the three, possibly due to its small size relative to the number of human residents. *Lepisiota* were densest on and near walls recently constructed around the forest perimeter and also just within the forest boundaries. In areas where very few other ants were encountered, *Lepisiota* ants were found reaching densities of several thousand per square meter over wide tracks of ground. During this, the dry season, the species was also prevalent, although at lesser densities, in open disturbed habitats where there was herbaceous cover or other signs of moisture.

The most compelling indication of the potentially invasive nature of this ant was the lack of aggression described in this article between individuals transferred to distant (up to 38 km) localities in the field, indicating supercolonies are not just present, but expansive, as seen in Argentine ants (Vogel et al., 2010). Therefore, the species may 1) be native and exhibiting the potential to become invasive (manifesting the invasive ant syndrome), 2) have recently expanded its range locally or exhibiting invasive traits following disturbances (i.e., a *local invasive* as seen in *T. sessile*, for example), or 3) in fact be a non-native within these recently modified landscapes undergoing infrastructural expansion. Using a combination of genetic and behavioral data, we investigate these contrasting hypotheses about the *Lepisiota* species. We examine the population genetic structure and diversity using genetic data derived from the mitochondrial cytochrome oxidase I subunit. We then augment this through the inclusion of pairwise aggression assays performed on colony pairs along a 100 km transect; thus assessing the presence and extent of supercolonies and their population structure.

Methods

Natural History

Reflecting the fact that the behavior of this genus in unknown, basic natural history documentation was an essential part of our program. DMS and MWM observed the ants for several hours a day whenever possible over ten days, mostly at Zhara Church Forest, to build a general description of this species' foraging behavior, movements, and other activities relevant to assessing the possibility of ecological dominance as a species.

Sample collection and bait transects

Lepisiota sp. was collected from 16 locations along a ~100 km transect following the road between Bahir Dar and Debre Tabor, Ethiopia in January 2012. Additional samples were collected at Zhara Church Forest (8 ha), Debresena Church Forest (11.5 ha), Gelawdios Church Forest (100 ha), and in the towns of Lalibela and Gonder resulting in a total of 28 sampling locations (Fig. 5.1, Appendix D). Samples were stored in 95% ethanol for genetic analysis, and a subset was kept alive for behavioral tests. Seven bait transects were installed at three of the Church Forests (Zhara, Gelawdios, and Debresena). Each transect was 100-200 m long with bait stations every 10 m. At Zhara Church Forest, we started the transect at a stone wall designed to fence in the forest and leading 200 m into the forest, with a second transect starting at the outside of the same wall leading through an open field outside of the forest into a patch of Eucalyptus forest ~150 m away. At Gelawdios Church Forest one transect was installed into the forest interior, one in the open field outside the forest, and one along the edge of the forest (each 100 m). At Debresena, both a forest and a field transect were installed (both 100 m). Baits consisted of ca. 2 cm^3 of sardines in oil placed directly on

the ground. Each bait station was marked and visited after one hour. All the ant species at the bait were recorded and voucher specimens were collected (see Table 5.1).

DNA extraction and genetic analysis

A total of 28 workers from 27 locations of *Lepisiota* sp. were analyzed (Appendix D, Fig. 5.1). We also sequenced one queen from colony ZS02 (ET040) and 10 alate queens from colony ZL02 (ET010) (Appendix D). Total genomic DNA was isolated from whole specimens using the Qiagen DNeasy blood & tissue extraction kit (QIAGEN, Valencia, CA). From each specimen, a 669 bp fragment of the mitochondrial Cytochrome Oxidase subunit I (COI) gene was amplified by PCR using primers LepF1 (5' - ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'- TAAACTTCTGGATGTCCAAAAAATCA-3') (Hajibabaei et al., 2006; Hebert et al., 2004). Polymerase chain reactions were performed in 20 µl volumes containing: 1X PCR buffer, 2 mM MgCl2, 100 mM dNTPs, 2 pM of each primer, 0.5 U Taq DNA polymerase (Apex, Genesee Scientific, San Diego), ~50 ng DNA template, and ddH_2O to 20 µl. PCR cycling conditions were comprised of an initial denaturation stage of 3 min at 94 °C, followed by 35 cycles each consisting of 30 sec at 94 °C, 30 sec at 45 °C, and 1 min at 72 °C. PCR products were visualized on a 2.5 % agarose gel to confirm samples contained a single band, and they were subsequently purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, CA). Purified products were then bi-directionally sequenced on an ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were visualized and edited in CLC Main Workbench (CLC bio, Aarhus, Denmark). Sequence variation was calculated with the

Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software (Tamura et al., 2013). Diversity parameters, including nucleotide diversity (π) and haplotype diversity (h), were computed with DnaSP 5 (Librado & Rozas, 2009). Genetic distances between haplotypes were reconstructed using a minimum-spanning network algorithm implemented in PopART 1.7 (epsilon = 0) (Bandelt et al., 1999). Trends in the demographic history of *Lepisiota* sp. were investigated using Tajima's D and Fu's Fs statistics. A significant negative value in these two tests indicates a recent population expansion event.

Aggression assays

While not all pairs could be tested due to logistical constraints, 22 pairwise experiments were conducted to assess aggression between ants from different localities. Ants were collected using aspirators and kept alive in plastic collection vials. Aggression was then assessed in the field by locating ant trails and positioning a vial containing ants from the testing location near the trail. Ants were allowed to exit the vial and interactions with ants from the trail were observed for 10 minutes. The response was recorded into three categories: 1) aggression – fights, pulling appendages and pointing abdomens (Fig. 5.2A), 2) low-level aggression, described here as alarm – raised abdomens and close examination of the introduced workers, occasional pulling on them, with subsequent acceptance by the resident ants (Fig. 5.2B), and 3) no aggression – the ants show no signs of aggression and intermix freely with the resident group.

Results

Natural History

Lepisiota sp. nests were found under rocks with multiple queens, alates and brood. In addition, the ants also entered unadorned holes in the ground, suggesting subterranean nesting. There were no dirt piles to indicate extensive excavation; thus the species may have been making use of available cavities. Queens were often running on trails alongside workers, and sometimes alongside workers carrying brood. The *Lepisiota* sp. was also common in an urban setting at the Ghion Hotel in the city of Bahir Dar. They did not enter the rooms in a noticeable way, though some came to the window sills and onto the concrete paths. They were particularly abundant in the parts of the garden most watered by the hotel staff. In fact, the *Lepisiota* sp. often came to the surface when water was poured on the earth, suggesting there was a reserve population belowground. Some *Lepisiota* sp. workers were collected in litter and moss 12 meters high in the Zhara forest canopy in 2010 (Neville Winchester, unpubl. data), and the workers often climbed herbaceous plants, notably African senna (*Senna didymobotrya*), a legume common on roadsides and in open areas generally. At a number of sites the ants were observed tending froghoppers (*Tettigometra* sp.), and in one instance a caterpillar (probably a lycaenid, Phil J. DeVries, pers. comm.). They were also seen carrying freshly killed insect prey such as caterpillars and termites, and were efficient at dispatching other ant species, among them species with workers larger than themselves such as *Pheidole* and *Camponotus*. To do so, they gathered around to pull the workers apart as they do foreign-colony ants of their own species.

Bait Transects

In general, ant presence at baits was low (no ants at 47 % of all bait stations) probably due to the overall low abundance of ant foragers during the dry season and the placement of some baits along transects in sunny, bone dry areas (Fig. 5.3, Table 5.1). However, the *Lepisiota* sp. was abundant on the ground surface at Zhara, both in the forest and a few meters out into the adjacent fallow field. In fact it was the only species found at bait stations except for one station in the field transect were the *Lepisiota* sp. was found nearby while a *Monomorium* ant species occupied the bait. At the other two church forests sampled, the *Lepisiota* sp. was only found at the field transect at Gelawdios, where it was present at 60 % of bait stations. The *Lepisiota* sp. occupied the bait (presence of 100+ workers) at 16 of the 45 stations that had ants at them (three transects) and was highly aggressive towards the other ant species encountered.

Genetic analysis

Nine unique COI haplotypes were identified across the sampled region (Table 5.1, Fig. 5.1). Eight haplotypes were found to be present in the main sampling area encompassing Bahir Dar and three Church forests, while a single haplotype (4) was only found in an individual from Lalibela (Mt. Asheton) (Fig. 5.1). The additional workers and queens sequenced possessed the same haplotype as the first worker sequenced from the location and were excluded from subsequent analysis to avoid inflation of haplotype frequency at a given site. Of the 10 polymorphic sites, 6 were parsimony-informative. All haplotypes were joined in a minimum-spanning network with one haplotype (1) comprising 37% of all individuals (Fig. 5.4). Overall mean sequence divergence across the 9 haplotypes was found to be 0.3 %.

Haplotype diversity (h) amounted to 0.718 (SD 0.087) and nucleotide diversity (π) was 0.003. Tajima's D (-0.657, p = > 0.10) and Fu's Fs (-2.301, p = 0.056) indicate that the population has not undergone a recent expansion.

Aggression assays

Our behavioral experiments revealed a pattern of non-aggression (but included an initial alarm response in three instances) between sampling locations of up to 38 km apart (Fig. 5.1), with the transferred ants intermingling with the local population just as they would have if they had been released exactly where they had been collected. We found no aggression between locations encompassing the area from Bahir Dar until 8.6 km past Zhara Church Forest, and only three locations within this area showed an aggressive response towards samples from Zhara (SC1, see Fig. 5.1). In addition we found no aggression over 6 km and 9 km (SC2 and SC3 respectively, Fig. 5.1).

Discussion

Lepisiota sp. as an ant with invasive species characteristics.

Our results indicate that this *Lepisiota* sp. behaves like an invasive ant and in fact meets the criteria often used to distinguish between species that are merely introduced from those that are both introduced and invasive (Abbott et al., 2007; Ascunce et al., 2011). However, it possesses the genetic signature of a native species. Based on our samples, the *Lepisiota* sp. in our study area has more mtDNA diversity than found in the continent-wide distributions of many invasive species (Ascunce et al., 2011).

Six traits associated with invasive ants were examined: general nesting, polygyny, budding, generalist diet, ecological dominance, and the presence of large colonies. No strong nesting preference was observed; nests were found both under rocks and in the ground, with the ants avoiding xeric areas. The species is polygynous, with numerous queens. Colony expansion through budding was not directly observed (by definition budding has to result in spread to sites that are completely unoccupied beforehand, which was difficult to assess). However, budding seems inevitable given the frequent encounter of queens along trails and the occupancy of so many nest sites. The ants did best under herbaceous cover or where there was some other indication of moisture. The fact that more ants came to the surface when the ground was moistened indicates the species might extend its presence over wider areas, with less fragmented populations during the rainy part of the year, as seen in Argentine ants ((N. E. Heller, Ingram, & Gordon, 2008; Holway & Suarez, 2006).

While a full diet study of *Lepisiota* sp. was not performed, it both killed prey (which would be a protein source for the growing larvae) and frequently tended sap-sucking insects. The availability of carbohydrate-rich resources like honeydew has previously been linked to the successful spread of invasive ants, providing the nutritional resources adult ants require for colony offense and defense against other ants (Holway et al., 2002). For their success against other *Lepisiota* supercolonies and other ants generally, their density suggests they can often depend on Lanchester's square law, which states that battles can be won by the side with sheer force of numbers in its favor, even against troops that are superior in fighting ability (Boswell, Franks, & Britton, 2001; Franks & Partridge, 1993). By use of this strategy, Argentine ants have eliminated most native ants in California, other than species active when conditions are unsuitable for the Argentine ants (Holway, 1999; Human & Gordon, 1999). In

its native range, where Argentine ants encounter many more (and hence individually relatively smaller) supercolonies, the species appears to have little influence on a diverse local ant fauna that is presumably adapted to its presence (Andrew V. Suarez et al., 1999). The fact that this *Lepisiota* sp. dominated, and at least in the dry season excluded, most other ants is therefore another likely sign of its invasive status.

Bait transects indicated the ecological dominance of the *Lepisiota* sp. wherever that species was present. Dominance was most pronounced at Zhara Church Forest. Zhara is the smallest, most disturbed of the three forests sampled. In addition to the church itself, there is a human settlement within the forest and until recently there were no latrines available. As a result the forest has been polluted (fertilized) by human waste, and the undergrowth had grown dense and weedy due to the recent exclusion of grazing animals. The only ant species to control bait stations when the *Lepisiota* sp. was in the general area was a *Monomorium* species that took over a single station in a parched open field where the *Lepisiota* sp. was scarce. The *Lepisiota* sp. was entirely absent at Debresena and only present in the field outside Gelawdios, both of which were less overgrown. This indicates that the species may be more successful in at least partially disturbed environments.

A lack of aggression was observed in transfers of the *Lepisiota* sp. over distances of up to 38 km. The introduced ants merged seamlessly with the local population, as expected under the assumption of supercolony structure (Nicole E. Heller, Sanders, & Gordon, 2006; Moffett, 2012; Pedersen, Krieger, Vogel, Giraud, & Keller, 2006). Yet other transfers resulted in clear and extreme aggression towards the intruders. This is evidence for a unified identification of ants across wide areas, as expected when there are supercolonies with definite territorial boundaries and memberships. Presumably this is due to the sharing of

cuticular hydrocarbon profiles, however this has yet to be determined for this species. In Argentine ants, battles between supercolonies are ongoing and involve massive mortality along a narrow band that extends for kilometers where large supercolonies abut (M. L. Thomas, Payne-Makrisâ, Suarez, Tsutsui, & Holway, 2006). Tracking down these frontiers to observe fighting between supercolonies is labor-intensive (M. L. Thomas et al., 2006) and unfortunately our time in Ethiopia did not allow for this.

We predict there will also be no ambiguity about colony boundaries or membership in this *Lepisiota* species, with supercolonies meeting along similarly well-defined battle lines wherever conditions allow fighting at the surface. Because colony membership in ants is based on behavioral responses to cuticular hydrocarbons, there is no reason the patterns of mitochondrial genetic variation could not be decoupled from variation at the loci encoding for, or at least influencing, the hydrocarbons and the behaviors required to recognize them (Neil Tsutsui, pers. comm.). This could explain, for example, the significant local population structuring found by (Ingram & Gordon, 2003) within California's large Argentine ant supercolonies, which are nevertheless unified, with lethal aggression only along supercolony borders.

Three of the *Lepisiota* transfers revealed a low level of aggression, with the local ants examining the newcomers and sometimes pulling on them, described here as *alarm* rather than aggression. Because intermediate levels of aggression are sometimes mentioned in the literature (e.g., Suarez, et al. 1999), we think it useful to consider here alternative possibilities for what such responses might mean. One option is that our hypothesis is incorrect, in that intermediate responses and population structuring are indicators that the supercolonies are not distinct and cleanly separated entities. In this view, fusion might be possible between ant

supercolonies, as has been demonstrated between colonies of some basal termite taxa in which workers are capable of transforming into reproductives following death of original queen(s) and king(s) (e.g., (Howard, Johns, Breisch, & Thorne, 2013). However, the underlying dynamics surrounding reported colony mergers for more derived termites are "difficult to evaluate" (Thorne & Traniello, 2003) and as far as is known, occur rarely if at all between large mature colonies of higher termites (family Termitidae) in nature (Barbara Thorne, pers. comm.). Such a finding would be a remarkable if shown for *Lepisiota*.

An alternative hypothesis is that the identity of supercolony members (their hydrocarbon profiles) shifts gradually across space so that while there is no aggression locally, members from distant parts of a supercolony will show some uncertainty about each other. This might produce the social equivalent of a ring species, an analogy that suggests the population can still be considered part of the same colony. Surprisingly, Argentine ant super-colonies do not show any evidence of this sort of shift in identity across their vast territories, and so the workers from the enormous western Europe supercolony, will not fight with the ants that occupy much of southern California and therefore belong to the same cross-continental supercolony (Van Wilgenburg et al., 2010).

Much more probably, the alarm response with *Lepisiota* sp. was instead the result of a mundane fact: stress induced through the manipulation of the specimens during transfer to geographically distant sites. Such conditions might cause the transferred ants to sometimes appear abnormal when inspected by the local ants. Transfer Nr. 8 (Tab. 3) of ants from the same location collected earlier in the day provide support for this argument. Unfortunately, the logistics of the projects underway in Ethiopia at that time did not permit returning to these sites to repeat aggression assays, however our uncertainty about interpreting low levels

of aggression does not negate the fact that the ants across many widely separated sites exhibited no aggressive interactions and therefore should be considered part of the same colony (i.e., a supercolony).

Lepisiota as a likely "local invasive."

Differentiating between ants exhibiting the invasive ant syndrome in their native range from those that are actual invasives can be difficult when a new species is encountered. Ants within the genus *Lepisiota* (a genus in need of considerable taxonomic revision, Peter Hawkes, pers. comm.) have already revealed the potential to be invasive. In 2005 an invasion of the "browsing ant" (*L. frauenfeldi*) was discovered at the airport in Guam and subsequently eradicated (Hoffmann et al., 2011). In 2013 this species was found covering a 60 ha area around Perth airport, where it may have arrived five years earlier, and a year later a 10 ha outbreak was located 6 km away in the suburb of Belmont; while this year another infestation of the same species led to the partial shut-down at the port of Darwin, Australia (the Perth populations have been eradicated) (Mark Widmer, pers. comm.). Similarly, *L. incisa* has been reported from Kruger National Park, South Africa, where it is thought to have arrived in the 1990's (Sithole et al., 2010). That species is most abundant in habitats associated with human disturbance and development, where it is behaviorally dominant to all other ants, including endemics. Judging from the slight, if any, aggressive response toward ants transferred between sites hundreds of kilometers apart around Kruger (Caldera 2004, cited in (Sithole et al., 2010), it appears that the region is occupied by a single supercolony — presumably derived from the initial colony that arrived and spread widely into this previously uninhabited area. At one site, however, the workers were hostile to con-specifics

from elsewhere (Sithole et al., 2010), which tells us that a second supercolony has arrived and established a toehold in the region. The similarities between the *Lepisiota* species at Kruger National Park and the species described in this present study could indicate that supercolony behavior, and the concomitant potential to invade new terrain, whether locally or by jump-dispersal, may be a common attribute of the genus.

Based on our results of this Ethiopian species, it appears that the genetic evidence does not support the introduced-status of *Lepisiota* in our study area. The results paint a picture different from that usually seen for invasive ants; non-aggression was not associated with low genetic diversity. A similar pattern was found in the native range of *Formica exsecta* where six haplotypes were recovered within a single supercolony over a distance of 400 m (Seppä et al., 2012). Some researchers have claimed that the low genetic diversity within ant supercolonies, the outcome of their propagule pressure, would be pivotal to a supercolony staying together as a cooperative unit over large areas (e.g., (Neil D. Tsutsui & Suarez, 2003). This *Lepisiota* sp. confirms that an ant society (supercolony) with a diverse population can remain united as long as the members accurately identify each other as belonging to the same society (Moffett, 2012).

If this *Lepisiota* sp. had been introduced from a remote part of the world (or at least a distant part of Africa), as has been the case with many invasive species studied to date, the diversity of its mitochondrial DNA would have had to represent as many as 8 independent colonization events to an area of approx. 2,600 km^2 (assuming each introduction was derived from a propagule representing a single colony); a very unlikely scenario. Rather, the number of different supercolonies, their moderate sizes (for an invasive species), and their genetic diversity leads us to propose that this *Lepisiota* sp., while in fact native to nearby parts of

eastern Africa may have expanded its range regionally to become a local invasive of this particular part of Ethiopia, aided by having traits typical of most invasive ant species (e.g., showing the invasive ant syndrome of (Cremer et al., 2008)]). The mosaic genetic structure we see within each supercolony could be explained by short-range dispersal of small groups of colony mates between different parts of the supercolony range (Seppä et al., 2012).

The favored plant visited by this *Lepisiota* sp, African senna, is native to Eastern Africa but a recent arrival to the study area, showing up soon after the road had been paved (AWE, pers. comm.). African senna is a common invasive plant in many parts of the world, but therefore apparently, like *Lepisiota*, also what we have called a *local invasive*. Although the resources on other plants might have contributed as well, the range extension of African senna in particular may have facilitated the spread of the ant, or at least increased its numbers, with the plant's honeydew-producing residents providing nutritional resources, the equivalent of fuel at gas stations, required in an otherwise barren landscape.

It is worrisome that parts of Zhara Church Forest harbor a thick ground cover of weedy herbs, and also the *Lepisiota* sp., which does well among these plants and dominates other ants, suggesting ecological issues could arise from such competition. The ancient Church Forests have been effective repositories for biodiversity in a region heavily populated by people for centuries (Cardelús et al., 2013). The recent exclusion of cattle, the only ungulates to replace the once extensive fauna of native grazers, from some Church Forests could contribute to the flourishing and overgrown understory, with a cascade of negative effects, a *Lepisiota* population explosion being among them.

In conclusion, while the behavioral data supports a suite of traits that are commonly observed in invasive species (listed a-f above, supercolony structure included), the genetic

data reveals a contrasting pattern; that of a species that is regionally genetically diverse, but with limited actual population genetic structure. This pattern is characteristic of species dispersed into new areas by human-mediated means (e.g., Booth et al., 2011; Saenz et al., 2012), therefore where propagules establish, the genetic diversity is dispersed somewhat randomly across the landscape. The presence of genetically diverse neighboring sites that fail to initiate aggression may therefore result from a decoupling of the mechanisms linked to colony recognition, thus potentially permitting subsequent supercolony formation following introduction. We therefore conclude that while this *Lepisiota* sp. possesses the characteristics indicative of an invasive species, it is more likely to be a species native elsewhere within the region/country, transported to the area following infrastructural development, and establishing due to evincing the syndrome typical of invasive ants. This syndrome may identify the species as a potentially significant invader if transport routes and transport vectors present themselves into new areas. In light of these findings, combined with recent invasions of *Lepisiota* spp. into other countries, this genus may represent a potentially informative model system to study the evolution of invasiveness in ants. The fact is that many invasive species must have begun by evolving the means to efficiently move around the native environment before human commerce gave them the chance to "go international" and before environmental degradation opened up opportunities for them to become ecologically dominant.

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Table 5.1. Bait transect length, start time and habitat notes.

Transect	Length	Date	Start time	Habitat notes
Zhara - Forest	20 m	6-Jan-12	12:00	forest interior, several open areas and sunny patches
Zhara - Field	15 m	7-Jan-12	11:30	10 stations on open harvested field, 5 stations inside Eucalypus forest
Gelawdios - Forest	10 m	9-Jan-12	14:00	forest interior
Gelawdios - Field	11 m	10-Jan-12	10:45	open area with many rocks
Gelwadios - Edge	12 m	10-Jan-12	12:40	along forest edge
Debresena - Forest	13 m	12-Jan-12	14:30	forest interior
Debresena - Field	14 m	12-Jan-12	16:00	5 stations in harvested straw field, 5 stations in plowed fallow field

Table 5.2. Aggression assays summary.

Trial	Resident loc.	Test loc.	Date	Response	Notes
1	GH	GF	13-Jan-12	aggression	fighting right away
2	GH	ZW	13-Jan-12	low-level alarm	
3	ZS1	ZW	13-Jan-12	no aggression	low number of ants transferred
4	ZS1-2	ZW	13-Jan-12	aggression	first of two trials
5	ZS1-2	ZW	13-Jan-12	aggression	second trial: overall weaker response
6	ZS2	ZW	13-Jan-12	aggression	response clearer than in trials 4 & 5
7	ZS2-3	ZW	13-Jan-12	aggression	
8	ZW	ZW	13-Jan-12	low-level alarm	transfer of ZW sample collected earlier in the day
9	ZL1	ZL5	6-Jan-12	no aggression	
10	ZC	ZW	7-Jan-12	no aggression	
11	DS5	ZW	13-Jan-12	no aggression	
12	ZW	ZS6	13-Jan-12	aggression	
13	ZW	D1	13-Jan-12	aggression	high number of ants at ZW
14	D1	ZW	13-Jan-12	aggression	
15	ZS5	ZS6	13-Jan-12	no aggression	
16	ZS6	D1	13-Jan-12	aggression	a lot of inspection before fighting
17	ZS5	D1	13-Jan-12	aggression	
18	DS3	D1	13-Jan-12	no aggression	
19	DS2	D1	13-Jan-12	low-level alarm	alarm response mostly with dead ants from vial
20	DE	D1	12-Jan-12	no aggression	
21	D1	GF	13-Jan-12	aggression	
22	D1	GH	13-Jan-12	aggression	

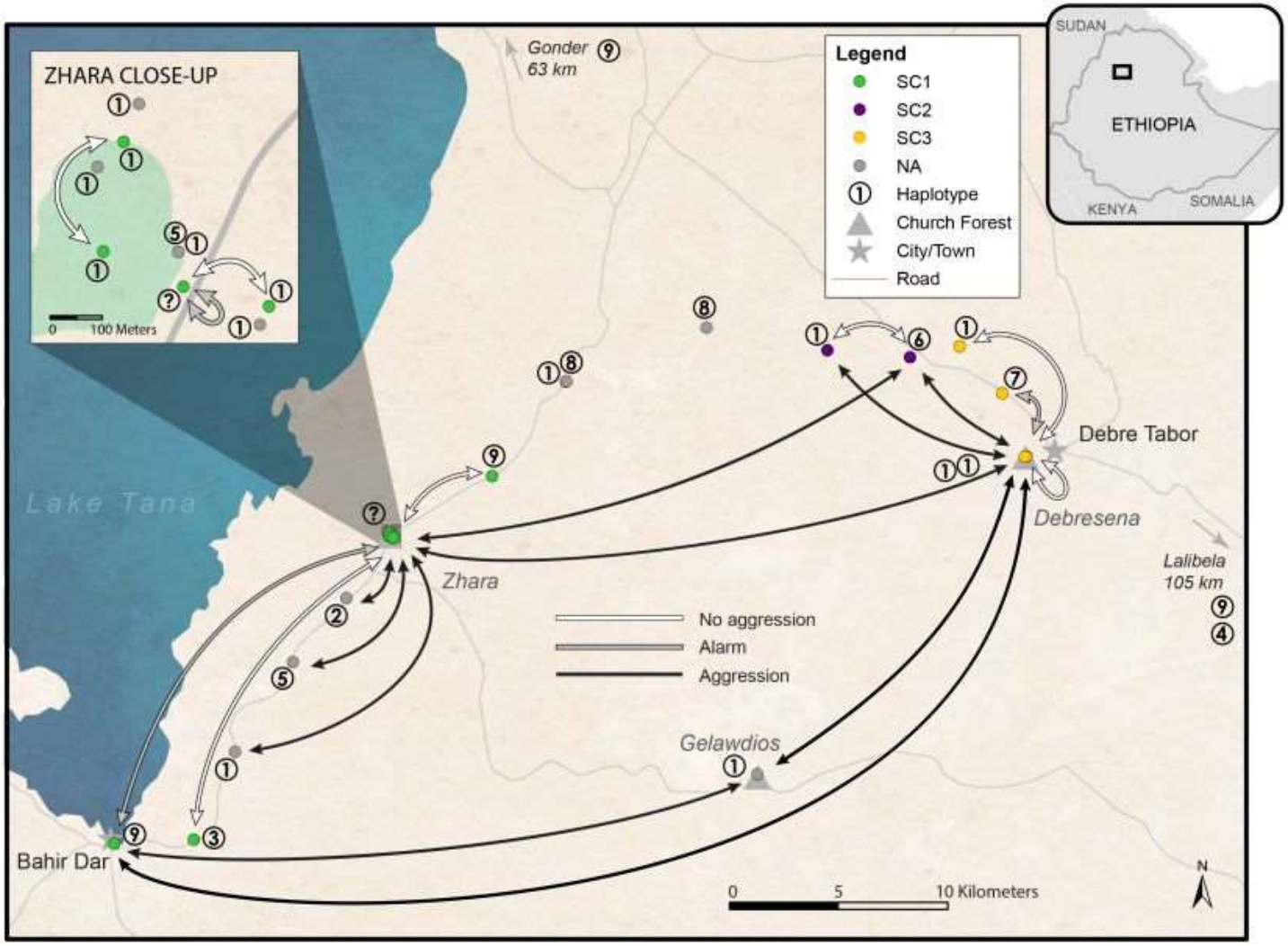

Figure 5.1. Sampling map including results from aggression assays and haplotypes. Arrows indicated results from aggression assays between locations. Locations colored the same show supercolony identity indicated by no aggression.

Figure 5.2. (A) Aggression & (B) Alarm. Photo credit: M. Moffett.

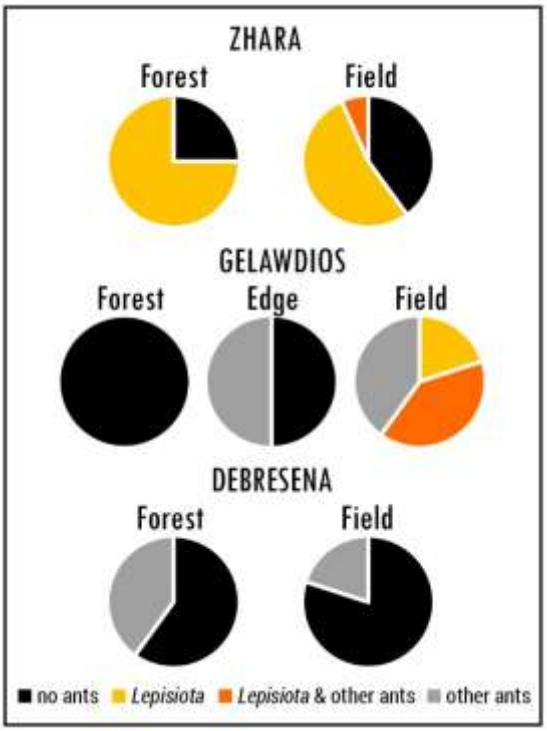

Figure 5.3. Percentage of baits occupied by no ants, *Lepisiota* alone, *Lepisiota* & other ants, and just other ants in three church forests. *Lepisiota* was the dominant ant in Zhara compared with two other forests.

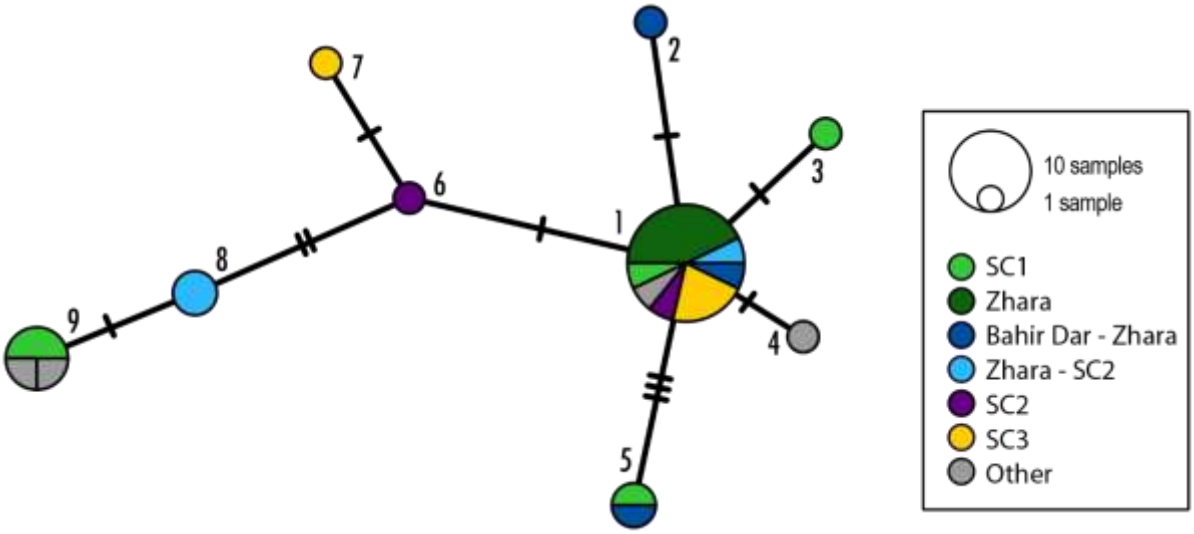

Figure 5.4. Minimum-spanning haplotype network for *Lepisiota* sp.. Size of nodes represent number of individuals per haplotype, tick marks between nodes indicate number of mutations between haplotypes.

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APPENDICES

Appendix A. List of samples and locations of *O. relictus* collected in Florida.

ID	Ridge	Group	mtDNA	morph.	color	Date	Lat	Long
B-01	BR	BR-S				6/9/2012	28.75062777	-82.49103106
B-02	BR	BR-S				6/9/2012	28.75088476	-82.49123843
B-03	BR	BS-S	1w			6/9/2012	28.7509897	-82.49134152
B-04	BR	BS-S				6/9/2012	28.75275786	-82.50050135
B-05	BR	BR-C				6/10/2012	28.85450788	-82.42988884
B-06	BR	BR-C				6/10/2012	28.85453872	-82.4298601
B-07	BR	BR-C				6/10/2012	28.8490977	-82.42941921
B-08	BR	BR-C	1w		3w	6/10/2012	28.84942442	-82.42955466
B-09	BR	BR-S				6/11/2012	28.75298409	-82.50009156
B-10	BR	BR-S				6/12/2012	28.75063774	-82.49087306
B-11	BR	BR-S				6/12/2012	28.75060966	-82.49102159
B-12	BR	BR-C				6/12/2012	28.85337657	-82.42973336
B-13	BR	BR-S				7/2/2012	28.75064897	-82.49113206
B-14	BR	BR-S	1w			7/2/2012	28.75058234	-82.49088831
B-15	BR	BR-S	1w			7/2/2012	28.75295433	-82.49651391
B-16	BR	BR-S				7/2/2012	28.75291209	-82.49672405
B-17	BR	BR-S	1w		2w	7/2/2012	28.75294536	-82.50008603
B-18	BR	BR-S	1w		3w	7/2/2012	28.74842299	-82.50037688
B-19	BR	BR-C		10w		7/4/2012	28.85488574	-82.42982665
B-20	BR	BR-C	1w	10w		7/4/2012	28.85335294	-82.42970386
B-21	BR	BR-C				7/4/2012	28.84959374	-82.42961367
B-22	BR	BR-N		10w		7/6/2012	29.03420566	-82.52915589
B-23	BR	BR-N				7/6/2012	29.03424589	-82.52913754
B-24	BR	BR-N	1w	10w		7/6/2012	29.0340148	-82.52931255
B-25	BR	BR-N	1w		3w	7/6/2012	29.03428118	-82.52891659
B-26	BR	BR-N	1w			7/6/2012	29.03243247	-82.53014806
MalesFL12-083	BR	BR-C				6/10/2012	28.85353415	-82.42974568
OREL-25	BR	BR-C				7/6/2011	28.8545833	-82.4299
OREL-26	BR	BR-C	1w			7/6/2011	28.8533667	-82.4296667
OREL-27	BR	BR-C	1w			7/6/2011	28.84975	-82.4295667
OREL-28	BR	BR-C				7/7/2011	28.84825	-82.4295167
OREL-29	BR	BR-C	2w			7/7/2011	28.8491	-82.42945
ORELB_01	BR	BR-C				11/10/2011	28.8534167	-82.4297
ORELB_02	BR	BR-C				11/10/2011	28.8533667	-82.4296833
O-01	LWR	LWR-N				6/17/2012	28.35858705	-81.6528446
O-02	LWR	LWR-N				6/17/2012	28.35866768	-81.6528389
O-03	LWR	LWR-N	1w	10w		6/17/2012	28.3595684	-81.65315498
O-04	LWR	LWR-N				6/17/2012	28.35961257	-81.65321055
O-06	LWR	LWR-N			3w	6/17/2012	28.35829091	-81.65385327
O-07	LWR	LWR-N	1w		3w	6/17/2012	28.35822587	-81.65526403
OBRU-10	LWR	LWR-S				7/1/2011	27.1701667	-81.3534
OREL-01	LWR	LWR-S	1w			6/21/2011	27.1797333	-81.3501833
OREL-02	LWR	LWR-S	1w			6/21/2011	27.1795833	-81.3497167
OREL-03	LWR	LWR-S				6/22/2011	27.1795833	-81.3501333
OREL-04	LWR	LWR-S				6/22/2011	27.1795333	-81.35035
OREL-05	LWR	LWR-S				6/22/2011	27.1795667	-81.3505333
OREL-06	LWR	LWR-S				6/23/2011	27.1864333	-81.3359167

Appendix A Continued

OREL-07	LWR	LWR-S	2w			6/23/2011	27.18645	-81.33535
OREL-08	LWR	LWR-S				6/23/2011	27.1851	-81.3368333
OREL-09	LWR	LWR-S				6/25/2011	27.1847833	-81.3480167
OREL-10	LWR	LWR-S				6/25/2011	27.18505	-81.3476833
OREL-11	LWR	LWR-S	1w			6/25/2011	27.186	-81.34745
OREL-12	LWR	LWR-S				6/25/2011	27.1831167	-81.3482833
OREL-13	LWR	LWR-S				6/25/2011	27.18175	-81.3480833
OREL-14	LWR	LWR-S				6/26/2011	27.1836167	-81.3528167
OREL-15	LWR	LWR-S	1w			6/26/2011	27.1837833	-81.3533833
OREL-16	LWR	LWR-S	1w			6/28/2011	27.1849833	-81.3491333
OREL-17	LWR	LWR-S				6/29/2011	27.2042667	-81.3648333
OREL-18	LWR	LWR-S				6/29/2011	27.2037	-81.3635333
OREL-19	LWR	LWR-S	1w			6/29/2011	27.1933167	-81.3424
OREL-20	LWR	LWR-S				7/1/2011	27.1646	-81.3537
OREL-21	LWR	LWR-S				7/1/2011	27.1645667	-81.3547167
OREL-22	LWR	LWR-S				7/1/2011	27.1645667	-81.3544
OREL-23	LWR	LWR-S				7/2/2011	27.1424333	-81.3554
OREL-24	LWR	LWR-S	1w			7/2/2011	27.1355667	-81.3551167
ORELO_01	LWR	LWR-N				11/9/2011	28.3922333	-81.6093167
ORELO_02	LWR	LWR-N				11/9/2011	28.3924333	-81.6091
ORELO_03	LWR	LWR-N				11/9/2011	28.3923667	-81.6088333
ORELO_04	LWR	LWR-N				11/9/2011	28.392071	-81.610229
W-01	LWR	LWR-S		10w		6/20/2012	27.17954379	-81.35056123
W-02	LWR	LWR-S				6/20/2012	27.1795064	-81.35029201
W-03	LWR	LWR-S	1w			6/20/2012	27.17950305	-81.35031656
W-04	LWR	LWR-S				6/22/2012	27.21054191	-81.34953244
W-05	LWR	LWR-S				6/22/2012	27.19031143	-81.3360069
W-06	LWR	LWR-S	1w		3w	6/22/2012	27.19016944	-81.33600539
W-07	LWR	LWR-S	1w			6/22/2012	27.1885721	-81.34809435
W-08	LWR	LWR-S	1w			6/23/2012	27.17535057	-81.36079889
W-09	LWR	LWR-S				6/24/2012	27.14451372	-81.35760211
W-10	LWR	LWR-S	1w		3w	6/24/2012	27.14490482	-81.35757194
W-11	LWR	LWR-S	1w			6/24/2012	27.15096837	-81.35755895
W-12	LWR	LWR-S				6/24/2012	27.16453297	-81.3536047
W-13	LWR	LWR-S	1w			6/24/2012	27.16371071	-81.35360738
W-14	LWR	LWR-S				6/26/2012	27.20420251	-81.36471441
W-15	LWR	LWR-S	1w			6/26/2012	27.20426487	-81.36473763
W-16	LWR	LWR-S			3w	6/26/2012	27.13698685	-81.32510288
W-17	LWR	LWR-S	1w			6/26/2012	27.13678787	-81.32495401
W-18	LWR	LWR-S				6/26/2012	27.13412376	-81.32682477
W-19	LWR	LWR-S	1w			6/26/2012	27.13413172	-81.32676736
W-20	LWR	LWR-S	1w			6/27/2012	27.21931499	-81.38155868
W-21	LWR	LWR-S				6/27/2012	27.21924274	-81.38162448
W-22	LWR	LWR-S				6/27/2012	27.21998395	-81.38103146
W-23	LWR	LWR-S	1w			6/27/2012	27.22095952	-81.3813338
W-24	LWR	LWR-S(C)	1w			6/27/2012	27.28719626	-81.31891209
W-25	LWR	LWR-S(C)		10w		6/27/2012	27.28720766	-81.31921761
W-26	LWR	LWR-S(N)	1w	10w		6/29/2012	27.44710553	-81.3771353

Appendix A Continued

W-27	LWR	LWR-S(N)				6/29/2012	27.44717075	-81.375271
W-28	LWR	LWR-S(N)	1w			6/29/2012	27.44518658	-81.37683774
W-29	LWR	LWR-S(C)		10w		6/29/2012	27.33422569	-81.3437843
W-30	LWR	LWR-S(C)	1w		3w	6/29/2012	27.33788657	-81.352262
W-31	LWR	LWR-S(C)				6/29/2012	27.30046774	-81.42453404
W-32	LWR	LWR-S(C)	1w			6/29/2012	27.30047227	-81.4246011
W-33	LWR	LWR-S(C)	1w			6/29/2012	27.30062909	-81.42452801
W-34	LWR	LWR-C		10w		6/30/2012	27.98380684	-81.49677062
W-35	LWR	LWR-C	1w			6/30/2012	27.98410968	-81.49660064
W-36	LWR	LWR-C	1w		3w	6/30/2012	27.98417816	-81.49597401
W-37	LWR	LWR-S(N)				7/20/2015	27.53343	-81.4133
W-38	LWR	LWR-S(N)				7/20/2015	27.53403	-81.41283
W-39	LWR	LWR-S(N)	1w			7/20/2015	27.53472	-81.41355
W-40	LWR	LWR-S(N)				7/20/2015	27.53457	-81.41353
W-42	LWR	LWR-S(N)				7/20/2015	27.66432	-81.39572
W-43	LWR	LWR-C				7/21/2015	27.82827	-81.46727
W-44	LWR	LWR-C				7/21/2015	27.8286	-81.46733
W-45	LWR	LWR-C				7/21/2015	27.83353	-81.46658
W-46	LWR	LWR-C	1w			7/21/2015	27.83328	-81.46657
W-47	LWR	LWR-C	1w			7/21/2015	27.83167	-81.46293
W-48	LWR	LWR-C				7/21/2015	27.83177	-81.46315
W-49	LWR	LWR-N	1w			7/22/2015	28.39215	-81.60937
W-50	LWR	LWR-N	1w			7/22/2015	28.39242	-81.60953

Appendix B. List of samples and locations of *Odontomachus* in Borneo.

Species	mtDNA	nucDNA	msts	col	morph	CHC	Code	Alt. (m)	Place	Location	Latitude	Longitude	Date	Collector
latidens	x	x					Od025/MJ19769	822	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.043309977	114.8755703	8-Mar-12	D.M. Sorger
latidens	x	x					Od026/MJ19770	891	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042198621	114.8782484	8-Mar-12	D.M. Sorger
latidens	x	x					Od027/MJ19767	1122	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.039822938	114.8828515	10-Mar-12	D.M. Sorger
latidens	x	x					Od047/MJ19768	618	Borneo, Sarawak	Mt. Api, Mulu NP	4.129792023	114.893743	16-Mar-12	D.M. Sorger
latidens	x	x					Olat01/MJ19766	472	Borneo, Sarawak	Mt. Api, Mulu NP	4.13169	114.89354	23-Jul-12	D.M. Sorger
malignus	x	x					MJ13287		Palau	Ulong Island				Czekanski-Moir
malignus	x	x					Od056/MJ19771	0	Borneo, Sarawak	Bako NP	1.72382053	110.4451713	31-Mar-12	D.M. Sorger
rixosus	x	x					MCZ-0041		Cambodia	Ko Khong Thma Bang			2007	G. Alpert
rixosus	x	x					MJ13493		Malaysia	Pahang Kuala Lompat NP			25-Aug-92	D. Furth
rixosus	x	x					MJ13514		Cambodia	Ko Khong Thma Bang			2002	L. Alonso
saltans	x		x	x	x		Od(13)001	57	Borneo, Sarawak	Mulu NP	4.04635	114.81572	7-Jul-13	D.M. Sorger
rixosus			x		x		Od001	67	Borneo, Sarawak	Fairy Caves, Kuching	1.381653	110.117486	5-Feb-12	D.M. Sorger
rixosus	x		x				Od004	53	Borneo, Sarawak	Mulu NP	4.042410264	114.8135004	8-Feb-12	D.M. Sorger
rixosus	x	x	x		x	x	Od006	54	Borneo, Sarawak	Mulu NP	4.040381927	114.8151387	10-Feb-12	D.M. Sorger
rixosus	x		x				Od007	61	Borneo, Sarawak	Mulu NP	4.04165891	114.8134223	10-Feb-12	D.M. Sorger
rixosus	xx		x	x	x	x	Od008	64	Borneo, Sarawak	Mulu NP	4.037741711	114.8130497	10-Feb-12	D.M. Sorger
rixosus	xx		x		x		Od012	45	Borneo, Sarawak	Niah NP	3.816769	113.771654	16-Feb-12	D.M. Sorger
rixosus	x						Od014	28	Borneo, Sarawak	Niah NP	3.81633684	113.7714725	17-Feb-12	D.M. Sorger
rixosus	x		x		x		Od016	34	Borneo, Sarawak	Niah NP	3.816075	113.771621	17-Feb-12	D.M. Sorger
rixosus	x		x				Od041	137	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136495199	114.8909934	14-Mar-12	D.M. Sorger
rixosus	xx		x		x		Od042	138	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136564098	114.891146	14-Mar-12	D.M. Sorger
saltans	x		x		x		Od044	158	Borneo, Sarawak	Kerangas, Camp 5, Mt. Api, Mulu NP	4.148510993	114.888161	15-Mar-12	D.M. Sorger
rixosus	xx		x	x	x	x	Od045	158	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136255225	114.8934552	15-Mar-12	D.M. Sorger
rixosus	x		x				Od046	163	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136463935	114.8932261	15-Mar-12	D.M. Sorger
rixosus	x		x		x		Od048	62	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136685217	114.8932924	16-Mar-12	D.M. Sorger
rixosus	x		x		x		Od049	57	Borneo, Sarawak	Mulu NP	4.020726699	114.82718	23-Mar-12	D.M. Sorger
rixosus						x	Od050	57	Borneo, Sarawak	Mulu NP	4.040189479	114.8143249	23-Mar-12	D.M. Sorger
rixosus	x		x	x	x	x	Od051	41	Borneo, Sarawak	Mulu NP	4.039162779	114.8147374	25-Mar-12	D.M. Sorger
rixosus	xx		x		x		Od052	31	Borneo, Sarawak	Santubong, Kuching	1.759494497	110.3209159	28-Mar-12	D.M. Sorger
rixosus				x	x		Orix01	57	Borneo, Sarawak	Mulu NP	4.04032	114.81512	7-Jul-13	D.M. Sorger
rixosus	x		x				Orix02	53	Borneo, Sarawak	Mulu NP	4.0402	114.81445	7-Jul-13	D.M. Sorger
rixosus			x	x	x		Orix03	55	Borneo, Sarawak	Mulu NP	4.03915	114.81453	7-Jul-13	D.M. Sorger
rixosus	x		x				Orix06	44	Borneo, Sarawak	Mulu NP	4.03767	114.81301	12-Jul-13	D.M. Sorger
rixosus	x						Orix10	150	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.13653	114.89266	24-Jul-13	D.M. Sorger
saltans	x	x					MJ14117	390	Borneo, Kalimantan	Kayan river, Malinau Region, Long Jelet	2.687506	115.805967	8-Jun-08	M. Janda
saltans	x		x		x		Od005	50	Borneo, Sarawak	Mulu NP	4.024621015	114.8228009	9-Feb-12	D.M. Sorger
saltans	x		x	x	x	x	Od019	553	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042537333	114.8714882	8-Mar-12	D.M. Sorger
saltans	x		x				Od020	554	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042492993	114.8715187	8-Mar-12	D.M. Sorger
saltans	x						Od021	544	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042492742	114.8715764	8-Mar-12	D.M. Sorger
saltans	x		x				Od022	544	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042643281	114.8713534	8-Mar-12	D.M. Sorger
saltans	x		x				Od023	618	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042872526	114.8727039	8-Mar-12	D.M. Sorger
saltans	x		x				Od024	545	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042471116	114.8712853	8-Mar-12	D.M. Sorger
saltans	xx		x			x	Od028	676	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.043284412	114.8738712	10-Mar-12	D.M. Sorger
saltans	x		x		x		Od029	658	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.043356497	114.8738184	10-Mar-12	D.M. Sorger
saltans	xx	x	x			x	Od030	635	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042903958	114.8730547	10-Mar-12	D.M. Sorger
saltans	x		x		x		Od031	624	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042917369	114.872863	10-Mar-12	D.M. Sorger
saltans					x		Od032	493	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042278919	114.8699327	10-Mar-12	D.M. Sorger
saltans	xx		x			x	Od033	481	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042325523	114.8696001	10-Mar-12	D.M. Sorger
saltans	xx		x	x	x	x	Od034	360	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.046633067	114.8644355	10-Mar-12	D.M. Sorger
saltans	xx		x			x	Od035	272	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.049395323	114.8600325	11-Mar-12	D.M. Sorger
saltans	x		x		x		Od036	274	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.048964744	114.8603996	11-Mar-12	D.M. Sorger
saltans	xx		x			x	Od037	314	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.047825309	114.8627655	11-Mar-12	D.M. Sorger
saltans	x		x		x		Od038	311	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.047844168	114.8626692	11-Mar-12	D.M. Sorger
saltans	x		x		x		Od039	285	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.048186904	114.8615738	11-Mar-12	D.M. Sorger
saltans	xx		x	x	x	x	Od040	205	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.050511457	114.8573188	11-Mar-12	D.M. Sorger
saltans	x		x				Od043	184	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136618329	114.8908823	14-Mar-12	D.M. Sorger
dunni	x			x	x		Od053	319	Borneo, Sarawak	Kubah NP	1.608883683	110.1888041	29-Mar-12	D.M. Sorger
simillimus	x	x					MJ23375		Moluccas	Ternate Island				M. Janda
simillimus	x						Od011	48	Borneo, Sarawak	Niah NP	3.816865319	113.7717598	16-Feb-12	D.M. Sorger
simillimus	x						Od054	20	Borneo, Sarawak	outside Kuching	1.421253271	110.3262521	30-Mar-12	D.M. Sorger
simillimus	x						Od055	17	Borneo, Sarawak	outside Kuching	1.421345221	110.3263854	30-Mar-12	D.M. Sorger

Appendix C. List of samples and locations of *Odontomachus saltans*, *O. rixosus*, and *O. dunni* in Borneo.

Species	mtDNA	msts	morph	CHC	Haplotype	Code	Alt. (m)	Place	Location	Latitude	Longitude	Date	Collector
rixosus	1w				10	MCZ-0041		Cambodia	Ko Khong Thma Bang			2007	G. Alpert
rixosus	1w				9	MJ13493		Malaysia	Pahang Kuala Lompat NP			25-Aug-92	D. Furth
rixosus	1w				10	MJ13514		Cambodia	Ko Khong Thma Bang			2002	L. Alonso
saltus	1w	9w	2w		6	Od(13)001	57	Borneo, Sarawak	Mulu NP	4.04635	114.81572	7-Jul-13	D.M. Sorger
rixosus		12w	10w			Od001	67	Borneo, Sarawak	Fairy Caves, Kuching	1.381653	110.117486	5-Feb-12	D.M. Sorger
rixosus	1w	28w			11	Od004	53	Borneo, Sarawak	Mulu NP	4.042410264	114.8135004	8-Feb-12	D.M. Sorger
rixosus	1w	12w	5w	10w	15	Od006	54	Borneo, Sarawak	Mulu NP	4.040381927	114.8151387	10-Feb-12	D.M. Sorger
rixosus	1w	28w			15	Od007	61	Borneo, Sarawak	Mulu NP	4.04165891	114.8134223	10-Feb-12	D.M. Sorger
rixosus	2w	12w	10w	10w	15	Od008	64	Borneo, Sarawak	Mulu NP	4.037741711	114.8130497	10-Feb-12	D.M. Sorger
rixosus	2w	12w	10w		13	Od012	45	Borneo, Sarawak	Niah NP	3.816769	113.771654	16-Feb-12	D.M. Sorger
rixosus	1w				13	Od014	28	Borneo, Sarawak	Niah NP	3.81633684	113.7714725	17-Feb-12	D.M. Sorger
rixosus	1w	12w	10w		13	Od016	34	Borneo, Sarawak	Niah NP	3.816075	113.771621	17-Feb-12	D.M. Sorger
rixosus	1w	12w			15	Od041	137	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136495199	114.8909934	14-Mar-12	D.M. Sorger
rixosus	2w	12w	10w		15	Od042	138	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136564098	114.891146	14-Mar-12	D.M. Sorger
saltus	1w	12w	2w		7	Od044	158	Borneo, Sarawak	Kerangas, Camp 5, Mt. Api, Mulu NP	4.148510993	114.888161	15-Mar-12	D.M. Sorger
rixosus	2w	12w*	10w	10w	15	Od045	158	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136255225	114.8934552	15-Mar-12	D.M. Sorger
rixosus	1w	12w			15	Od046	163	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136463935	114.8932261	15-Mar-12	D.M. Sorger
rixosus	1w	12w	10w		15	Od048	62	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136685217	114.8932924	16-Mar-12	D.M. Sorger
rixosus	1w	12w	10w		14	Od049	57	Borneo, Sarawak	Mulu NP	4.020726699	114.82718	23-Mar-12	D.M. Sorger
rixosus				10w		Od050	57	Borneo, Sarawak	Mulu NP	4.040189479	114.8143249	23-Mar-12	D.M. Sorger
rixosus	1w	12w	10w	10w	15	Od051	41	Borneo, Sarawak	Mulu NP	4.039162779	114.8147374	25-Mar-12	D.M. Sorger
rixosus	2w	12w	10w		12	Od052	31	Borneo, Sarawak	Santubong, Kuching	1.759494497	110.3209159	28-Mar-12	D.M. Sorger
rixosus			2w			Orix01	57	Borneo, Sarawak	Mulu NP	4.04032	114.81512	7-Jul-13	D.M. Sorger
rixosus	1w	12w			15	Orix02	53	Borneo, Sarawak	Mulu NP	4.0402	114.81445	7-Jul-13	D.M. Sorger
rixosus		12w	2w			Orix03	55	Borneo, Sarawak	Mulu NP	4.03915	114.81453	7-Jul-13	D.M. Sorger
rixosus	1w	28w			15	Orix06	44	Borneo, Sarawak	Mulu NP	4.03767	114.81301	12-Jul-13	D.M. Sorger
rixosus	1w				15	Orix10	150	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.13653	114.89266	24-Jul-13	D.M. Sorger
saltus	1w				5	MJ14117	390	Borneo, Kalimantan	Kayan river, Malinau Region, Long Jelet	2.687506	115.805967	8-Jun-08	M. Janda
saltus	1w	12w	5w		2	Od005	50	Borneo, Sarawak	Mulu NP	4.024621015	114.8228009	9-Feb-12	D.M. Sorger
saltus	1w	28w	10w	10w	1	Od019	553	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042537333	114.8714882	8-Mar-12	D.M. Sorger
saltus	1w	12w			1	Od020	554	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042492993	114.8715187	8-Mar-12	D.M. Sorger
saltus	1w				1	Od021	544	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042492742	114.8715764	8-Mar-12	D.M. Sorger
saltus	1w	20w			3	Od022	544	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042643281	114.8713534	8-Mar-12	D.M. Sorger
saltus	1w	12w			1	Od023	618	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042872526	114.8727039	8-Mar-12	D.M. Sorger
saltus	1w	1l, 3w,4q			1	Od024	545	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042471116	114.8712853	8-Mar-12	D.M. Sorger
saltus	2w	17w		10w	1	Od028	676	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.043284412	114.8738712	10-Mar-12	D.M. Sorger
saltus	1w	12w	10w		1	Od029	658	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.043356497	114.8738184	10-Mar-12	D.M. Sorger
saltus	2w	11w,1q		10w	1	Od030	635	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042903958	114.8730547	10-Mar-12	D.M. Sorger
saltus	1w	12w	2w		1	Od031	624	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042917369	114.872863	10-Mar-12	D.M. Sorger
saltus			5w			Od032	493	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042278919	114.8699327	10-Mar-12	D.M. Sorger
saltus	2w	18w		10w	1	Od033	481	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.042325523	114.8696001	10-Mar-12	D.M. Sorger
saltus	2w	17w,1q	10w	10w	1	Od034	360	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.046633067	114.8644355	10-Mar-12	D.M. Sorger
saltus	2w	11w,1q		10w	4	Od035	272	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.049395323	114.8600325	11-Mar-12	D.M. Sorger
saltus	1w	18w,2q	10w		1	Od036	274	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.048964744	114.8603996	11-Mar-12	D.M. Sorger
saltus	2w	12w		10w	2	Od037	314	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.047825309	114.8627655	11-Mar-12	D.M. Sorger
saltus	1w	12w,1q	10w		1	Od038	311	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.047844168	114.8626692	11-Mar-12	D.M. Sorger
saltus	1w	18w	5w		1	Od039	285	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.048186904	114.8615738	11-Mar-12	D.M. Sorger
saltus	2w	2q	10w	10w	1	Od040	205	Borneo, Sarawak	Mt. Mulu, Mulu NP	4.050511457	114.8573188	11-Mar-12	D.M. Sorger
saltus	1w	2q			1	Od043	184	Borneo, Sarawak	Camp 5, Mt. Api, Mulu NP	4.136618329	114.8908823	14-Mar-12	D.M. Sorger
dunni	1w				8	Od053	319	Borneo, Sarawak	Kubah NP	1.608883683	110.1888041	29-Mar-12	D.M. Sorger

**3q were included but their individual code was unknown*

Appendix D. List of samples and locations of *Lepisiota* sp. in Ethiopia.

Code	Vial	mtDNA	Haplotype	collected	Location	Alt. (m)	Lat	Long	Notes
GH	ET016	1w	9	8-Jan-12	Ghion Hotel, Bahir Dar	1780	11.596972	37.385582	on ground at edge of concrete path
ZS01	ET015	1w	3	7-Jan-12	near Bahir Dar, on way to Zhara	1873	11.599377	37.438370	on roadside
ZS1-2	ET053	1w	1	13-Jan-12	between Debre Tabor and Bahir Dar	1896	11.657675	37.465493	
ZS02	ET040	1w, 1q	5	11-Jan-12	between Debre Tabor and Bahir Dar	1818	11.717039	37.503864	under rock and on plant with yellow flowers (Mimosa sp.)
ZS2-3	ET050	1w	2	13-Jan-12	between Debre Tabor and Bahir Dar	1901	11.758932	37.539395	
ZW	N/A	N/A	N/A	6-Jan-12	Zhara Church Forest, at wall	1920	11.800063	37.567653	
ZL01	ET009	1w	1	6-Jan-12	Zhara Church Forest	1922	11.800054	37.568947	
ZL02	ET010	1w, 10q	5	6-Jan-12	Zhara Church Forest	1910	11.800054	37.568948	nest under rock with alates and multiple queens
ZL03	ET011	1w	1	7-Jan-12	Zhara Church Forest	1910	11.801514	37.567554	
ZL04	ET012	1w	1	7-Jan-12	Zhara Church Forest	1908	11.801945	37.568003	
ZL05	ET013	1w	1	7-Jan-12	Zhara Church Forest	1911	11.802593	37.568267	at wall
ZL06	ET014	1w	1	13-Jan-12	Zhara Church Forest	1911	11.799458	37.569032	in open field, ca. 100m from forest wall
ZC	ET020	1w	1	8-Jan-12	outside Zhara Church Forest, across road	1904	11.799123	37.570499	Eucalyptus forest, in dry riverbed
ZE	ET036	1w	1	8-Jan-12	outside Zhara Church Forest, across road	1910	11.798806	37.570346	Eucalyptus forest
DS05	ET033	1w	9	13-Jan-12	between Debre Tabor and Bahir Dar	1792	11.839730	37.635725	
ZS03	ET034	1w	1	11-Jan-12	between Debre Tabor and Bahir Dar	1808	11.902334	37.684748	with froghoppers on yellow-flower plant
ZS04	ET049	1w	8	11-Jan-12	between Debre Tabor and Bahir Dar	1811	11.902566	37.685182	across street from ZS03, on yellow-flower plant
DS04	ET045	1w	8	13-Jan-12	between Debre Tabor and Bahir Dar	1838	11.938083	37.777999	
ZS05	ET028	1w	1	11-Jan-12	between Debre Tabor and Bahir Dar	1975	11.922832	37.858068	on yellow-flower plant, no froghoppers, only a few ants present
ZS06_01	ET046	1w	6	11-Jan-12	between Debre Tabor and Bahir Dar	2055	11.918482	37.912698	in agricultural field, under rock, very dry area
ZS06_02	ET031	1w	6	13-Jan-12	between Debre Tabor and Bahir Dar	2055	11.918482	37.912698	same as ZS06_01 (duplicate collection)
DS03	ET041	1w	1	13-Jan-12	between Debre Tabor and Bahir Dar	2255	11.925489	37.945463	on yellow-flower plant, on roadside near secondary forest
DS02	ET042	1w	7	13-Jan-12	between Debre Tabor and Bahir Dar	2439	11.894392	37.973882	on yellow-flower plant (different species than seen before)
DE	ET051	1w	1	12-Jan-12	Debresena Church Forest	2651	11.852790	37.989962	under rock
D01	ET027	1w	1	12-Jan-12	Debresena Church Forest	2681	11.852734	37.988680	nest under rock
GF	ET019	1w	1	9-Jan-12	outside Geladwios Church Forest	2430	11.642658	37.811672	on open grassfield under rock
MA	ET058	1w	4	17-Jan-12	near Lalibela, Mt. Asheton	2719	12.029738	39.057542	on ground
GC	ET067	1w	9	18-Jan-12	Gondor Castle, inside royal enclosure	2225	12.609290	37.470137	on tree (bean family)
LC	ET066	1w	9	16-Jan-12	Lalibela churches	2471	12.031239	39.045793	