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DNA BARCODING A COLLECTION OF ANTS (HYMENOPTERA: FORMICIDAE) FROM ISLA DEL COCO, COSTA RICA

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Abstract

Islands can be exceptionally sensitive to changes brought about by newly arrived species. Non-native ants on an island can cause catastrophic changes. Identifying these non-native species is taxonomically challenging because their geographic source is unknown. We DNA barcoded a 2011 collection of ants from Isla del Coco in the Pacific Ocean, about 500 km from the mainland of Costa Rica and Colombia. We compared these barcodes with those from Area de Conservación Guanacaste in northwestern (coastal) Costa Rica, and with ant barcodes from elsewhere in the Neotropics. We found 10 species from 7 genera in 3 subfamilies.

Key Words: invasive, introduced, DNA barcodes, *Wasmannia*, tropical island ants

Resumen

Las islas pueden ser excepcionalmente sensibles a los cambios provocados por las especies recien llegadas. Hormigas no nativas en una isla pueden provocar cambios catastróficos. La identificación de estas especies no nativas es taxonómicamente difícil porque su origen geográfico es desconocido. Nosotros hicimos el codigo de barras ADN de una colección de hormigas del 2011, de la Isla del Coco en el Océano Pacífico, a unos 500 kilómetros de la parte continental de Costa Rica y Colombia. Se compararon estos códigos de barras con los de Área de Conservación Guanacaste, en el noroeste (costa) Costa Rica, y con códigos de barras de hormiga en otros lugares del Neotrópico. Encontramos diez especies en siete géneros en tres.

Palabras Clave: invasoras, introducidas, códigos de barras de ADN, *Wasmannia*, hormigas de isla tropical

In an era of globalized trade, wild species are frequently introduced by humans to places previously unoccupied by those species. This introduction may have large negative consequences for the local biota (Levine 2008). Large databases have been established to record these non-native species. A database for introduced species is the Global Invasive Species Database (www.issg.org/ database/welcome/) where 5 of the 100 species listed are ants. The negative impact of these ants on mainland species of ants appears to be strong and is even more pronounced on islands (Lach & Hooper-Bùi 2009). A first step in understanding the potential effects of an introduced species on a resident fauna is to inventory that local fauna before and after the introduction. Unfortunately, for many species of arthropods, the taxonomic infrastructure necessary to conduct such an inventory is lacking (Gardner et al. 2008; Pawar 2003). One potential solution to this problem may be using DNA barcoding (Janzen et al. 2009) as an inventory tool. If the ant species from an area have been DNA barcoded, even without assigning scientific names (Smith & Fisher 2009), the barcodes of future ant collections can be compared to the DNA barcode library for the island and adjacent mainland area (which, unfortunately, may be as large as the entire world). Such faunistic comparisons can be made with any kind of tissue, rather than requiring tissue from a particular morph or life stage (Smith & Fisher 2009).

Isla de Coco is a 24 km^2 island approximately 480 km west of Costa Rica (N 05° 31' 08" W 87° 04' 18"). While the current human population is only a permanent ranger station with 15 staff, human impact on the island has been ongoing since the 1700's. Human impact began with the island's use as a staging post and water replenishment for mariners. There have been several previous inventories of Isla del Coco ants (detailed here: Solomon & Mikheyev 2005; Forel 1902, 1908; Wheeler 1919, 1933), the most recent in 2003. Solomon & Mikheyev (2005) identified several likely taxonomic mistakes in these original surveys [e.g., the presence of *Pseudomyrmex flavicornis* (Smith 1877), an obligate acacia-ant (Janzen 1966) despite the absence of its obligate host plant *Vachellia* (Fabaceae), formerly known as *Acacia*] and carried out a thorough analysis of the changes from 1902 to 2005. They concluded that much of this island fauna was "dominated" by non-native species.

Here we compare our 2011 inventory with the 2003 inventory, and begin to populate the DNA barcode database for this island, both for our own purposes and for future inventories.

MATERIALS AND METHODS

Ants were collected opportunistically and visually by hand and without using bait (RBS), and preserved in 95% ethanol, on 3 Dec 2011 (end of the long rainy season). Collecting occurred on 2 trails, the first to the Rio Genio waterfall (0-50 m asl), and the second to Cerro Iglesias, the highest point of the island, (0-635 m asl) (Fig. 1).

Ants were sorted to morphospecies at the University of Guelph. Representatives of each morphospecies were then point-mounted, photographed and sampled for DNA extraction. The tissue sampled was generally a single leg. However, in the case of very small ants, sometimes all the point-side legs were used as the sample tissue.

DNA extracts were prepared from the legs using a Macherey-Nagel 96-well NucleoSpin tissue extraction kit. The DNA extracts was re-suspended in 30 μL of dH$_2$O, and a 658-bp region near the 5′ terminus of the COI gene was amplified using a cocktail of standard barcoding region primers (C_LepFolF-C_LepFolR; Table 1) following established barcoding protocols (Smith et al., 2007; Fisher & Smith 2008; Smith et al. 2008). All sequences were derived from amplifications that produced single bands during agarose electrophoresis and none displayed systematic heteroplasmy. The resultant amplicons were uni-directionally sequenced with the LepF1 primer. All laboratory information for individual sequences can be retrieved from the Barcode of Life Data System [BOLD, (Ratnasingham & Hebert 2007)] using the Process IDs detailed in Supplementary Table 1. All sequence data is available on BOLD (www.barcodinglife.org) in the public dataset: Formicidae of Isla del Coco, Costa Rica (dx.doi.org/10.5883/DS-ASCOS), while the collection information and accessions (BOLD and GenBank (Benson et al. 2008)) for all specimens and sequences are listed in Supplementary Table 1.

Using MEGA5 (Tamura et al. 2011), we calculated the Kimura-2 Parameter (Kimura 1980) for intra- and inter-specific variability for the species from which we had collected more than one specimen (Table 2) and displayed this variation as a neighbor-joining phenogram (Saitou & Nei 1987) using MEGA5 and FigTree (Rambaut 2013) (Fig. 2). In addition to these calculations, DNA barcodes were compared to other ant DNA sequences using the Barcode of Life Data System (BOLD) ID engine. Here results are given in % similarity also calculated using K2P distances.

The DNA barcodes of the ants from Isla de Coco were compared with those of a larger ongoing inventory of the ants in Area de Conservación Guanacaste (ACG), northwestern Costa Rica

Fig. 1. Collecting sites on Isla del Coco, A) trail to Rio Genio, B) trail to Cerro Igelsias.

TABLE 1. COCKTAIL OF STANDARD BARCODING PRIMERS USED IN SEQUENCING A 658-BP THE COI GENE OF THE VARIOUS ANT SPECIES FROM ISLA DEL COCO.

Primer Code	Oligonucleotide (5' to 3')	Reference
C_LepFolF	LepF1:LCO1490	
LepF1	ATTCAACCAATCATAAAGATATTGG	(Hebert et al. 2004)
LCO1490	GGTCAACAAATCATAAAGATATTGG	(Folmer et al. 1994)
C_LepFolR	LepR1:HCO2198	
LepR1	TAAACTTCTGGATGTCCAAAAAATCA	(Hebert et al. 2004)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	(Folmer et al. 1994)

(Janzen et al. 2009; Smith et al. 2013), as well as to all other publically available collections from the Neotropics in GenBank and BOLD. Species identities were also compared with the names in earlier collection inventories of ants from Isla de Coco (Solomon & Mikheyev 2005).

Diversity estimates and confidence intervals were calculated using the Chao2 estimate (Chao et al. 2005), an estimate known to be sensitive to datasets with a large number of singletons and doubletons, using EstimateS (Colwell 2005).

RESULTS

Eighty-three ant specimens were point-mounted and 38 of these were photographed. All collection information is available online as a supplemental appendix in Florida Entomologist 96(4) (Dec 2013) at http://purl.fcla.edu/fcla/entomologist/browse. All 83 specimens were tissue-sampled and prepared for DNA extraction, and 96.4% of these yielded a COI sequence of at least 200 base pairs. All sequences analyzed here were derived from amplifications that displayed single bands during agarose electrophoresis, none displayed systematic heteroplasmy, nor the presence of stop codons, frameshift mutations or a high dN/dS ratios—leading us to conclude that these are sequences are mitochondrial and not NUMTs (Bensasson et al. 2001; Calvignac et al. 2011). Interpreting unidentified NUMTs as true mitochondrial sequences could result in the misplaced identification of cryptic species (Song et al. 2008), distinguishing between mitochondrial and NUMT's of recent origin can be challenging (Bertheau et al. 2011; Levitsky et al. 2013). Based on these tests performed, we are confident that the sequences analyzed here are mitochondrial in origin, however to allow for further testing in the future (when the database of DNA barcodes is that much larger), all data from which we derived our species hypotheses are publically available for further examination.

The average intra-specific variability (K2P) was 0.58% and the average inter-specific variation was 27% (Table 2). The contrast of this minor intra-specific variation with the large inter-specific variability is evident in a neighbor-joining phenogram (Fig. 2).

Ten species from 7 genera from 3 subfamilies were collected in our inventory of Isla del Coco: *Camponotus*, *Nylanderia* (Formicinae), *Odontomachus*, *Hypoponera* (Ponerinae), and *Solenopsis*, *Pheidole*, and *Wasmannia* (Myrmicinae) (Fig. 3).

Individuals with a sequence longer than 500 bp that have fewer than 1% ambiguous bases and are more than 2% divergent from other sequences already in the BOLD database receive a globally unique identifier, or GUI, (BOLD:LLL####) (the derivation of the GUI is described in Ratnasingham & Hebert 2013). Specimens grouped into these GUI are often, but not necessarily, the same specimens as would be grouped under the same traditional and morphologically-identified assigned taxonomic species name. In advance of all specimens being identified as having traditional names, however, these GUI can be used to search for specimens in BOLD.

Formicinae

Camponotus: Two morphologically-identified species were found (BOLD:ACE0809, BOLD:ACE0810) on each trail. The barcode of BOLD:ACE0809 is 8% divergent from *Camponotus novogranadensis* Mayr, while that of BOLD:ACE0810 is 10% divergent from *C. planatus*. Neither *C. planatus* (Roger) nor *C. novogranadensis* were recovered in the 2003 survey that collected *C. cocosensis* Wheeler and an unnamed *Camponotus* (reported as "sp2" in Solomon & Mikheyev 2005). Wheeler (1919) considered that the *C. cocosensis* major was most similar to *C. novogranadensis*. However as *C. cocosensis* was not already present in our data—the closest match is to *C. novogranadensis*. We suspect that our BOLD:ACE0809 is likely *C. cocosensis*.

Nylanderia: The most frequently collected ant on each trail was one species (BOLD:ACE0888) of *Nylanderia* that was 5% divergent from the interim species *Nylanderia* MAS004 from ACG dry forest. The Isla del Coco *Nylanderia* is morphologically similar to *Nylanderia guatemalensis* (Forel)*,* which ranges from Mexico to Colombia (at least). Wheeler (1919) viewed the Isla del Coco *Nylanderia* as being a sub-species called *Para-*

TABLE 2. INTRA-SPECIFIC (DIAGONAL) AND INTER-SPECIFIC (SUB-DIAGONAL) DIVERGENCES FOR THE DNA BARCODE REGION (K2P DISTANCE) OF SEVERAL ANT SPECIES FROM ISLA DEL COCO.

	Camponotus cf novogranadensis	Camponotus cf planatus	Nylanderia cf MAS004	Odontomachus cf ruginodis	Pheidole cf JTL194	Solenopsis cf MAS005	Wasmannia auropunctata
Camponotus cf novogranadensis	0.43%						
Camponotus cf planatus	20.79%	0.14%					
Nylanderia cf MAS004	26.5%	25.46%	0.80%				
Odontomachus cf ruginodis	32.53%	31.76%	26.38%	1.25%			
Pheidole cf JTL194	31.02%	28.08%	24.97%	30.26%	1.16%		
Solenopsis cf MAS005	27.40%	24.25%	26.17%	27.01%	21.04%	0.25%	
Wasmannia auropunctata	32.03%	25.61%	25.20%	32.03%	24.14%	23.91%	0.05%

trechina (Nylanderia) guatemalensis cocosensis. However, what he meant by "subspecies" is unclear and so in any modern revision of Isla del Coco ant taxonomy this would probably be called *Nylanderia cocosensis*. Again, we assume that the modern-day *N. cocosensis* to be the same as what Wheeler collected, but cannot know for certain until the holotype is compared morphologically and molecularly with the *Nylanderia* collected in our sample.

Myrmicinae

Pheidole: Three species of *Pheidole* were encountered (one more than in the 2003 inventory), BOLD:ACE2223, BOLD:ACE0710, BOLD:AAB8368,. The first two are known from single specimens. BOLD:AAB8368 is a 100% barcode match with the barcode of *Pheidole sagittaria* from ACG, and morphologically matches it as well. It is a species not previously reported on the island. BOLD:ACE0760 was collected multiple times but only on the trail to Rio Genio (along with BOLD:AAB8368). The single specimen of BOLD: ACE2223 was from the Iglesias trail.

Solenopsis: One species (BOLD:ACE0701) of *Solenopsis* was found on both trails. Its barcode is 5% different from that of an undescribed species of ACG *Solenopsis* (*Solenopsis* MAS005).

Wasmannia: One species (BOLD:AAA3842) of *Wasmannia* was encountered on the Rio Genio trail, and it is morphologically similar to *Wasmannia auropunctata* (Roger) *(*native to South and Central America)—and which has been barcoded from ACG and Belize. The little fire ant, *Wasmannia auropunctata,* an introduced species of concern for its ability to negatively affect native species (—especially on Pacific islands (Global Invasive Species Database). The *Wasmannia* recovered in our collections were similar to 1 of the 2 molecular operational taxonomic units [MOTU (Blaxter et al. 2005)] that are evident within surveys of ACG and Belize (searchable on BOLD using this unique GUI – BOLD:AAA3842). Within the ACG and Belize, there is another *Wasmannia* MOTU that is 5.7% divergent and two more MOTU collected from French Guiana (BOLD:AAA3837). Whether the name *Wasmannia auropunctata* actually contains more than 1 species has been discussed before (Longino & Fernandez 2007). Specifically on the Isla del Coco, Solomon & Mikheyev (2005) reported no morphological differences among their collections of what they called *W. auropunctata* and concluded that their collections represented only one species. They further concluded that, "Although this species is native to the Neotropics, its distribution on Coco Island strongly suggests that it was introduced, despite its presence as early as 1902" (Solomon & Mikheyev 2005). The exact similarity of the barcodes for *W. auropunctata* to other *W. auropunctata* from mainland Costa Rica
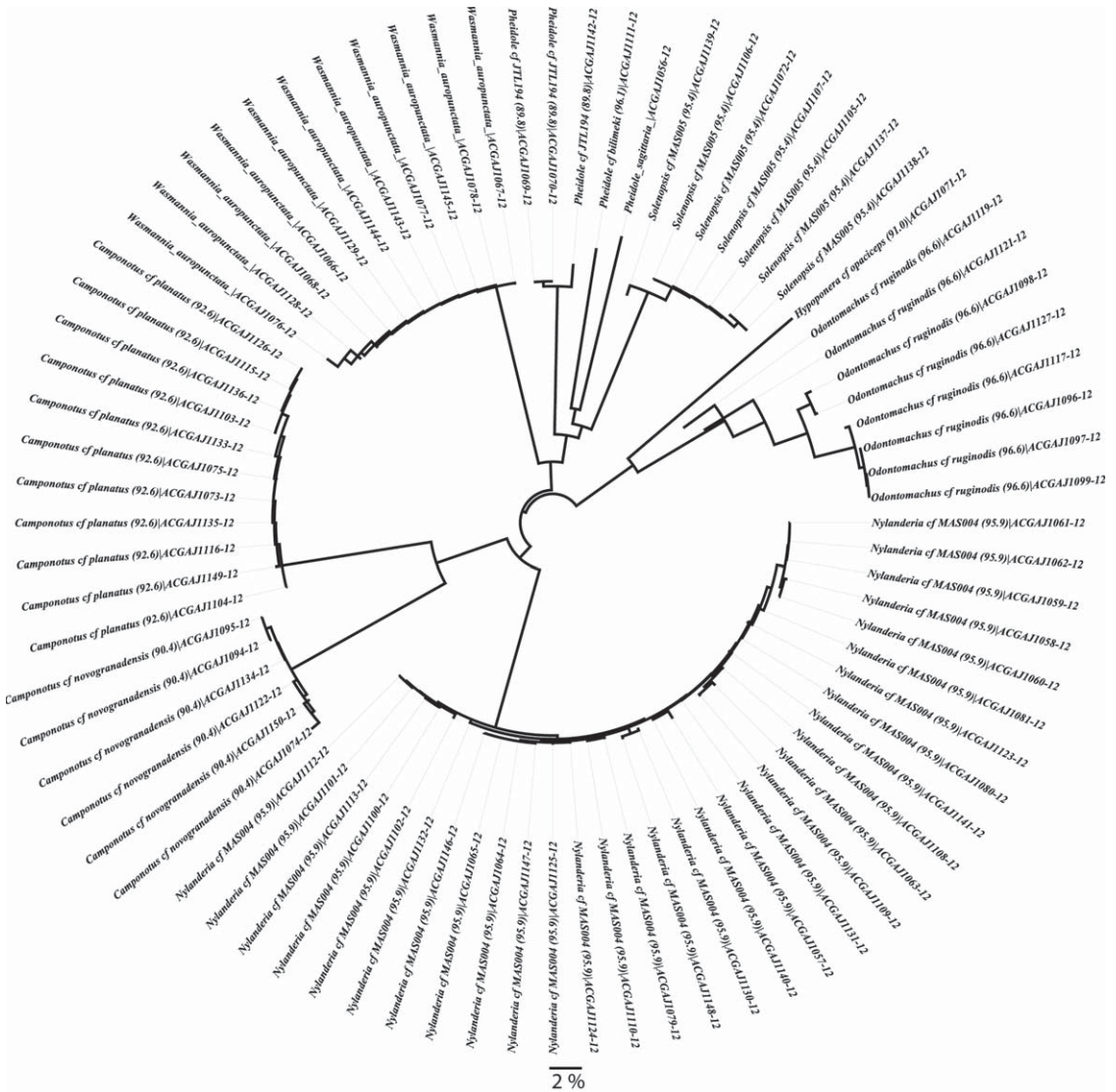
Fig. 2. Neighbor-joining tree of K2P distance for all 80 successfully barcoded ant specimens from Isla del Coco.

and Belize support the hypothesis that this population may have arrived on the island within the past several centuries.

Ponerinae

Odontomachus: One species of *Odontomachus*, encountered only on the Cerro Iglesias trail, was the 4th most abundant species in the sample, likely due to it being large and conspicuous. This species is morphologically very similar to *Odontomachus ruginodis* M. R. Smith from Nicaragua, but its barcode is 5% different from the island species. *Odontomachus ruginodis* was reported in 2003, and *O. haematodes* (L.) 1898 and 1905,

though the latter case may represent a misidentification of *O. ruginodis*.

Hypoponera: *Hypoponera opaciceps* (Mayr), reported in earlier collections from Isla del Coco, is a widely introduced species with a global distribution (Wilson & Taylor 1967). It was encountered one time on the Rio Genio trail. However, the barcode of this morphologically identified specimen is 9% divergent from that of *H. opaciceps* specimens collected from the US (Texas and Florida), Belize and French Polynesia (BOLD: AAI2326), which suggests either a high rate of barcode change due to the usual founder effects, novel selection on a small island, or truly some other species only visible through its barcode.
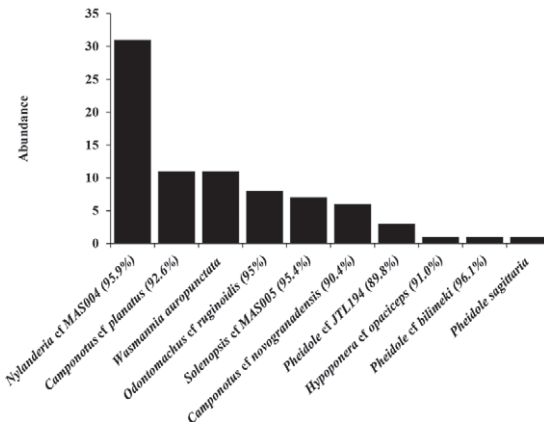
Fig. 3. Relative abundance of each ant species for sampling of ants on Isla del Coco, Costa Rica. Provisional species names (cf) include the percentage similarity to the closest species match in the BOLD database.

When we calculated the Chao2 estimate of species richness (Fig. 4), the projected mean richness was 12, but the 95% confidence intervals on that estimate were quite wide—with the higher bounds being up to 36 species. All we know for certain is that there are at least 10 species of ants living on Isla del Coco at this time. Given that ant community structure is grossly different on islands than it is on mainlands, and that there was no defined sampling protocol, it would be unwise to further conjecture about the number of species on the island. What we can say is that many of them have different DNA barcodes from those of what appear morphologically to be the same species on the mainland.

DISCUSSION

Islands are particularly sensitive to changes brought about by newly introduced species, however they arrive. When contemporarily non-
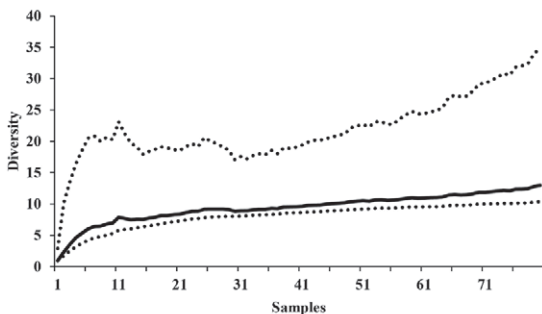

Fig. 4. Chao2 estimate of the ant species richness encountered (solid line) with 95% confidence intervals (dashed lines).

native ants are introduced to an island they can cause catastrophic changes (McGlynn 1999), as was seen on Christmas Island when *Anoplolepis gracilipes* F. Smith led to the displacement of a native 'keystone' species of red land crab, *Gecarcoidea natali* (pocock) (O'Dowd et al. 2003). Human transport of non-native ants to New Zealand is particularly feared (Ward et al. 2006). Ant identification for this and any other purpose can be challenging for many reasons. For instance, ant identification keys are biased strongly towards worker ants and other stages are very difficult to identify (Yoshimura & Fisher 2007; Fisher & Smith 2008). For understudied taxa in understudied areas, the ability to differentiate newly arriving ants from those of an unknown local fauna is limited; DNA barcoding may be the most reliable current method (Smith & Fisher 2009). Previous surveys of the ants of Isla del Coco, (Solomon & Mikheyev 2005) reported that 68% of the 19-species ant fauna was believed to be non-native.

It is important to consider whether morphologically similar ants from mainland and island populations that are 5% genetically divergent ought to be viewed as a different species from their insular morphological look-alikes. In our experience barcoding species in the neotropics (e.g. Smith et al. 2008; Janzen et al. 2009; Smith et al. 2012)—when specimens from a morphologically determined single species name are subsequently revealed to contain more than 2-5% genetic diversity it is strongly suggestive that there is cryptic diversity within that single name. If this is the case here, then 8 of the 10 species recorded here (cf-containing names in Fig. 3) are not yet accurately named. Since 2 of these are species names that have been previously reported on the island (*O. ruginodis, H. opacior*), this would suggest that these collections should be recognized as new species. Further collections and molecular work on this island, and on the Mesoamerican mainland are required to determine this.

The collection method is the principal difference between our brief inventory and the 2003 survey (Solomon & Mikheyev 2005). Solomon & Mikheyev (2005) are professional entomologists and used the ALL ant collection protocol (Agosti & Alonso 2000) for standardized collection leaf-litter ants, principally through mini-Winkler sifting. It is well known that opportunistic inventory such as this one may capture ants that are missed by active search [e.g., Solomno & Mikheyev (2005) found species only by active visual searching]. Our opportunistic survey clearly missed some of the smaller and more cryptic species prevalent in the ALL-based surveys, but it found some that may have been missed in 2003.

The sample size curve that was generated using DNA barcodes produced similar trends, but lower estimates of species richness than what has been reported earlier. In particular, species

previously reported from the island but not encountered by this brief inventory were collected near buildings [*Monomorium floricola* Jerdon*, Solenopsis invicta* Buren and *Tapinoma melanocephalum* (Fabricius)]. In one case, the molecular data allowed us to link the workers of *Odontomachus* cf. *ruginodis* to an alate male from the island. Without DNA data, such associations are challenging (Yoshimura & Fisher 2007; Fisher & Smith 2008).

It is our hope that by making our results public, that they will be used and augmented by others for research and monitoring of the ant fauna of Isla del Coco in the future. Island faunas, particularly in the Pacific, are likely to experience only greater and more regular anthropogenic visits and impacts. New species records for this island are still being generated. The importance of regular monitoring is called into tight focus in such a dynamic and unique island environment.

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