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Global domination by crazy ants: phylogenomics reveals biogeographical history and invasive species relationships in the genus *Nylanderia* (Hymenoptera: Formicidae)

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Abstract. *Nylanderia* (Emery) is one of the world's most diverse ant genera, with 123 described species worldwide and hundreds more undescribed. Fifteen globetrotting or invasive species have widespread distributions and are often encountered outside their native ranges. A molecular approach to understanding the evolutionary history and to revision of *Nylanderia* taxonomy is needed because historical efforts based on morphology have proven insufficient to define major lineages and delimit species boundaries, especially where adventive species are concerned. To address these problems, we generated the first genus-wide genomic dataset of *Nylanderia* using ultraconserved elements (UCEs) to resolve the phylogeny of major lineages, determine the age and origin of the genus, and describe global biogeographical patterns. Sampling from seven biogeographical regions revealed a Southeast Asian origin of *Nylanderia* in the mid-Eocene and four distinct biogeographical clades in the Nearctic, the Neotropics, the Afrotropics/Malagasy region, and Australasia. The Nearctic and Neotropical clades are distantly related, indicating two separate dispersal events to the Americas between the late Oligocene and early Miocene. We also addressed the problem of misidentification that has characterized species-level taxonomy in *Nylanderia* as a result of limited morphological variation in the worker caste by evaluating the integrity of species boundaries in six of the most widespread *Nylanderia* species. We sampled across ranges of species in the *N. bourbonica* complex (*N. bourbonica* (Forel) + *N. vaga* (Forel)), the *N. fulva* complex (*N. fulva* (Mayr) + *N. pubens* (Forel)), and the *N. guatemalensis* complex (*N. guatemalensis* (Forel) + *N. steinheili* (Forel)) to clarify their phylogenetic placement. Deep splits within these complexes suggest that some species names – specifically *N. bourbonica* and *N. guatemalensis* – each are applied to multiple cryptic species. In exhaustively sampling *Nylanderia* diversity in the West Indies, a 'hot spot' for invasive taxa, we found five adventive species among 22 in the region; many remain morphologically indistinguishable from one another, despite being distantly related. We stress that overcoming the taxonomic impediment through the use of molecular phylogeny and revisionary study is essential for conservation and invasive species management.

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Introduction

The taxonomic impediment

'The "habitus" of a species, as every taxonomist knows, is something one may take in at a glance, but be quite unable to express without wearisome prolixity.'

– William Morton Wheeler (1910).

The term 'taxonomic impediment' (Taylor, 1983) refers to the ongoing challenge of naming and characterizing vast numbers of species, namely those with inconspicuous diversity owing to broad distributions or limited morphological variation. Although ants are unusually well-characterized among insects, with over 13 000 species formally described, more than 7000 ant species are estimated to remain undescribed (Hölldobler & Wilson, 1990; AntWeb, 2019). More than a quarter of the ants on Earth lack formal descriptions because, in some cases, particular groups remain unstudied, whereas other groups are impeded by morphological crypsis; historical taxonomic confusion can compound both of these problems. Many species descriptions are more than a century old, are based only on the worker caste, and have not been revisited in higher-level revisionary study. Surprisingly, one of the most species-rich ant genera, *Nylanderia* (Emery, 1906) – which currently includes 123 extant species and 25 subspecies worldwide (Bolton, 2019) – is severely underdescribed, with an estimate of hundreds more species awaiting description (LaPolla *et al.*, 2011).

Phylogeny, taxonomy and species-level identification have long been characterized by confusion in this genus as a result of widespread morphological convergence and a high number of widely distributed adventive species. As in many genera that are species-rich and hard to identify, obstacles to resolving the systematics and clarifying the nomenclature of this group include:

1. **High species richness and abundance worldwide –** *Nylanderia* is one of the most commonly collected ant genera worldwide and includes hundreds of species (Ward, 2000; LaPolla *et al.*, 2011).
2. **Limited morphological variation –** there are few easily discretizable worker morphological characters between species, and variation within a species often overlaps considerably with that of other, similar species (Trager, 1984).
3. **Globetrotting species –** at least 15 species have been transported across the globe by humans and are easily mistaken for native species (Williams and Lucky, in press).

These challenges have long confounded attempts to clarify the biology and natural history of the genus at the species level. Recognition of the need to manage emerging invasive threats such as the tawny crazy ant, *N. fulva* (Mayr), has added new urgency to the need for establishing a solid taxonomic foundation in this lineage (Gotzek *et al.*, 2012). *Nylanderia fulva* is just one of several taxonomically difficult species that are accidentally transported along human trade routes. Resolving the systematics and clarifying the nomenclature of the genus is necessary so that non-native species can be distinguished from native species and rapidly identified before they can expand their ranges further.

The ant genus Nylanderia

Nylanderia and six closely related genera (collectively called the '*Prenolepis* genus-group') have a tumultuous taxonomic history that has only recently reached some level of stability (reviewed in LaPolla *et al.*, 2010; see also Lapolla *et al.*, 2012). LaPolla *et al.* (2010) found support in multigene sequence data for the monophyly of *Nylanderia*. Additional morphological characters distinguishing *Nylanderia* from other genera were discovered in a global taxonomic revision of the genus *Prenolepis* Say (Williams & LaPolla, 2016), and recent molecular phylogenetic and phylogenomic studies have continued to support the monophyly of *Nylanderia* (Blaimer *et al.*, 2015; Matos-Maraví *et al.*, 2018). Emery (1906) originally described Nylanderia over a century ago and a genus-wide taxonomic revision is needed because taxonomic confusion frequently leads to misidentified or unnamed specimens. Regional revisions have included taxonomic treatment of species in some areas where *Nylanderia* species richness is relatively low, including the Afrotropics (LaPolla *et al.*, 2011), the Nearctic (Kallal, 2012) and the West Indies (LaPolla & Kallal, 2019). Taxonomic revision is still needed for *Nylanderia* in regions where the genus is most diverse: Mesoamerica, South America, Southeast Asia and Australasia. These regions are major centres of *Nylanderia* species diversity, and Southeast Asia likely represents the biogeographical origin of the genus (Matos-Maraví *et al.*, 2018). In order to stabilize the chaotic taxonomy of this genus, nomenclature needs to reflect actual species boundaries and relationships among lineages. For this, a phylogeny with representative sampling from all major lineages worldwide is needed.

Nylanderia is a near-globally distributed genus, but it is not clear whether regional faunas represent monophyletic lineages and it is impossible to understand how geography has shaped them without first knowing the major clades. LaPolla *et al.* (2010) suggested that *Nylanderia* includes five biogeographically distinct clades: (i) Nearctic; (ii) Neotropics; (iii) Afrotropics; (iv) Indomalaya; and (v) Australasia, but this study was focused specifically on genus-level relationships and only sampled *c*. 24 of the estimated hundreds of *Nylanderia* species. The hypothesis that there are five major lineages corresponding to these regions must be tested in order to advance global revisionary study. Without a grasp on the global phylogeny it is difficult to place diagnostic morphological characters in a global context.

Taxonomic revisions and phylogenies over the past decade have brought a measure of much-needed clarity to the systematics of *Nylanderia* at the regional level, but more questions than answers persist about the origins and identities of some of the most widespread globetrotting species in the genus, such as: (i) how many times 'invasiveness' has arisen in *Nylanderia*, and to which major clade or clades these invasive lineages belong; (ii) whether the globetrotting species are monophyletic; (iii) whether species boundaries are reasonably delimited or if some species have multiple names applied to them based on

geographical region; and (iv) whether any of the five globetrotting species that are found in the West Indies are native to the region or if some or all of them arrived via human-mediated dispersal. LaPolla *et al.* (2010) proposed at least three independent origins for 'invasiveness' across *Nylanderia*, represented by: (i) *N. vividula* (Nylander), (ii) *N. fulva*, and (iii) *N. bourbonica* + *N. vaga*. Given that several other globetrotting *Nylanderia* species from across the world have not yet received phylogenetic treatment, additional independent lineages of globetrotting and invasive species across the genus are likely to exist. Among these unsampled species are *N. flavipes* (F. Smith) from temperate Asia (Palaearctic), *N. glabrior* (Forel) from Australasia and *N. jaegerskioeldi* (Mayr) from the Afrotropics. Additionally, six of the most widespread *Nylanderia* species belong to three major species complexes that all exhibit high overlap in intra- and interspecific morphological variation: (i) the *N. fulva* complex (including *N. fulva* and *N. pubens*); (ii) the *N. guatemalensis* complex (including *N. guatemalensis* and *N. steinheili*); and (iii) the *N. bourbonica* complex (including *N. bourbonica* and *N. vaga*). The *N. fulva* and *N. guatemalensis* complexes are native to the Neotropics: *N. fulva* is native specifically to central South America, but the specific native ranges of *N. pubens*, *N. guatemalensis* and *N. steinheili* remain undetermined. *Nylanderia bourbonica* is native to Southeast Asia and *N. vaga* is native to Australasia (Williams and Lucky, in press). With the exception of *N. vaga*, five of these six species all co-occur on islands across the West Indies. The West Indies has been a major centre for human trade and tourism for centuries and is consequently a hub for invasive species spread, with more than 550 non-native species documented across the region (Moses *et al.*, 2003). This region also is known for an especially high rate of endemism, which is particularly significant because globetrotting *Nylanderia* species can be difficult to distinguish from native species and from each other, despite being distantly related (Gotzek *et al.*, 2012; LaPolla & Kallal, 2019). Because characterization of morphology is difficult due to the limits of variation for *Nylanderia* in a global context, molecular data are expected to be particularly useful in resolving global phylogenetic relationships and the identities of both widespread globetrotting species and unknown native species alike, especially in regions where the genus is most species-rich and least characterized. Monophyly of *Nylanderia* and its phylogenetic placement among the *Prenolepis* genus-group genera has been strongly corroborated by Sanger datasets (LaPolla *et al.*, 2010; Lapolla *et al.*, 2012; Matos-Maraví *et al.*, 2018). However, a genus-wide revision informed by a reconstruction of the evolutionary history leading to the global biodiversity observed today will require inclusion of more species, with representative sampling from all major lineages across *Nylanderia*.

Ultraconserved elements (UCEs)

Over the past 5 years, ant systematics has been revolutionized by the development and increased availability of tools designed to capture genomic DNA from ants and other Hymenoptera through targeted enrichment of UCEs (Zhang *et al.*, 2019). The most recent Hymenoptera probe sets can capture up to as many as 2590 UCE loci from each specimen (Branstetter *et al.*, 2017c). The increased accessibility and versatility of UCEs – combined with open collaboration across the ant systematics community – has incited an upswing of studies cutting across multiple timescales between families of Hymenoptera (Blaimer *et al.*, 2015; Faircloth *et al.*, 2015; Branstetter *et al.*, 2017a, 2017c), and within subfamilies and tribes of Formicidae (Branstetter *et al.*, 2017b; Blaimer *et al.*, 2018; Borowiec, 2019). Studies resolving ant phylogeny within genera, species, and even at the population level are among the most recent phylogenomic successes with UCE data (Blaimer *et al.*, 2016; Ješovnik *et al.*, 2017; Pierce *et al.*, 2017; Prebus, 2017; Ward & Branstetter, 2017; Branstetter & Longino, 2019). In addition to defining the major lineages of *Nylanderia*, UCEs should also help to define species boundaries for widespread invasive species that have been challenging to identify throughout their non-native ranges. This is especially important in the context of invasive ant management.

Main objectives

The goal of this study is to characterize the evolutionary history of these ants and at the same time address uncertainties about the identity of several widespread globetrotting species, laying the foundations for a global revision of *Nylanderia* by using molecular genomic data. We present the first global phylogenomic reconstruction of *Nylanderia* based on UCE data and infer the pre-human processes that have led to the global distribution of the genus. We use this phylogeny to: (i) define and characterize the major biogeographical clades within *Nylanderia*; (ii) describe historical biogeography, including the age and origin of the genus and timing of events leading to its spread across the globe; (iii) determine the number of independent lineages of 'invasiveness' in the genus by resolving the origins of nine of the most widespread globetrotting *Nylanderia* species; and (iv) evaluate the phylogenetic relationships among *Nylanderia* species in a region – the West Indies – where morphological uniformity has impeded designation of species boundaries as well as native or non-native species status. Non-native species are frequently mistaken for native species (and vice versa), and we test the utility of UCEs in an area where non-native species are prevalent and can be compared to known local fauna. The West Indies make an excellent test case in this regard because the region has both native and non-native species that are somewhat known, but uncertainties remain about many species' origins and identities. If UCEs can help untangle problems here, then this approach can be applied in other regions – and in other taxa – where taxonomy is challenging and confounded by the spread of globetrotting species.

Materials and methods

Taxon sampling

We selected *Nylanderia* specimens to represent the greatest possible degree of genetic variation among species based on

locality, geographical distance and morphological variation (Table S1). Specimens represent seven major biogeographical regions of the world (from Cox, 2001): Nearctic, Neotropics, Palaearctic, Afrotropics, Malagasy, Indomalaya and Australasia (Table S2). Wallace's line is used as the boundary between Indomalaya and Australasia (Wallace, 1876). Our dataset includes 96 specimens collected across the globe that represent approximately 44 *Nylanderia* species. Of the 96 samples, 40 represent specimens of 17 native and non-native species (of 22 total) collected across the West Indies. We used additional sequence data from four *Nylanderia* species and 12 outgroup species (generated by Blaimer *et al.*, 2015), which were downloaded from the NCBI Sequence Read Archive.

DNA extraction

We extracted DNA from workers and, in one case, a queen using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, U.S.A.). For 23 of the 96 samples, we destructively extracted DNA from single pinned specimens that had multiple worker representatives from the same colony, ranging in collection year from 1988 to 2007. DNA also was extracted from three legs of one additional pinned specimen (with the rest of the body retained as a voucher), collected in 2012. For the remaining 71 samples, we destructively extracted DNA from single specimens preserved in 95% ethanol that had been dried just before extraction. Voucher specimens are deposited in the Smithsonian Institution National Museum of Natural History insect collection (USNM). See Table S1 for collection and locality data of specimens sampled in this study.

UCE sequence data collection

All wet laboratory work was conducted in the Laboratories of Analytical Biology (LAB) at the Smithsonian Institution's National Museum of Natural History (NMNH, Washington, DC, U.S.A.). To capture and enrich up to 2590 UCE loci from ants we followed the protocol from Branstetter *et al.* (2017c). To assess quality, we quantified the extracted DNA in each sample using a high sensitivity kit on a Qubit 2.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). To shear DNA to appropriate size, we fragmented the DNA to an average fragment distribution of 400–600 bp using a Qsonica Q800R sonicator (Qsonica LLC, Newton, CT, U.S.A.). We constructed libraries from the sheared genomic DNA using Kapa Hyper Prep library preparation kits (Kapa Biosystems Inc., Wilmington, ME, U.S.A.), ligated TruSeq adapter barcodes to either end of each fragment, and then amplified the barcoded libraries using PCR. As a final quality control measure following PCR amplification, the DNA concentration of genomic libraries was measured on a Qubit 2.0 fluorometer and visualized via gel electrophoresis.

The post-PCR libraries were purified in a 'speed-bead' clean-up step using a generic SPRI substitute (Rohland & Reich, 2012), and then pooled together in 12 pools of eight libraries each at equimolar concentrations, with final concentrations of 127–170 ng/μL. We followed the MYcroarray MYbaits protocol (Blumenstiel *et al.*, 2010) for target enrichment of the pooled DNA libraries but instead used a 1:4 (baits: water) dilution of the custom ant-specific 'hym-v2-ants" probes (ArborBiosciences, Ann Arbor, MI, U.S.A.) developed by Branstetter *et al.* (2017c). We ran the hybridization reaction of the RNA probes to the sequencing libraries at 65°C for 24 h. All enriched library pools were bound to streptavidin beads (Dynabeads MyOne Streptavidin T1, Life Technologies, Inc., Carlsbad, CA, U.S.A.) and washed.

We quantified and verified enrichment success of each pool using quantitative (q)PCR (CFX96 Touch, Bio-Rad Laboratories, Hercules, CA, U.S.A.), and then combined all 12 pools into a single pool-of-pools, which was size-selected to 300–500 bp using a BluePippin (SageScience, Beverly, MW, U.S.A.). Sample quality was checked using high-sensitivity D1000 tape on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, U.S.A.). The final pool-of-pools was sequenced on an Illumina HiSeq 2500 (125 cycled paired-end, Illumina Inc., San Diego, CA, U.S.A.) at the University of Utah High-Throughput Genomics Center.

UCE data processing and alignment

We used the PHYLUCE v1.6.6 pipeline (Faircloth, 2015) to process the UCE data, using largely default settings except the following: the cleaned reads were assembled using ABySS v2.1.1 (Jackman *et al.*, 2017) with nucleotide sequence length (*k*-mer) set to 35. Alignments were trimmed using a wrapper script of Gblocks (Castresana, 2000) using the following settings: b1 = 0.5, b2 = 0.5, b3 = 12, b4 = 7. For our final datasets we selected a 75% complete data matrix retaining 787 loci (File S1) and a 90% complete matrix retaining 45 loci (File S2). The summary statistics for the matrices were calculated using the Alignment Manipulations and Summary (AMAS) script written by Borowiec (2016) and can be found in Table 2. Trimmed reads for all generated sequences in this study are available from the National Center for Biotechnology Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra; PRJNA553590).

The mean DNA concentration of our samples following extraction was 0.818 ng/μL (0.077–6.51 ng/μL), and 25.734 ng/μL (2.27–65.8 ng/μL) for post-PCR libraries. The sample with the lowest concentration was the specimen from which DNA was extracted from only three legs of a worker (NylaM106), and the sample with the highest concentration was the one from which DNA was extracted from a queen (NylaM63). The mean number of raw reads was 4 083 359 (89921–22 819 138). See Table 1 for a summary of UCE processing and sequencing statistics (a full summary of statistics for each specimen can be found in Table S3).

Phylogenomic analyses

We used three different partitioning strategies to reconstruct phylogeny under the maximum-likelihood (ML) criterion in

Table 1. Ultraconserved element sequencing statistics

	Extract conc. (ng/μL)	Post-PCR conc. (ng/μL)	Raw reads	Contigs	Total bp	Mean length	Min length	Max length	Median length
Mean	0.818	25.734	493 801 992	1635.8	769 094	456.056	101.321	1457.063	447.955
Min	0.077	2.27	10 926 359	468	122 547	194.684	101	490	185
Max	6.51	65.8	2 704 291 405	2330	1 827 384	809.921	106	3032	872
SD	0.692	15.792	526 781 341	573.4	379 024	141.439	0.851	554.063	144.769
95% CI	0.138	3.159	105 376 279	114.7	75 819.2	28.293	0.17	110.834	28.959

CI, confidence interval; SD, standard deviation.

Table 2. Alignment Manipulations and Summary (AMAS) statistics for alignment matrices

Alignment	Length	Total cells	Missing %	Proportion variable	Parsimony-informative
75% internal	316 557	35 454 384	32.708	0.256	0.136
90% internal	17 838	1 997 856	24.17	0.28	0.142

IQ-TREE v1.69 (Nguyen *et al.*, 2015): (A1) single concatenated alignment, (A2) one partitioned per locus ($n = 787$), and (A3) using a partitioning scheme determined in PartitionFinder2 ($n = 446$; Lanfear *et al.*, 2017). For analyses A1 and A2 the best models of nucleotide substitution were determined with the Bayesian Information criterion (BIC) in IQ-TREE, whereas the number of partitions in A3 was determined using the Sliding-Window Site Characteristics (SWSC) based on site entropy (Tagliacollo & Lanfear, 2018). To assess nodal support, we performed a Shimodaira–Hasegawa approximate likelihood-rate test (SH-aLRT; Guindon *et al.*, 2010) with 1000 replicates using the '-alrt' command in IQ-TREE. Additionally, 1000 ultrafast bootstrap replicates (UFBoot; Hoang *et al.*, 2017) were conducted using '-bb', along with '-bnni' to reduce risk of overestimating branch supports. Only nodes with support values of SH-aLRT ≥0.90 and UFBoot ≥0.95 were considered robust.

We also inferred species trees under the multi-species coalescent (MSC) model in ASTRAL-III v5.6.2 (Zhang *et al.*, 2018). Gene trees of each locus were estimated in IQ-TREE for all 787 loci. The individual loci were not partitioned, and the best models of nucleotide substitution were selected using BIC in IQ-TREE using all available models. Support was assessed by annotating the MSC tree with local posterior probabilities (Sayyari & Mirarab, 2016), with ≥0.95 considered as strong support.

Divergence date estimation

We inferred divergence dates within *Nylanderia* using the 90% complete reduced dataset and 46 taxa, with a single sample selected for each species to reduce computational time in BEAST2 (Bouckaert *et al.*, 2014). Topological constraints based on strongly supported relationships recovered in both the ML and MSC analyses were applied as priors to further reduce computational burden. The analyses were run under the unpartitioned HKY substitution model, uncorrelated molecular clock with branch lengths drawn from a lognormal distribution (relaxed clock lognormal; starting value for clock rate = 1e-9), and with a birth–death tree prior. Lognormal calibration priors were assigned using two *Nylanderia* fossil species (reviewed in LaPolla & Dlussky, 2010) and their median ages based on calibrations performed by Matos-Maraví *et al.* (2018): (i) *N. vetula* LaPolla and Dlussky (17.1 Ma) and (ii) *N. pygmaea* (Mayr) (38.9 Ma). However, unlike Matos-Maraví *et al.* (2018), we assigned *N. vetula* (from Dominican amber) to a clade of species from the Dominican Republic (DR Clade I) rather than to the Nearctic clade based on strong morphological similarity with the newly described *N. fuscaspecula* LaPolla & Kallal (2019). An 'empty' analysis without data (sampling only from the marginal prior) was conducted to ensure the informativeness of the priors (reviewed in Bromham *et al.*, 2018). We ran two independent MCMC chains for over 250 million generations, and convergence was determined in Tracer v1.7 (Rambaut *et al.*, 2018) when the combined effective sample size (ESS) for all parameters was >200. At least 10% of the burn-in was discarded, and the trees were summarized as maximum clade credibility (MCC) trees using LogCombiner v2.4.8 and TreeAnnotator v2.4.8.

Biogeographical analysis

We reconstructed the global biogeographical history of *Nylanderia* using the R package BioGeoBEARS (Matzke, 2013) with the birth–death tree from the divergence dating analysis as the input. We coded seven major biogeographical regions (Cox, 2001) for the analysis: Nearctic (N), Neotropical (T), Palaearctic (P), Afrotropical (E), Malagasy (M), Indomalaya (O) and Australasia (U). Wallace's line was used as the boundary between Indomalaya and Australasia (Wallace, 1876). Because we wanted to infer the deep-level historical biogeographical patterns of species across *Nylanderia* and avoid confounding this by the presence of non-native species,

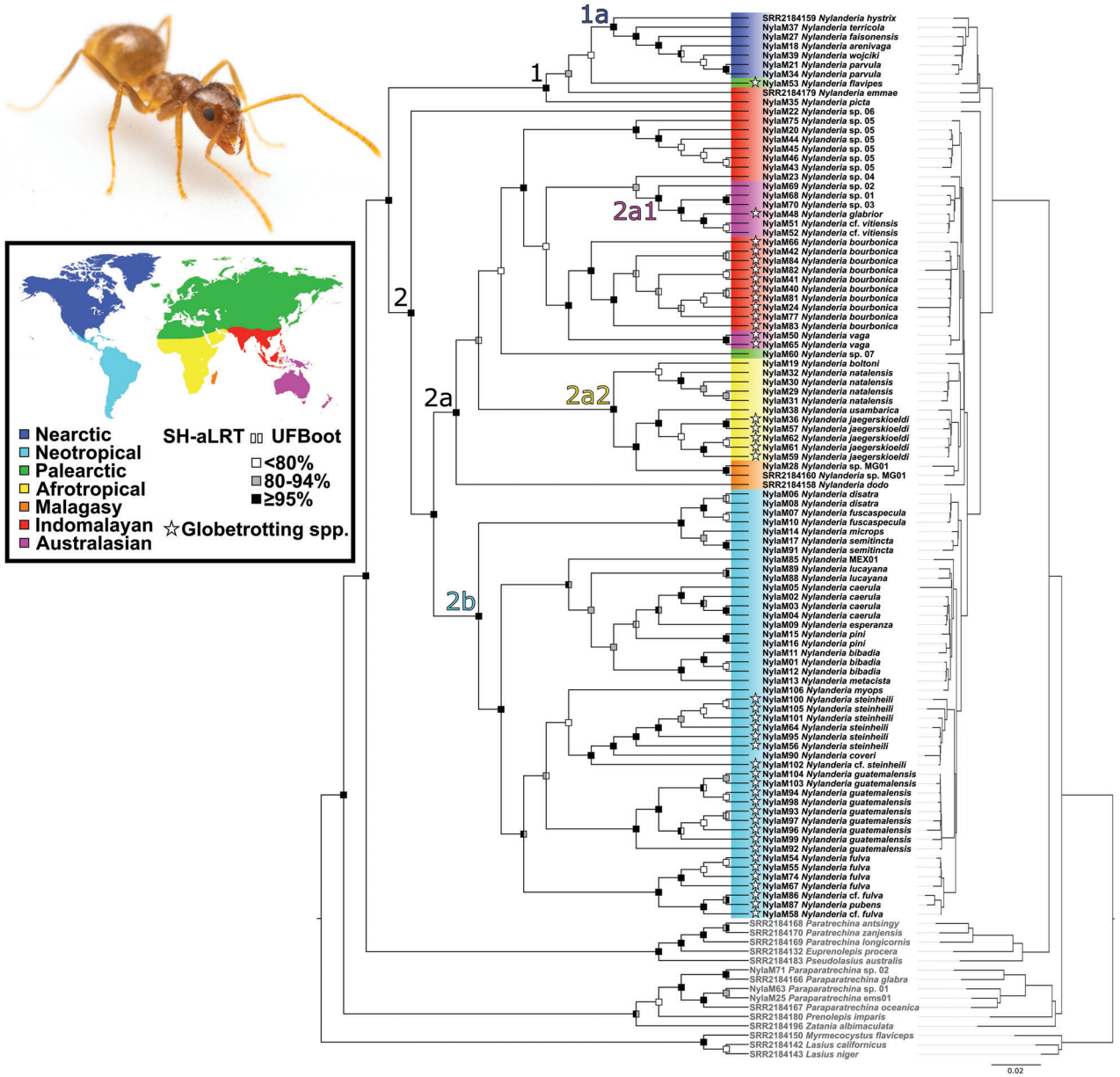

Fig. 1. Maximum-likelihood phylogeny of *Nylanderia* generated in IQ-TREE from the 75% complete SWSC partitioned UCE matrix, with cladogram depicted on the left and phylogram depicted on the right. Biogeography of terminal taxa is based on inferred native ranges and may not indicate collecting locality for known non-native species (denoted with a star). Node support is provided in SH-aLRT (Shimodaira-Hasegawa approximate likelihood ratio test) and UFBoot (ultrafast bootstrap) values. For both support values, <80% is considered weak support and ≥95% is considered strong support. Photo by Y. M. Zhang.

we assigned all taxa to their purported native biogeographical regions and not necessarily to their collection localities. See Table S2 for the biogeography data matrix we used as an input for BioGeoBEARS. We implemented both the two-parameter dispersal and extinction cladogenesis (DEC) model (Ree & Smith, 2008) and a modified version of that model (DEC + J) including a 'j' parameter that allows for 'jump dispersal' or founder-event speciation (Matzke, 2014). For each analysis we set Max_range_size = 7 and jstart = 0.0001.

Results

Phylogenetic reconstruction

The ML (Fig. 1) and MSC (Fig. 2) analyses strongly supported the monophyly of *Nylanderia* and a sister relationship of the genus to *Pseudolasius* + (*Paratrechina* + *Euprenolepis*). Two major clades within *Nylanderia* were strongly supported: (i) an Indomalayan–Holarctic clade (Fig. 1, Clade 1) consisting of a

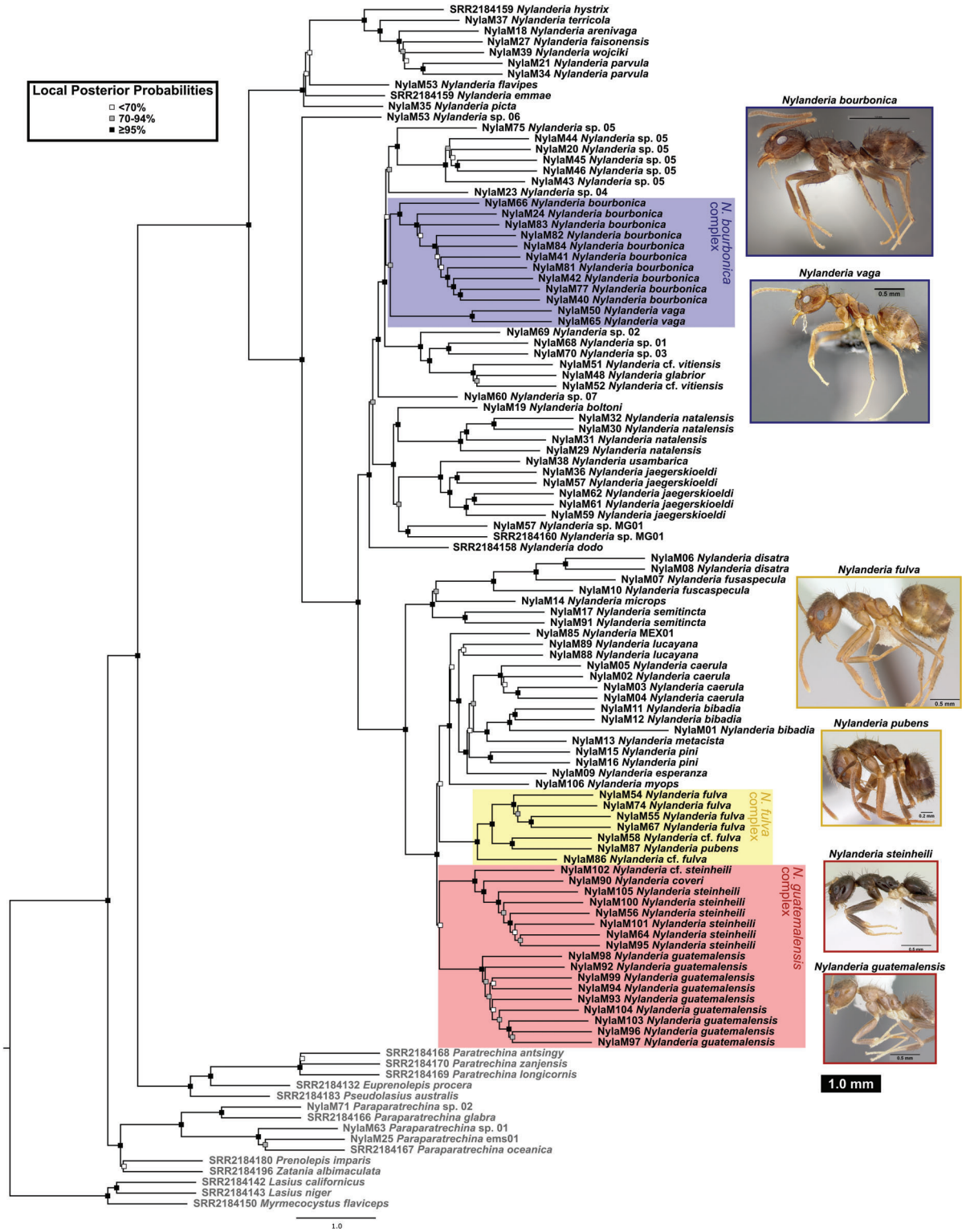

Fig. 2. Species tree analysis of *Nylanderia* generated in ASTRAL-III from the 75% complete UCE matrix. Node support is provided as local posterior probabilities. Values <70% are considered weak support, and those ≥95% are considered strong support. Images of the following specimens are to scale and were downloaded from www.AntWeb.org: FMNHINS0000062866 (*N. bourbonica*; photo by Gracen Brilmyer), CASENT0171069 (*N. vaga*; photo by Eli M. Sarnat), CASENT0173491 (*N. fulva*; photo by April Nobile), CASENT0104862 (*N. pubens*; photo by April Nobile), CASENT0104221 (*N. guatemalensis*; photo by April Nobile) and CASENT0173233 (*N. steinheili*; photo by April Nobile).

polytomy of Indomalayan and Palaearctic species and a Nearctic clade (Fig. 1, Clade 1a); and (ii) a clade including all other taxa (Fig. 1, Clade 2), consisting of an isolated Indomalayan taxon, an Eastern Hemisphere clade (Fig. 1, Clade 2a), and a Neotropical clade (Fig. 1, Clade 2b). An Australasian clade (Fig. 1, Clade 2a1) and an Afrotropical + Malagasy clade (Fig. 1, Clade 2a2) were both strongly supported within the Eastern Hemisphere clade. *Nylanderia dodo* (Donisthorpe), a species endemic to Madagascar, did not group with other species from Madagascar. *Nylanderia vaga* from Australasia did not group with other species from the Australasian clade and was resolved as sister to *N. bourbonica*, which has historically been considered a Southeast Asian species (Williams and Lucky, in press).

Biogeographical analysis and divergence dating

The DEC and DEC + J models from the biogeographical analysis had log-likelihood scores of −61.73 and − 47.84, respectively (see Fig. S2 for comparison), meaning that DEC + J was the highest scoring model (Fig. 3). The Indomalayan realm was determined to be the biogeographical origin of *Nylanderia* under the DEC + J model. This model supported two independent dispersals to the Americas; one to the Nearctic and one to the Neotropics. The independent arrival of two separate lineages to Madagascar also was supported by DEC + J, whereas DEC instead supported one arrival to Madagascar and subsequent dispersal to mainland Africa. Although the DEC + J model supported the independent arrival of *N. vaga* to Australasia from other Australasian species, the DEC model provided support for a single arrival to the region (albeit with low certainty). See Fig. S1 for the resulting tree from the dating analysis, and Table 3 for divergence dates of major biogeographical events and globetrotting species groups.

Globetrotting and invasive species relationships

The nine globetrotting species that we sampled belong to five independent lineages (Figs 1 and 2), represented by: (i) *N. flavipes*; (ii) *N. glabrior*; (iii) *N. bourbonica* + *N. vaga*; (iv) *N. jaegerskioeldi*; and (v) *N. guatemalensis* + *N. steinheili* + *N. fulva* + *N. pubens*. *Nylanderia flavipes*, which is a temperate woodland species from East Asia (i.e. Palaearctic origin), was recovered as sister to the Nearctic clade, albeit with weak support. However, *N. flavipes* was strongly supported as being more closely related to Nearctic species than to other Palaearctic or Indomalayan species. *Nylanderia jaegerskioeldi*, a widespread Afrotropical species, was recovered as monophyletic and as most closely related to other Afrotropical species with strong support. *Nylanderia bourbonica* (native to Southeast Asia) and *N. vaga* (native to Australasia) were recovered with strong support as reciprocally monophyletic sister species. *Nylanderia glabrior* (Forel) was recovered as belonging to a group of Australasian species distinct from the lineage that includes *N. vaga* and *N. bourbonica*, resolving as most closely related to *N. vitiensis* (Mann). Two closely related clades of globetrotting Neotropical species also were recovered: one including *N. fulva* and *N. pubens* (the *N. fulva* complex), and one including *N. guatemalensis* and *N. steinheili* (the *N. guatemalensis* complex). The *N. fulva* complex was strongly supported as a monophyletic group and relationships within it differed slightly between the ML and MSC analyses, but the latter overall provided much higher support, with *N. fulva* specimens from reported non-native populations across the southeastern United States recovered as monophyletic. The monophyly of the *N. guatemalensis* complex was weakly supported in all analyses, with the integrity of two distinct clades within the complex strongly supported. Both of these clades included specimens historically identified as *N. guatemalensis* and *N. steinheili*. The placement of *N. myops* (Mann) within this complex was particularly contentious; in the ML analysis it was resolved with weak support as being most closely related to *N. steinheili* and *N. coveri* LaPolla and Kallal within the *N. guatemalensis* complex, whereas in the MSC analysis it was resolved with strong support outside this clade.

West Indian species relationships

Two major lineages of globetrotting species found in the West Indies included these five species: (i) *N. bourbonica*; (ii) *N. fulva*; (iii) *N. pubens*; (iv) *N. guatemalensis*; and (v) *N. steinheili* (Fig. 4). *Nylanderia bourbonica* was recovered with strong support as most closely related to other Indomalayan and Australasian species, whereas the other four species were recovered with strong support in the Neotropical clade as being more closely related to species from Mexico, Cuba, The Bahamas and a Dominican Republic clade (DR Clade II; Fig. 4). The ML analysis recovered the *N. fulva* complex and the *N. guatemalensis* complex as sisters with moderate to strong support (Fig. 1), and the MSC analysis recovered the *N. fulva* complex as being more closely related to West Indian species than to the *N. guatemalensis* complex, albeit with weak support (Fig. 2). All five globetrotting species in the West Indies were collected in areas with low elevation (<400 m above sea level) and human habitation across Florida and the West Indies, whereas most native species (excepting *N. coveri*, *N. lucayana* LaPolla and Kallal, and *N. esperanza* LaPolla and Kallal) were collected in areas with moderate to high elevations (≥900 m a.s.l.; Fig. 4).

Discussion

Phylogeny

We present the first extensive global phylogeny of *Nylanderia*, which supports the monophyly and phylogenetic placement of *Nylanderia* within the *Prenolepis* genus-group, corroborating previous molecular phylogenetic studies (LaPolla *et al.*, 2010; Blaimer *et al.*, 2015; Matos-Maraví *et al.*, 2018). In most cases, clades are associated with major biogeographical regions,

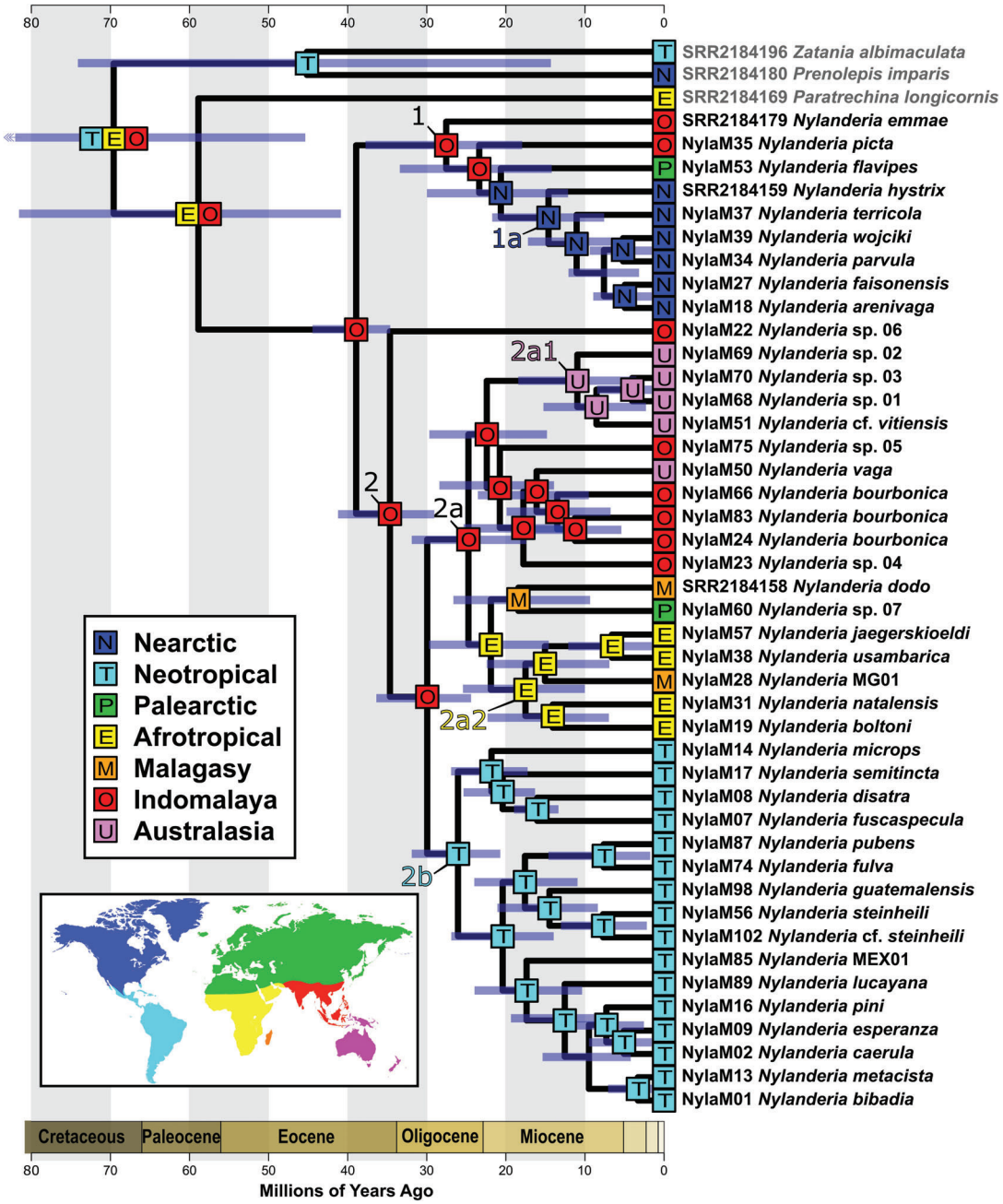

Fig. 3. Chronogram constructed from BEAST2 and BioGeoBEARS (DEC + J) analyses. Blue bars on nodes depict 95% HPD (high posterior density) values.

including resolution of four major biogeographical clades in the Nearctic, Neotropics, Afrotropics + Malagasy, and Australasia, which are all nested within an ancestral Indomalayan group. The Palaearctic species are nonmonophyletic and distantly related, with one lineage more closely related to the Nearctic clade and another more closely related to Indomalayan and Australasian species.

LaPolla *et al.* (2010) recovered similar clades, but their sampling scheme was unable to determine nestedness within a larger Indomalayan group. However, the phylogeny by Matos-Maraví *et al.* (2018) was able to resolve this nested relationship, in addition to the finding that there are at least three independent clades of Indo-Pacific (i.e. Indomalayan + Australasian) *Nylanderia*. Likewise, they also found that the *Nylanderia* native to Madagascar are nonmonophyletic, with *N. dodo* a distant relative to other species from the region. Our results highlight the independent arrival of *N. dodo* to Madagascar from the lineage which includes other Afrotropical and Malagasy

Table 3. Divergence dates for major biogeographical events and globetrotting species groups. Biogeographical clades correspond to those labeled in Figs 1 and 3

Biogeography	Mean (Ma)	95% HPD (Ma)
Nylanderia (Indomalaya)	39.6	34.8–44.6
Nearctic (Clade 1a)	14.9	7.8–21.9
Australasia (Clade 2a1)	11.2	3.7–18.6
Afrotropical + Malagasy (Clade 2a2)	22.3	14.8–29.9
Neotropical (Clade 2b)	26.5	20.9–32.0
Species/species groups		
N. bourbonica complex	16.4	9.7–23.7
N. fulva + *N. guatemalensis* complex	17.9	11.2–24.2
N. fulva complex	7.8	2.0–14.8
N. guatemalensis complex	14.8	8.6–21.3

HPD, high posterior density.

species. *Nylanderia dodo* has particularly unusual morphology for *Nylanderia* and strongly resembles *Pseudolasius* Emery (1887) species. In fact, it was originally described in *Pseudolasius*, but later moved to *Acropyga*, and then finally to *Nylanderia* based on molecular evidence and probable absence of a major worker caste, for which *Pseudolasius* are typically known (LaPolla, 2002; LaPolla *et al.*, 2010). Unlike all other known *Nylanderia* species, *N. dodo* has a reduced palpal formula of 5:3 (maxillary:labial) compared to the typical 6:4 observed in all other *Nylanderia* species. Queens of *N. dodo* also have reduced, vestigial wings, a trait perhaps associated with insular living. No other *Nylanderia* species, including many others endemic to islands, are known to have brachypterous alates. Queens of the socially parasitic species *N. deceptrix* Messer et al. are not truly brachypterous, but they do have smaller wings than those typical for other *Nylanderia* species (Messer *et al.*, 2016).

Divergence dating and historical biogeography

We determine the origin of *Nylanderia* to be Southeast Asia, with crown age estimates placing it as a post-Eocene Optimum radiation. This is older than estimates by Blaimer *et al.* (2015) and Matos-Maraví *et al.* (2018), whose analyses suggested ages of *c*. 25 Ma and 29 Ma, respectively, making their estimates much younger than ours and of the estimated age of the Baltic amber fossil species *N. pygmaea* (38.9 Ma). Matos-Maraví *et al.* (2018) also proposed an Oriental and Palaearctic (including continental Southeast Asia and Malay Peninsula) origin for crown group *Nylanderia*, which is consistent with our determination of an Indomalayan origin.

The Nearctic clade and the Neotropical clade are distantly related, indicating that the Americas were colonized in two separate radiations from Southeast Asia: the first arriving in the Neotropics and the second in the Nearctic. Our estimates place the Nearctic crown clade in the mid-Miocene and the Neotropical crown clade in the late Oligocene to early Miocene. These two clades may have dispersed to the Americas from East Asia through the Beringian land bridge, which has been a frequent route for the interchange of terrestrial organisms over geological time (Marincovich & Glenkov, 1999). The close relationship between the Palaearctic species *N. flavipes* (native to temperate East Asia) and the Nearctic clade, and the observation that *Nylanderia* also is relatively depauperate across the western Palaearctic (West Asia and Europe) additionally support dispersal across Beringia. Given that the Neotropical clade is older and appears to have most recently descended from an Indomalayan lineage, this raises the question as to why no trace of this lineage has been found in the Nearctic. One hypothesis for this observation is that the first radiation of *Nylanderia* to the Americas went extinct in the Nearctic, but only after members of this lineage reached the Neotropics. However, a simpler explanation is that perhaps our sampling of the Nearctic species was not broad enough to capture a North American relative that is most closely related to the Neotropical clade – a possibility that could be addressed with expanded sampling. These two American lineages also are supported by differences in morphology. Nearctic species have a glossy cuticle overall and little to no pubescence, whereas Neotropical species have relatively dense pubescence across the cuticle (Kallal, 2012).

We present the DEC + J model (Fig. 3) and not the DEC model, but a comparison of the two can be seen in Fig. S2. Although the DEC + J model scored higher than the DEC model, caution must be taken in interpretation of these results. The trend in biogeographical analysis has been to choose the DEC + J model over the DEC model because it accounts for founder-event speciation and often has a higher log-likelihood. However, the DEC + J model has recently received criticism as a poor representative of founder-event speciation and there are potential flaws in using statistical comparisons of likelihood to choose one over the other. Also, neither DEC nor DEC + J account for time when modeling ancestral node range inheritance (Ree & Sanmartín, 2018).

Globetrotting and invasive species

By including representatives from nine of the most widespread human-transported species we also clarify globetrotting and invasive species relationships, test their monophyly, and enumerate the total independent lineages in which they are included. LaPolla *et al.* (2010) proposed at least three separate origins of 'invasiveness' within *Nylanderia*, represented by *N. fulva/pubens*, *N. vividula* and *N. bourbonica/vaga* (alongside a fourth one outside *Nylanderia*, represented by *Paratrechina longicornis*). Our phylogeny includes at least five distinct lineages of globetrotting species, represented by *N. flavipes*, *N. glabrior*, *N. bourbonica/vaga*, *N. jaegerskioeldi*, and *N. fulva/pubens* + *N. guatemalensis/steinheili*. However, although our ML analysis (Fig. 1) provides moderate to strong support for the sister relationship between the *N. fulva* and *N. guatemalensis* complexes, our MSC analysis (Fig. 2) does not resolve this relationship and provides only weak support for the sister placement of the *N. fulva* complex with a clade of other Neotropical species. Although our sampling does not include *N. vividula*, its sister species *N. terricola* (Buckley) (LaPolla *et al.*, 2010) is represented in our phylogeny and would place *N. vividula* firmly in the Nearctic clade as an additional

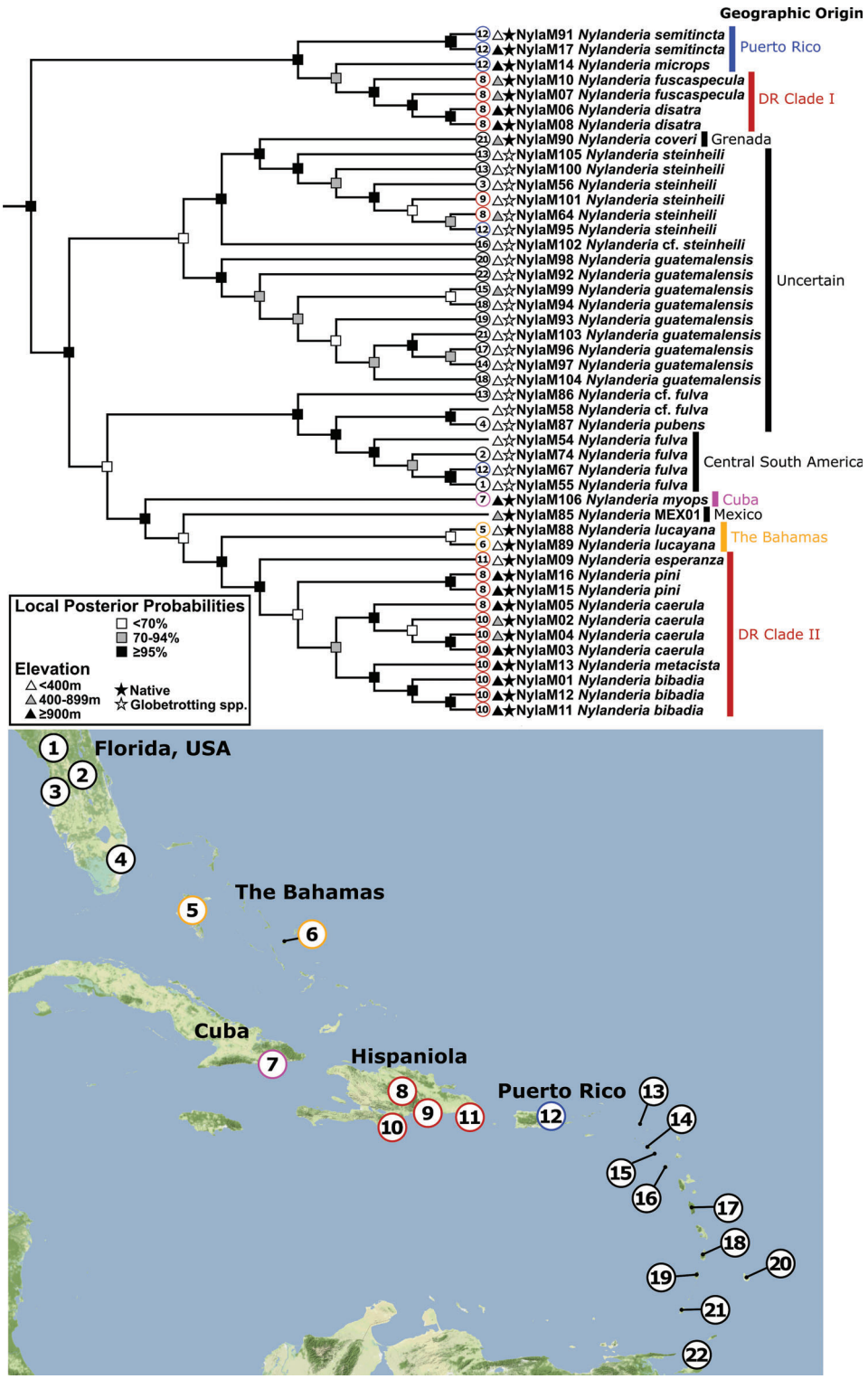

Fig. 4. Species tree analysis of Neotropical *Nylanderia* species constructed using ASTRAL-III, with numbers in circles corresponding to specimen collection localities on the map of the West Indies: yellow, Bahamas; pink, Cuba; red, Hispaniola; blue, Puerto Rico; black, from elsewhere within the region. Triangles correspond to elevation of collecting locality: white, <400 m a.s.l.; grey, 400–899 m a.s.l.; black, ≥900 m a.s.l. White stars, species with non-native records; black stars, native species. Node support is provided as local posterior probabilities. Values <70% are considered weak support, and those ≥95% are considered strong support.

distinct lineage of globetrotting species. Still at least three other adventive species, *N. amia* (Forel), *N. clandestina* (Mayr) and *N. tasmaniensis* (Forel), remain unsampled and with undetermined origins and each may represent additional independent globetrotting lineages (Williams and Lucky, in press).

Both the ML (Fig. 1) and MSC (Fig. 2) analyses support *N. bourbonica* and *N. vaga* as reciprocally monophyletic species, but more global sampling of this complex is needed, especially of individuals that best fit the description of *N. vaga*. Estimates for the most recent common ancestor of the *N. bourbonica* complex place it roughly in the mid-Miocene. In our biogeographical analysis (Fig. 3), we coded the Indomalayan realm as the native range of *N. bourbonica* as established in the literature (Williams and Lucky, in press). However, the true native range of *N. bourbonica* is still poorly understood as this species has been widespread for hundreds of years and its morphological and geographical species boundaries remain unclear. *Nylanderia bourbonica* may be native across Southeast Asia and Australasia, and *N. vaga* is considered native to some parts of Australasia (mainland Australia and nearby islands, including Papua New Guinea and parts of Indonesia) and non-native to others. The apparent sister relationship between *N. bourbonica* and *N. vaga* may lend support to the hypothesis that *N. bourbonica* – or at least one or more unnamed species in the complex – is native to Australasia. Increased sampling of populations across this complex is necessary to establish the actual native ranges of species within this complex, however many there may be. Relatively deep splits in the *N. bourbonica* complex suggest that some specimens of what are currently designated as *N. bourbonica* could represent undescribed taxa, the species boundaries for which remain unexplored. Re-delimitation of species boundaries in this complex may reveal more globetrotting species that are currently undescribed. Additionally, *N. bourbonica* is considered the most widespread *Nylanderia* species and the breadth of its geographical range may be exaggerated in part due to poorly understood species limits.

Sister placement of the *N. guatemalensis* complex with the *N. fulva* complex is moderately to strongly supported in the ML analysis (Fig. 1) and is contradicted (with weak support) in the MSC analysis (Fig. 3). Monophyly of the *N. guatemalensis* complex is weakly supported in both analyses. The ML analysis (Fig. 1) shows two distinct clades within the *N. fulva* complex: one composed of samples identified as *N. fulva* based on diagnostic behavioural and male genitalic characters, and the other including one sample identified as *N. pubens* in addition to two tentatively labelled *N*. cf. *fulva* because there are no male specimens for verification. However, the MSC analysis (Fig. 2) shows a different topology, with one *N*. cf. *fulva* sister to the rest of the group. Full resolution of these groups will require much broader range sampling and should ideally include representation of male specimens as sample vouchers for comparison of genitalic characters.

Poor delimitation persists in the *N. guatemalensis* and *N. fulva* complexes, and both are likely to include undescribed species. Workers of the recently described *N. coveri*, which may be endemic to Grenada, are nearly indistinguishable from *N. guatemalensis* (LaPolla & Kallal, 2019) and this species is most closely related to *N. steinheili* (Figs 1 and 2). Distinct male genitalic characters have supported the description of this new species. Scrutinous observation of male genitalia in light of phylogenomic results may be the key to clarifying *Nylanderia* species limits, especially in species complexes that exhibit subtle variation in worker morphology.

Nylanderia of the West Indies

The West Indies provide an exemplary test case for comparing relationships between *Nylanderia* species in a region with strong representation from native and globetrotting species alike. The ages of terrestrial biota found in the West Indies are salient for testing hypotheses of Caribbean biogeography; a particularly contentious subject because the region has been shaped by a variety of geological and meteorological events since the Cretaceous (Pindell, 1994). These events may implicate dispersal, vicariance, or both, as contributing mechanisms to West Indian biodiversity, depending upon the age and origin of the taxonomic group of interest. Several *Nylanderia* species, including *N. fulva* and *N. bourbonica*, are well-adapted to frequent disturbance and marginal habitats (Matos-Maraví *et al.*, 2018; Williams and Lucky, in press), which could explain the combined high diversity of native West Indian species and prevalence of globetrotting species. Although *N. fulva* is native to central South America and subsequently arrived in northern South America, the West Indies and the southeastern United States, it remains uncertain as to whether *N. pubens*, *N. guatemalensis* and *N. steinheili* are native to the West Indies or were transported there from South America by human trade.

The Dominican Republic is home to two relatively distantly related clades of native (possibly endemic) species. One of these clades is most closely related to two Puerto Rican species, *N. microps* (M. Smith) and *N. semitincta* LaPolla and Kallal (Fig. 4). This Dominican clade (DR Clade I; Fig. 4) includes two recently described species: *N. fuscaspecula* LaPolla and Kallal and *N. disatra* LaPolla and Kallal (LaPolla & Kallal, 2019). The other Dominican Republic clade (DR Clade II), which includes *N. esperanza*, is most closely related to *N. lucayana* from The Bahamas. *Nylanderia coveri* from Grenada was recovered with strong support within the *N. guatemalensis* complex. *Nylanderia esperanza*, *N. lucayana* and *N. coveri* are morphologically similar to and easily confused with the much more widespread and possibly non-native *N. guatemalensis* (LaPolla & Kallal, 2019). All species with non-native records that are closely associated with disturbed or coastal habitats or that are known to spread via human-mediated dispersal are most often found at elevations near sea level. Almost all native and endemic West Indian species are found at moderate to high elevations. *Nylanderia esperanza*, *N. lucayana* and *N. coveri* are the only three exceptions, which is cause for concern because pocket populations of these three native species could easily be misdiagnosed as *N. guatemalensis*. If their identities are mistaken, native and endemic species to the region could become the unfortunate targets of well-meaning, but ill-informed, pest management practices.

Nylanderia fulva, which is native to central South America, is nested well within the West Indian species. However, phylogenetic placement of the South and Central American species relative to West Indian species will require more comprehensive taxon sampling from the former two regions. The biogeographical origins of *N. pubens* and of the *N. guatemalensis* complex also will require greater breadth in Neotropical taxon sampling to resolve. Biogeographical reconstruction at the scale of the West Indian islands will require more comprehensive sampling of Mesoamerican and South American *Nylanderia* to fully capture the extent to which lineages have traveled to and from the mainland since *Nylanderia* arrived in the Neotropics.

Taxonomic preparedness

Ants have been accidental travellers along human trade routes since the outset of globalization in the 1400s. Since then, intercontinental introductions have coincided with major events in recent human history, especially increases in global trade (Gotzek *et al.*, 2015; Bertelsmeier *et al.*, 2017). More than 240 ant species from around the world have been transported outside their native ranges (Bertelsmeier *et al.*, 2017), with more than 15 *Nylanderia* species among them (Williams and Lucky, in press). These species have spread unchecked in part because management efforts are historically reactive and species-level identification can be difficult during early stages of invasion, particularly when many species in the same genus are undescribed, and non-native and native species bear strong resemblance to one another (Krushelnycky *et al.*, 2005; Gotzek *et al.*, 2012). Proactive management relies on taxonomic clarity for fast and reliable identification and to link species names to information such as diagnostic morphological characters, biology, natural history and distribution. Early recognition of problematic species at ports of entry can reduce the frequency of introduction events and ensuing propagule pressure (Suarez *et al.*, 2005; Suarez & Tsutsui, 2008), but taxonomic tools – and especially a reliable species name and diagnosis – must first be made available to facilitate rapid identification.

One example of the taxonomic impediment interfering with invasive species prevention, management and control is the arrival of the tawny crazy ant (*N. fulva*) to Houston, Texas in 2002. This invasive pest from central South America was initially identified as *N. pubens* (or '*N*. sp. nr. *pubens*'); a misidentification that persisted during the following decade as it spread across the southeastern United States (Gotzek *et al.*, 2012). Only after this species was properly identified, ten years after introduction, was it determined to be a new invasive species that needed to be managed differently from the less problematic *N. pubens*. This example echoes historic misidentifications of other invasive ants such as the red imported fire ant, *Solenopsis invicta* Buren (Wetterer, 2013), and the tropical fire ant, *Solenopsis geminata* (Fabricius) (Wetterer, 2011). These histories underscore the importance of taxonomic preparedness as the foundation of prevention and control because they highlight a need for development of practical identification tools that permit a proactive approach to invasive species monitoring. Widespread species in *Nylanderia*, especially the *N. fulva*, *N. guatemalensis* and *N. bourbonica* species groups that are prevalent across the West Indies, need to be resolved so that these species can be quickly and accurately detected at ports of entry.

Our results cast some light on evolutionary relationships within these complexes but, as they do not include full range sampling, they are insufficient on their own for species-level delimitation. Increased population-level sampling and molecular study alongside rigorous, morphologically driven revisionary taxonomy of the Neotropical, Indomalayan, and Australasian faunas are all imperative to resolve species boundaries in these especially difficult complexes. Nomenclatural revision and the discovery of useful identifying characters for these problematic taxa will help overcome the taxonomic impediment and encourage preparedness for the advancing spread of adventive *Nylanderia* species along global trade routes.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. Partitioning scheme for the 75% complete data matrix.

File S2. Partitioning scheme for the 90% complete data matrix.

Fig. S1. Birth-death tree of extant taxa, with median ages and bars representing 95% HPD on the nodes. Scale bar at bottom of figure represents Ma.

Fig. S2. Comparison of DEC and DEC + J models of *Nylanderia* biogeographical history.

Table S1. Collection and locality data for specimens sampled in this study.

Table S2. Biogeography data matrix used as input for biogeographical analysis with BioGeoBEARS.

Table S3. Summary of UCE data collected in this study.

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