Title: Relatedness within colonies of three North American species of carpenter ants (Subgenus: *Camponotus*) and a comparison with relatedness estimates across Formicinae

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Running Head: Genomic relatedness in three *Camponotus* species

ABSTRACT

Understanding a haplodiploid species' social structure and quantifying relatedness among individuals are both important when designing sampling schemes or identifying potential biases in population genetics studies. However, it is not always possible to accurately identify social structure of study species in the field, or to collect large numbers of individuals from a single colony to estimate relatedness with methods that rely on accurate estimation of allele frequencies. Here, we assessed the utility of allele frequency-free inference of relationships in haplodiploid ant colonies, while using limited sample sizes. Using genome-wide single nucleotide polymorphism data, we measured intracolony relatedness and kinship estimates consistent with full sister relationships among workers in three Nearctic species: *Camponotus herculeanus*, *C. laevissimus*, and *C. modoc*. Notably, the allele frequency-free inference of relationships clearly demonstrated these full sister relationships without ambiguity; this result suggests the utility of these methods for identifying closely related individuals in population genetics studies of haplodiploid organisms. We additionally performed a literature review of relatedness estimates in the subfamily Formicinae both as a compiled resource and to place our results in context within this larger clade of ants. Our results suggestive of *Camponotus* colonies founded by a lone singly mated queen are consistent with previously published relatedness estimates in the genus *Camponotus* that have generally shown high intra-colony relatedness.

INTRODUCTION

The reproductive strategies of ants are varied; colonies may have one or multiple reproductive females (monogyny and polygyny, respectively), and these females may have mated with one (monandry) or multiple partners (polyandry) (Boomsma 2007, 2009). Understanding social structure is particularly important when designing sampling schemes or identifying potential biases in population genetics studies (Rosenberg and Nordborg 2006). When species' specific social organization is not available in the literature or easily assessed by excavating colonies, using genetic methods to estimate relatedness and/or kinship among individuals in or between colonies may provide insight about a given colony's or species' social structure.

However, a relatively small proportion of ant species have estimates of genetic relatedness in the literature; in particular, the ant subfamily Formicinae is diverse, with 51 extant genera and 3030 described species (Ward, et al. 2016), but only a small fraction of genera and species within this complex have estimates of genetic relatedness in the literature (Appendix 1). As such, researchers that want to study population genetics, adaptation, or speciation in understudied eusocial species may desire estimates of kinship and/or relatedness of their samples. Because it is not always possible or feasible to extract large colonies to count queens or obtain large sample sizes per colony (e.g., when utilizing museum collections), methods able to identify whether samples are closely related while using small sample sizes are necessary.

Most methods for estimating relatedness have utilized large sample sizes with various genotyping information among individuals (Queller and Goodnight 1989), including genotypes measured from variation in allozymes, microsatellites, and single-nucleotide polymorphisms (SNPs). However, most methods for relatedness estimation require the availability of or ability to infer accurate population allele frequencies (Waples, et al. 2019). Recently, Waples and colleagues (2019) demonstrated that three allele-frequency free ratios measured from pairwise biallelic SNP panels could accurately identify parent-offspring or sibling relationships in diploid organisms. This allele-frequency free method has the potential utility to identify closely related females in haplodiploid ant colonies, even with limited sample sizes.

Here, we used small sample sizes and whole-genome sequencing in three *Camponotus* species in the subgenus *Camponotus*—*C. herculeanus*, *C. laevissimus*, and *C. modoc*—to address the effectiveness of large SNP panels to estimate relatedness and kinship. We chose these three species for several reasons: (1) these species have either no intracolony genetic relatedness estimates published in the literature or none from the Nearctic (see *C. herculeanus* reference below). (2) The nest structure of this subgenus is suggestive of monogynous colonies (Akre, et al. 1994; Fowler 1986; Pricer 1908), and this generality is supported by one relatedness study in Palearctic colonies of *C. herculeanus* (Seppä and Gertsch 1996). This sets up our expectations of finding full sisters in intracolony sampling. Finally, (3) these species are some of the focal taxa in our research program for future population genomics and genome evolution studies, and it will be useful to know if we can identify closely related individuals, even when including small sample sizes for any given population or species.

Overall, our goals for this study are threefold. First, we aimed to determine whether allele-frequency free methods could clearly identify full sisters within colonies with low sample sizes. Second, we aimed to assess the correlation between allele-frequency free kinship estimates with genetic relatedness measures that use estimated allele frequencies. Third, we compiled genetic relatedness estimates in the Formicinae from the literature (1) as a resource for future studies looking at genetic relatedness in this group, (2) to identify whether patterns of relatedness exhibited phylogenetic signal, and (3) to place the relatedness estimates from our three focal species in context with previous studies.

METHODS

Sampling, lab work, and sequencing

For this study, we sampled three colonies each from three *Camponotus* species: *C. herculeanus*, *C. laevissimus*, and *C. modoc* (Table 1). Within species, all colonies had a minimum of 400 km distance between them. All colony identification numbers in Table 1 are associated with voucher specimens housed in the Invertebrate Zoology Collection of the Natural Science Research Laboratory, Museum of Texas Tech University. Specimens were identified using a combination of known habitat associations and published keys (Mackay 2019).

We used six major worker ant specimens per colony for genetic analyses. Whole ant specimens were destructively sampled by pulverization with liquid nitrogen, followed by DNA extraction with a QIAGEN (Hilden, Germany) DNeasy blood and tissue kit per manufacturer recommendations. We used an Invitrogen (Carlsbad, California) Qubit to quantify DNA extractions, followed by concentration standardization. We sent standardized DNA extractions to the Texas Tech University Center for Biotechnology and Genomics for standard Illumina shotgun sequencing library creation. Sequencing libraries were checked via quantitative PCR and a trial sequencing run on an Illumina MiSeq (nano run). After quality checks, the samples were sequenced on a partial lane of an S4 flow cell on an Illumina NovaSeq6000.

Genotyping and variant filtering

We quality filtered our raw sequencing data using the ‘bbduk’ command of the bbmap package (Bushnell 2014). We extracted mitochondrial DNA (mtDNA) reads using the ‘bbsplit’ command from the bbmap package and several Formicinae mitochondrial genomes (Table S1). We aligned our filtered reads to a *Camponotus* sp. reference genome (Manthey, et al. 2022) using the ‘BWA-MEM’ command in BWA (Li and Durbin 2009). We used samtools v1.4.1 (Li, et al. 2009) to convert the BWA output SAM file to BAM format and measure sequencing coverage depth per individual. Next, we used the Genome Analysis Toolkit (GATK) v4.1.0.0 (McKenna, et al. 2010) to clean, sort, and add read groups to the BAM files. To genotype our samples, we used GATK in three steps: (1) genotype each individual (function: 'HaplotypeCaller'), (2) create a database for each chromosomal segment (function: 'GenomicsDBImport'), and (3) group genotype all individuals together (function: 'GenotypeGVCFs'). We then used VCFtools v0.1.14 (Danecek, et al. 2011) to filter our sites with the following restrictions: (1) minimum site quality of 20, (2) minimum genotype quality of 20, (3) minimum depth of coverage of five for a variant to be included per individual, (4) a maximum mean depth of coverage across all individuals of 50, and (5) sites that were biallelic. From this filtering, we kept only single nucleotide polymorphisms (SNPs) and thinned our dataset to have two output SNP datasets: (1) thinned to a minimum distance between SNPs of 50 kbp or (2) 100 kbp. We thinned our datasets to reduce the impacts of linkage on relatedness estimates and to assess how different thinning strategies impacted our results.

Relatedness and kinship estimation

For each species, we estimated relatedness using methods that both do and do not rely on allele frequency estimates. First, we used the R package 'related' (Pew, et al. 2015) to estimate relatedness among all individuals sampled per species using the methods of Li et al. (1993) and Wang (2002). These methods incorporate estimated allele frequencies from the empirical SNP datasets for each species for estimating relatedness. We used these two measures of relatedness because they are relatively more reliable for use with biallelic loci (Gruber, et al. 2012).

We also estimated relationships between individuals using an allele-frequency free method (Waples, et al. 2019) that uses three ratios: R0, R1, and the KING-robust kinship estimator (Manichaikul, et al. 2010). Each ratio represents different relationships of genome-wide allelic patterns between pairs of individuals (Waples, et al. 2019). R0 is a ratio with fixed difference counts in the numerator; barring genotyping errors, we expect R0 to equal zero when estimated between full sisters from a singly mated queen because they should always share their paternal haplotypes. R1 is a ratio with shared heterozygous site counts in the numerator; R1 should have higher values the more closely related individuals are. The KING-robust kinship estimator is a ratio that incorporates both fixed difference counts and shared heterozygous sites in the numerator to estimate kinship on a continuous scale. In diploids, we would expect values of the KING-robust kinship estimator around 0.25 for parent-offspring or full sibling pairs, values around zero for unrelated individuals, and negative values for pairs of individuals in different, structured populations. Here, we may expect full sisters from a singly mated queen to have KING-robust kinship estimator values higher than 0.3.

We categorized pairs of individuals with the R0 ratio ≤ 0.01 and the KING-robust kinship > 0.3 as full sisters, and pairs with R0 > 0.1 and the KING-robust kinship < 0.1 as weakly related or unrelated.

To assess the robustness of relatedness estimates based on allele frequency estimates, we measured the association between the Li et al. (1993), Wang (2002), and KING-robust kinship estimators. If the estimators reliant on allele frequencies are still robust with large numbers of SNPs and small individual sample sizes (here, N = 18 per species), we would expect a strong positive association between each of these measures.

mtDNA haplotypes

We created haplotype networks for the cytochrome oxidase subunit 1 (COI) mtDNA gene for each species in this study for two reasons. First, as a sanity check for pairwise relatedness values similar to full sibling expectations, we’d expect the individuals would have the same mtDNA due to having the same female parent. Second, in pairwise comparisons with relatedness estimates less than expectations for full siblings, we would expect they’d have the same mtDNA haplotype if they had the same female parent, but they may or may not have the same mtDNA haplotype if they have the same male parent.

We used mtDNA reads for each individual—previously extracted using the ‘bbsplit’ script—in Geneious (BioMatters Ltd.) to assemble mitogenomes. We used the Geneious “Live Annotate” feature with the reference Formicinae mitogenomes (Table S1) to annotate the new assemblies. We extracted 1481 bp of the COI region for all individuals to create haplotypes because (1) these regions had high sequencing coverage and (2) the COI gene is common in mtDNA barcoding studies in ants. We only used 1481 bp of the COI gene because of incomplete mtDNA assemblies in a couple individuals. We aligned the COI gene fragment for each of the three species separately using MAFFT (Katoh and Standley 2013) and created a minimum spanning haplotype network for each species’ COI sequences using PopArt (Leigh and Bryant 2015).

Non-comprehensive Formicinae relatedness literature review

From January to March 2021, we performed a literature review of relatedness studies in the ant family Formicinae for several reasons. First, we wanted to compile a relevant list of literature about genetic relatedness estimates that will be useful in the future to researchers interested in estimating genetic relatedness in ants. Second, this would help inform us how our relatedness estimates in three *Camponotus* species correspond with previous research in the subfamily. Lastly, we wanted to test if there was phylogenetic signal of relatedness at the genus-level across this clade.

For each of 51 extant genera in this ant family (Ward, et al. 2016), we searched Google Scholar with the search terms ‘[genus]’ and ‘relatedness’ including only results that contained both terms. We manually scanned search results for those potentially relevant to within-colony relatedness for each genus. We looked at a maximum of the first 500 results for each search.

We compiled these results (Appendix 1) and plotted per genus relatedness using ggplot2 (Wickham 2011) and ggtree (Yu, et al. 2017).

We estimated the phylogenetic signal of the mean and variance of relatedness values for each genus using the R package ‘picante’ and the function “phylosignal.” For the phylogeny, we used a pruned genus-level phylogenetic hypothesis from Blaimer et al. (2015). We did not estimate phylogenetic signal at the species level because of the lack of a species-level phylogeny for the Formicinae.

RESULTS

Relatedness and kinship in three Nearctic carpenter ant species in the subgenus Camponotus

We sequenced between ~5 Gbp to 13 Gbp per individual, resulting in ~9 to 26× alignment coverage relative to the reference genome (Table S1). From these reads, we extracted mitochondrial DNA (mtDNA) and created haplotype networks for each species. We found that each colony exhibited a single COI haplotype (Fig. S1), suggestive but not conclusive of monogynous colonies.

We estimated relatedness and kinship between workers using two SNP datasets: thinned so SNPs were separated by a minimum of (1) 50 kbp or (2) 100 kbp (Table S2 for datasets’ characteristics). Most colonies exhibited pairwise relatedness between workers around 0.75 (Fig. 1; Table 2), similar to expectations of full siblings. Two colonies of *C. laevissimus* showed worker-worker relatedness values higher than that expected from full siblings (Fig. 1; Table 2; samples C-088, C-092), suggestive of some inbreeding.

Using allele frequency-free methods, all intracolony pairwise comparisons clearly showed full-sister relationships, with a lack of fixed differences and KING-robust kinship estimates > 0.3 (Fig. 2; Table 2). Intercolony comparisons indicated the members of the different colonies were either unrelated or exhibited population genetic structure (Fig. 2; Table 2).

All relatedness and kinship estimates were consistent between the differently thinned SNP datasets (all r > 0.99; Fig. S2). The two relatedness estimators (Wang 2002 and Li et al., 1993) were strongly positively associated (Fig. 3). Additionally, the allele frequency-based relatedness and allele frequency-free kinship estimators were strongly positively associated (Fig. 3). Overall, the conclusions drawn from the allele frequency-based relatedness estimates and the allele frequency-free ratio tests were consistent.

Literature review of Formicinae relatedness

We searched the scientific literature for studies using genetic markers to estimate within-colony worker-worker relatedness in the Formicinae. In total 15 of 51 extant genera in the Formicinae have genetic worker-worker relatedness estimates in the literature (Fig. 4; Appendix 1). Per genus, we found between one and 20 species with relatedness estimates, ranging from <1% to ~75% of described species in each genus (Fig. 4; Appendix 1). In total, we recovered 63 species with genetic-based worker-worker relatedness estimates.

Using tests for phylogenetic signal, we did not find a quantitative trend across the phylogeny for mean or variance of relatedness estimates at the genus level (Fig. 4; mean relatedness k = 0.432, p = 0.859; relatedness variance k = 0.696, p = 0.396). Qualitatively, within genera, there were some notable trends; for example, all studied *Camponotus* species exhibited > 50% mean worker-worker relatedness values within colonies (Fig. 4). In contrast, most species studied in *Formica* showed mean worker-worker relatedness values below 60% (Fig. 4). The Formicinae species studied to date show a wide range of reproductive strategies, with some species exhibiting strict monogyny and monandry, and others showing widespread polygyny. Of note, some species showed some colonies with high relatedness and others with low relatedness. Because we only report species' mean within-colony relatedness values here, species with mixed reproductive strategies generally exhibit intermediate mean values of relatedness (Fig. 4).

DISCUSSION

Here, we used genome-wide SNPs to show that all sampled intracolony workers from three Nearctic *Camponotus* species are full sisters (Fig. 1; Fig. 2; Table 2). Even with small sample sizes per colony, this is suggestive that *C. herculeanus*, *C. laevissimus*, and *C. modoc* often have colonies founded by a lone, single-mated queen. Furthermore, each of the colonies showed a single mtDNA COI haplotype, indicative of a single matrilineage (Fig. S1). Here, we use these results to further discuss (1) the utility of different estimators for identifying closely related individuals with small sample sizes, and (2) the context of these three species' relatedness estimates in overall trends of relatedness in the Formicinae.

Identifying closely-related individuals with small sample sizes

Using methods that do and do not use allele frequencies, we identified concordant results that all intracolony workers sampled are full sisters (Fig. 1; Fig. 2; Table 2). Even with our small sample sizes—17–18 individuals per species—we could unambiguously identify pairwise comparisons of individuals that were full sisters. Notably, the allele frequency-free method, particularly the combination of the R0 and KING-robust kinship ratios, demonstrated unambiguously that all intracolony samples were full sisters. In haplodiploid mating systems, we expect that workers will lack any fixed differences at biallelic SNPs if they are full sisters; this pattern manifests in a R0 ratio of zero barring any genotyping errors. Using thousands of SNPs, we clearly showed this trend across all intracolony comparisons (Fig. 2). These results suggest the utility of using large SNP panels, even with small sample sizes, to identify parent-offspring or full sister pairs in haplodiploid systems.

We additionally found that conclusions drawn from the methods reliant on estimated allele frequencies were generally consistent with conclusions from the allele frequency-free methods (Fig. 1; Fig. 3). The relatedness estimates and KING-robust kinship values were strongly positively associated (Fig. 3). In *C. laevissimus*, some intracolony comparisons had higher relatedness values suggestive of inbreeding, but the KING-robust kinship values were not equally inflated (Fig. 1; Fig. 3); this is suggestive of a potential bias in the genetic relatedness estimates in *C. laevissimus* due to small sample sizes. Notably, *C. laevissimus* has lower effective population sizes and genome-wide genetic diversity than the two other species studied here (Manthey, et al. 2022); this relatively lower diversity may be associated with this potential bias.

Relatedness trends across Formicinae

In the Formicinae, a relatively small proportion of the genera (~30%) and very few species (~2%) have published estimates of relatedness (Appendix 1). Many of the species with relatedness estimates have been studied because they are of management interest or have unusual ecological traits. For example, *Formica lugubris* was studied in a conservation genetics context (Gyllenstrand and Seppä 2003) and several other species—including *Anoplolepis gracilipes*, *Lasius neglectus*, and *Nylanderia fulva*—have been studied in regions where they are introduced or invasive (Boomsma, et al. 1990; Drescher, et al. 2007; Eyer, et al. 2018; Gruber, et al. 2012). In other cases, within-colony relatedness has been studied to better understand population structure and variation in mating strategies in relatively common ant species from different regions of the world (e.g., several species of *Formica* studied by Helanterä and Sundström 2007).

There is a broad range of variation in within-colony relatedness estimates across the Formicinae, with no clear trends (i.e., no phylogenetic signal) of relatedness at the genus level (Fig. 4). Within genera with relatively more species with relatedness estimates—*Formica* (n = 20), *Camponotus* (n = 10), and *Cataglyphis* (n = 6)—there are some qualitative trends; most *Formica* have within-colony worker-worker relatedness estimates below 60% and all *Cataglyphis* estimates exhibit below 50% within-colony relatedness. In contrast, all within-colony relatedness estimates in *Camponotus* are above 50%. Several genera with within-colony relatedness estimates for three or four species showed a wide range of values, including *Lasius* (0.14 – 0.65), *Polyrhachis* (0.12 – 0.67), and *Proformica* (0.14 – 0.67). Even within the Camponotini tribe (*Camponotus* and *Polyrhachis* here) within-colony relatedness values range between 0.12 and 0.87. Additionally, in some species, there may be variation in mating system in different parts of their geographic ranges. For example, in *Nylanderia fulva*, within-colony relatedness was estimated as 0.57 in the native geographic range versus 0.04 in areas where the species has been introduced.

Highly variable within-colony relatedness estimates, even between closely-related species and populations, may be representative of both biological reality and imprecise relatedness estimates. The Formicinae are highly variable in life history traits and reproductive strategies (Hölldobler and Wilson 1990), and much of the within-colony relatedness variation likely reflects real differences in mating strategies and within-colony population structure. Additionally, because most relatedness estimates for formicines have been obtained using microsatellites or allozyme data (see Appendix 1), there are likely wide bands of uncertainty around most of these relatedness estimates due to the small amount of total genetic variation measured.

Within *Camponotus*, previous work has quantified within-colony worker-worker relatedness in ten *Camponotus* species belonging to four subgenera: *Myrmothrix*, *Myrmamblys*, *Tanaemyrmex*, and *Camponotus* (Appendix 1). Mean relatedness estimates across the genus range from 0.551 to 0.873 (Fig. 4; Appendix 1). Genetic relatedness estimates in our three focal Nearctic species in the subgenus *Camponotus* are consistent with generally high intracolony relatedness estimates in this genus.

AUTHOR CONTRIBUTIONS

All authors contributed to data analysis and writing and revision of the manuscript. JDM and JPH performed fieldwork. JDM performed DNA extractions. JCG and JDM took ant specimen photographs.

DATA ACCESSIBILITY

All raw sequencing data is at NCBI's SRA repository with an accession under BioProject # PRJNA874018. Mitochondrial DNA sequences are uploaded to NCBI GenBank (OQ325048-OQ325100). Code used for analyses in this project is available on GithHub: github.com/jdmanthey/camponotus_relatedness_revised.

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Collection of the Museum of Texas tech University houses voucher specimens of the colonies used in this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FIGURES AND TABLES

Figure 1. Study taxa and kinship / relatedness estimates. (A) Study taxa and their phylogenetic relationships (modified from Manthey, et al. 2022). (B + C) Within-colony worker-worker pairwise kinship (B) and relatedness (C) estimates for each of the colonies sampled here. Values shown for the 50 kbp thinned SNP dataset. If colonies have a lone, singly mated queen producing workers, we expect a kinship value > 0.3 and relatedness values ~ 0.75. Ant photos by JCG and JDM from specimens housed at the Invertebrate Zoology Collection of the Museum of Texas Tech University.

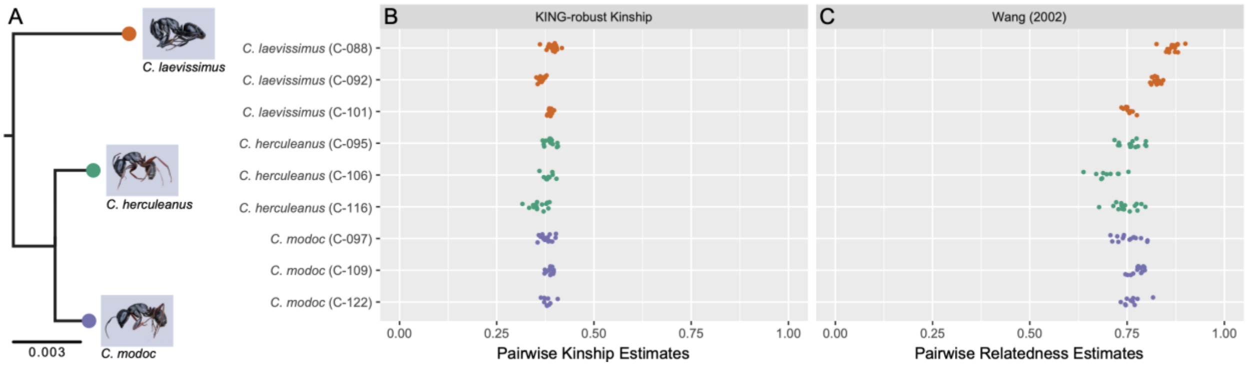

Figure 2. Kinship using allele frequency-free methods. Combinations of R0, R1, and the KING-robust kinship ratios were used to classify relationships in pairwise comparisons (see METHODS). If colonies have a lone, singly mated queen producing workers, intracolony pairwise comparison values should be ~0 for the R0 ratio, higher R1 values for more closely related individuals, and KING-robust kinship values > 0.3. Values shown for the 50 kbp thinned SNP dataset.

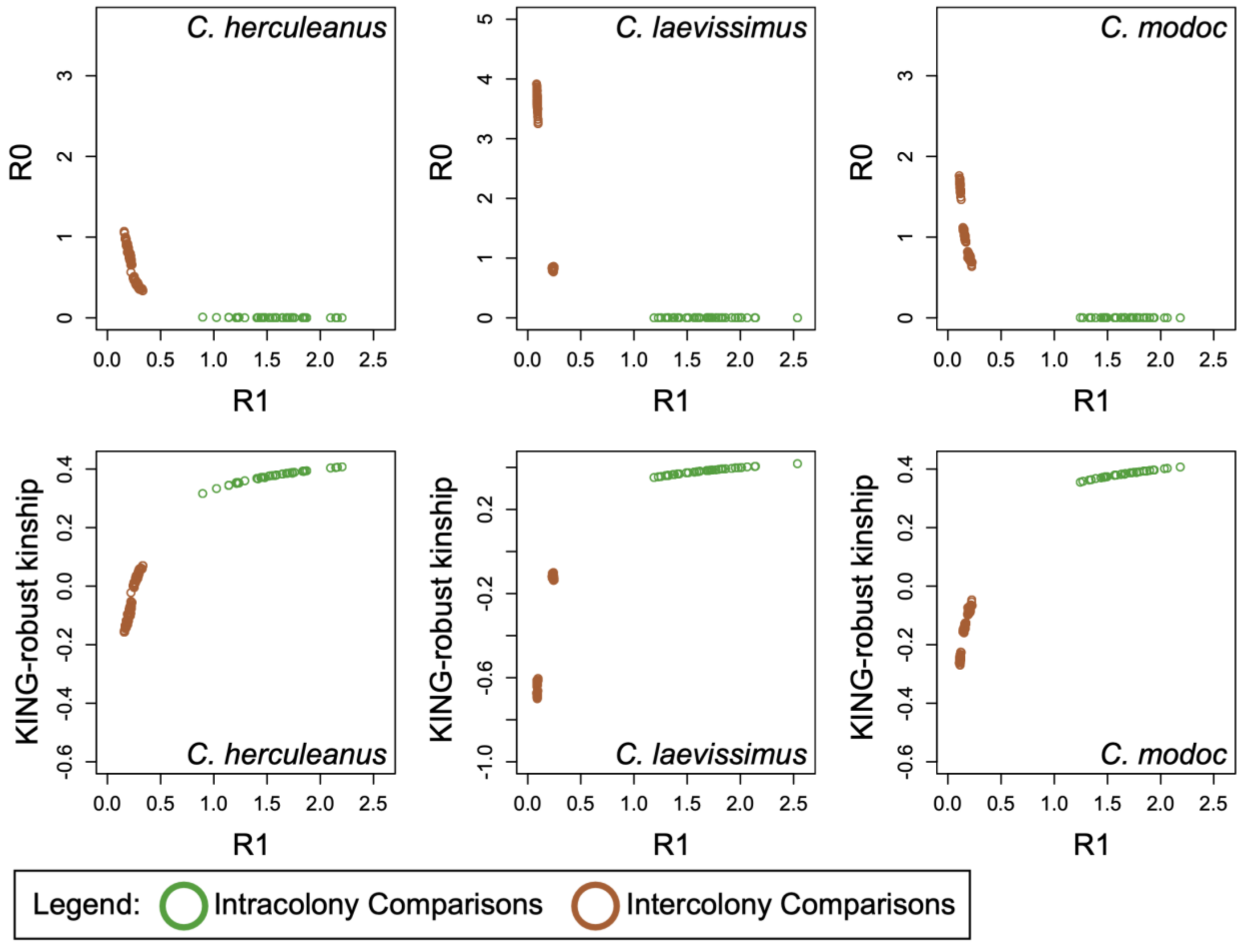

Figure 3. Associations among relatedness and kinship estimators. Positive values indicate comparisons among related individuals, values near zero indicate comparisons among unrelated individuals, and negative values indicate comparisons among individuals in different genetically structured populations.

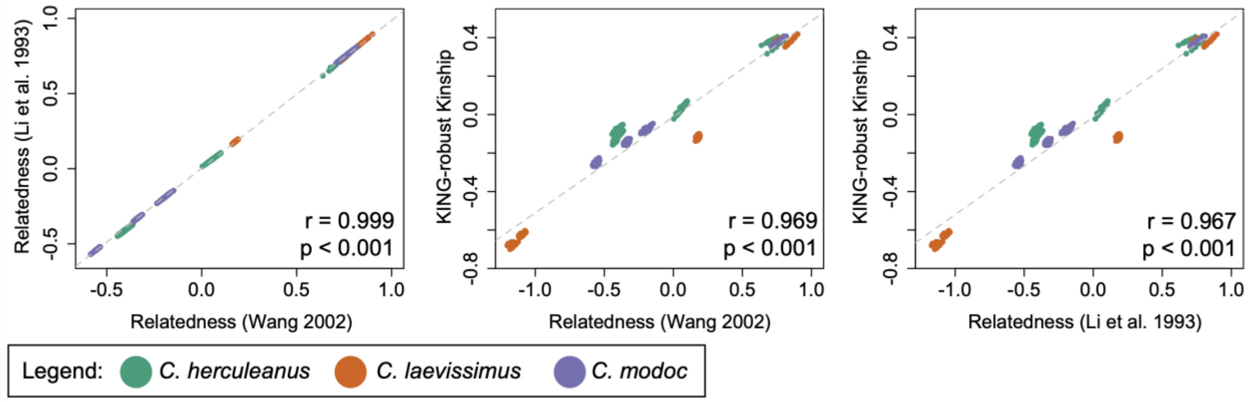

Figure 4. Within-nest worker-worker relatedness estimates from literature review. Estimates are mean values per species (see Appendix 1 for specific values and references). A slight y-axis jitter was added to values to reveal multiple points with similar values. The phylogeny is a pruned genus-level phylogenetic hypothesis from Blaimer et al. (2015), including only genera with genetic relatedness estimates from the literature. At the tips, we present the number of species with genetic relatedness estimates, and the approximate percentage of the described species in each genus with genetic relatedness estimates. In *Nylanderia*, two estimates are presented for the same species, one in the native range (R = 0.57) and one in the introduced range (R = 0.04; arrow). Of note, only 15 of 51 extant genera in the Formicinae have within-colony worker-worker genetic relatedness estimates in the literature.

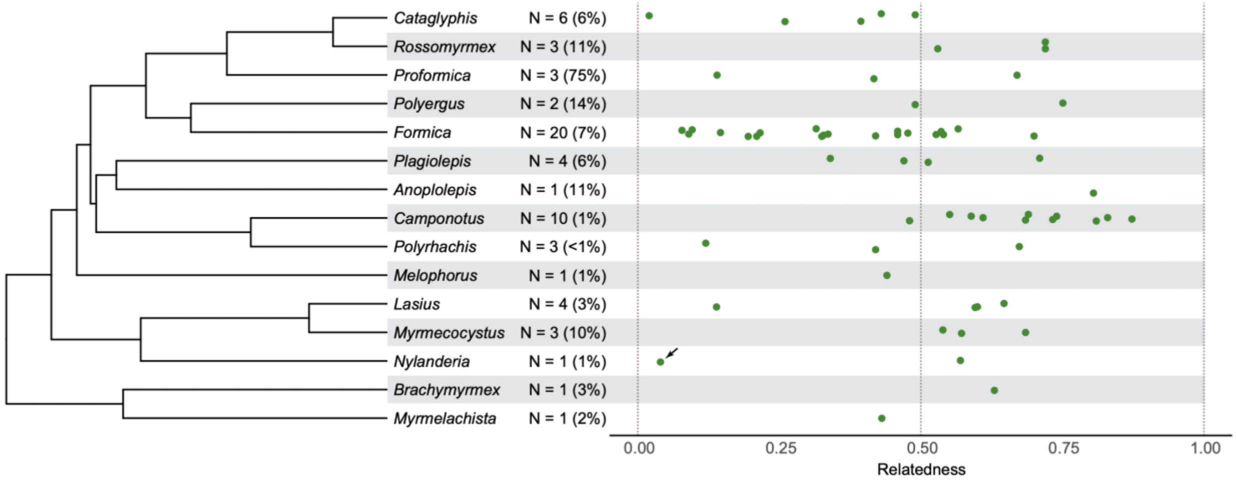

Table 1. Species and localities of colonies used for this study. For each colony, we genotyped six individuals.

Catalog #	Colony ID	Species	Latitude	Longitude	Elevation
TTU-Z_247357	C-095	*C. herculeanus*	40.551	-110.993	2762
TTU-Z_247360	C-106	*C. herculeanus*	43.874	-114.637	2341
TTU-Z_247362	C-116	*C. herculeanus*	41.347	-106.184	2838
TTU-Z_247355	C-088	*C. laevissimus*	33.577	-108.914	1955
TTU-Z_247356	C-092	*C. laevissimus*	40.309	-110.699	2153
TTU-Z_247359	C-101	*C. laevissimus*	43.822	-115.338	1448
TTU-Z_247358	C-097	*C. modoc*	40.592	-110.992	2480
TTU-Z_247361	C-109	*C. modoc*	44.631	-113.789	1936
TTU-Z_247363	C-122	*C. modoc*	38.837	-105.997	2612

Table 2. Relatedness and kinship estimates per colony. Mean pairwise intracolony worker-worker relatedness and kinship estimates for nine colonies studied here (see **METHODS**). Values in parentheses indicate full range of pairwise values for each colony. Values shown for the 50 kbp thinned SNP dataset.

Colony ID	Species	**Relatedness Wang (2002)**	**Relatedness Li et al. (1993)**	**KING-robust Kinship**
C-095	*C. herculeanus*	0.76 (0.72–0.80)	0.76 (0.71–0.79)	0.38 (0.37–0.41)
C-106	*C. herculeanus*	0.70 (0.64–0.75)	0.68 (0.62–0.74)	0.38 (0.36–0.40)
C-116	*C. herculeanus*	0.75 (0.68–0.80)	0.74 (0.67–0.79)	0.36 (0.32–0.39)
C-088	*C. laevissimus*	0.87 (0.83–0.90)	0.86 (0.82–0.90)	0.39 (0.36–0.42)
C-092	*C. laevissimus*	0.83 (0.81–0.84)	0.82 (0.80-0.84)	0.37 (0.35–0.38)
C-101	*C. laevissimus*	0.75 (0.74–0.78)	0.74 (0.72–0.76)	0.39 (0.38–0.40)
C-097	*C. modoc*	0.75 (0.71–0.80)	0.75 (0.70–0.80)	0.38 (0.36–0.40)
C-109	*C. modoc*	0.78 (0.75–0.80)	0.77 (0.73–0.79)	0.39 (0.37–0.40)
C-122	*C. modoc*	0.76 (0.74–-0.82)	0.76 (0.73–0.81)	0.38 (0.36–0.41)

Appendix 1. Summary of the scientific literature review for studies using genetic markers to estimate within-colony worker-worker relatedness in the Formicinae.

Genus	Species	# Colonies Genotyped	Mean Relatedness	References
Anoplolepis	*gracilipes*	93	0.805	(Drescher, et al. 2007; Drescher, et al. 2010; Gruber, et al. 2012; Thomas, et al. 2010)
Brachymyrmex	*patagonicus*	50	0.63	(Eyer, et al. 2020)
Camponotus	*chilensis*	16	0.733	(Eaton and Medel 1994)
Camponotus	*consobrinus*	?	0.61	(Fjerdingstad and Crozier 2006)
Camponotus	*festinatus*	31	0.69	(Goodisman and Hahn 2005)
Camponotus	*herculeanus*	45	0.589	(Seppä and Gertsch 1996)
Camponotus	*klugii*	10	0.81	(Muna 2008)
Camponotus	*nawai*	1	0.83	(Satoh, et al. 1997)
Camponotus	*ocreatus*	16	0.74	(Goodisman and Hahn 2004)
Camponotus	*renggeri*	22	0.551	(Azevedo-Silva 2017)
Camponotus	*rufipes*	35	0.685	(Azevedo-Silva 2017)
Camponotus	*yamaokai*	4	0.873	(Satoh, et al. 1997)
Cataglyphis	*aenescens*	18	0.48	(Cronin, et al. 2016)
Cataglyphis	*cursor*	69	0.394	(Fournier, et al. 2008; Pearcy and Aron 2006)
Cataglyphis	*emmae*	74	0.43	(Jowers, et al. 2013)
Cataglyphis	*niger*	12	0.02	(Leniaud, et al. 2011)
Cataglyphis	*sabulosa*	29	0.49	(Timmermans, et al. 2008)
Cataglyphis	*savignyi*	20	0.26	(Leniaud, et al. 2011)
Formica	*aquilonia*	424	0.078	(Helanterä and Sundström 2007; Mäki-Petäys, et al. 2005; Pamilo 1982, 1993; Pamilo, et al. 2005; Schultner, et al. 2014; Schultner, et al. 2016; Vanhala, et al. 2014)
Formica	*aserva*	14	0.42	(Savolainen and Seppä 1996)

Formica	*cinerea*	417	0.315	(Goropashnaya, et al. 2001; Helanterä and Sundström 2007; Schultner, et al. 2014; Zhu, et al. 2003)
Formica	*exsecta*	712	0.459	(Brown and Keller 2000; Goropashnaya, et al. 2007; Haag-Liautard, et al. 2008; Helanterä and Sundström 2007; Kümmerli and Keller 2007a, b, 2008; Liautard and Keller 2001; Martin, et al. 2012; Pamilo 1991; Pamilo and Rosengren 1984; Seppä, et al. 2004; Seppä, et al. 2012; Sundström, et al. 2003; Vitikainen, et al. 2011; Vitikainen, et al. 2015)
Formica	*fennica*	10	0.09	(Schultner, et al. 2014)
Formica	*fusca*	449	0.477	(Bargum, et al. 2007; Bargum and Sundström 2007; Chernenko, et al. 2013; Hannonen, et al. 2004; Hannonen and Sundström 2003; Helanterä, et al. 2011; Helanterä and Sundström 2007; Pamilo 1983; Schultner, et al. 2014)
Formica	*lemani*	216	0.566	(Gardner, et al. 2007; Schultner, et al. 2014; Seppä, et al. 2009)
Formica	*lugubris*	171	0.146	(Bernasconi, et al. 2005; Gyllenstrand and Seppä 2003; Mäki-Petäys, et al. 2005; Procter, et al. 2016)
Formica	*paralugubris*	83	0.096	(Chapuisat, et al. 1997; Chapuisat and Keller 1999; Holzer, et al. 2009)
Formica	*picea*	145	0.216	(Helanterä and Sundström 2007; Pamilo 1982; Rees, et al. 2010)
Formica	*podzolica*	137	0.336	(DeHeer and Herbers 2004)

Formica	*polyctena*	96	0.325	(Gyllenstrand, et al. 2004; Helanterä and Sundström 2007; Pamilo 1982; Seifert, et al. 2010)
Formica	*pratensis*	133	0.527	(Beye, et al. 1998; Helanterä, et al. 2016; Helanterä and Sundström 2007; Pirk, et al. 2001)
Formica	*pressilabris*	10	0.21	(Pamilo and Rosengren 1984; Schultner, et al. 2014)
Formica	*rufa*	101	0.54	(Gyllenstrand, et al. 2004; Helanterä and Sundström 2007; Seifert, et al. 2010; Skaldina and Sorvari 2020)
Formica	*sanguinea*	315	0.459	(Fernández-Escudero, et al. 2002; Pamilo 1981; Pamilo and Seppä 1994; Pamilo and Varvio-Aho 1979; Schultner, et al. 2014; Seppä, et al. 1995)
Formica	*selysi*	123	0.536	(Chapuisat, et al. 2004; Rosset, et al. 2007)
Formica	*subintegra*	19	0.7	(Savolainen and Seppä 1996)
Formica	*truncorum*	415	0.328	(Elias, et al. 2005; Gyllenstrand, et al. 2005; Helanterä and Sundström 2007; Schultner, et al. 2014; Seppä, et al. 1995; Sundström 1993)
Formica	*yessensis*	5	0.195	(Kidokoro-Kobayashi, et al. 2012)
Lasius	*austriacus*	16	0.6	(Steiner, et al. 2007)
Lasius	*flavus*	8	0.596	(Boomsma, et al. 1993)
Lasius	*neglectus*	62	0.139	(Boomsma, et al. 1990)
Lasius	*niger*	558	0.647	(Boomsma and Van der Have 1998; Fjerdingstad, et al. 2002; Van der Have, et al. 1988)
Melophorus	*bagoti*	14	0.44	(Lecocq de Pletincx and Aron 2020)

Myrmecocystus	*depilis*	57	0.685	(Hoelldobler, et al. 2011; Kronauer, et al. 2003)
Myrmecocystus	*mendax*	139	0.539	(Eriksson, et al. 2019; Eriksson 2018)
Myrmecocystus	*mimicus*	36	0.572	(Hoelldobler, et al. 2011; Kronauer, et al. 2003)
Myrmelachista	*schumanni*	2	0.431	(Malé, et al. 2020)
Nylanderia	*fulva* (introduced range)	41	0.04	(Eyer, et al. 2018)
Nylanderia	*fulva* (native range)	21	0.57	(Eyer, et al. 2018)
Plagiolepis	*barbara*	66	0.71	(Thurin, et al. 2011)
Plagiolepis	*pallescens*	26	0.47	(Thurin, et al. 2011)
Plagiolepis	*pygmaea*	112	0.513	(Thurin, et al. 2011; Trontti, et al. 2005; Trontti, et al. 2007)
Plagiolepis	*schmitzii*	60	0.34	(Thurin, et al. 2011)
Polyergus	*breviceps*	14	0.49	(Savolainen and Seppä 1996)
Polyergus	*rufescens*	4	0.751	(Brunner, et al. 2005)
Polyrhachis	*australis*	1	0.42	(Fjerdingstad and Crozier 2006)
Polyrhachis	*moesta*	4	0.674	(Sasaki, et al. 1996)
Polyrhachis	*robsoni*	37	0.12	(van Zweden, et al. 2007)
Proformica	*korbi*	2	0.14	(Tinaut, et al. 2010)
Proformica	*longiseta*	269	0.417	(Fernández-Escudero, et al. 2002; Sanllorente, et al. 2015; Seppä, et al. 2008; Tinaut, et al. 2010)
Proformica	sp.	2	0.67	(Tinaut, et al. 2010)
Rossomyrmex	*anatolicus*	5	0.72	(Tinaut, et al. 2010)
Rossomyrmex	*minuchae*	7	0.72	(Tinaut, et al. 2010)
Rossomyrmex	*quandratinodum*	7	0.53	(Tinaut, et al. 2010)

SUPPLEMENTAL FIGURES AND TABLES

Figure S1. Haplotype networks for each species. Each color indicates a single colony. Size of circles is proportional to sample size for each haplotype. Number of dashed lines indicate number of inferred substitutions differing between haplotypes.

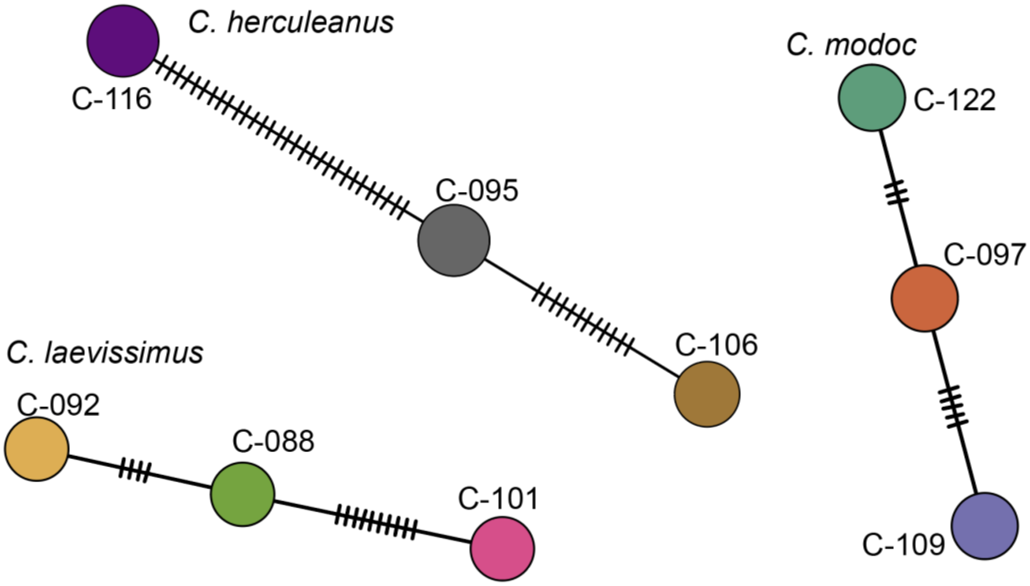

Figure S2. Comparison of relatedness and kinship estimates using differently thinned SNP datasets (see **METHODS**). All correlation coefficients > 0.99. KrK = KING-robust kinship estimator.

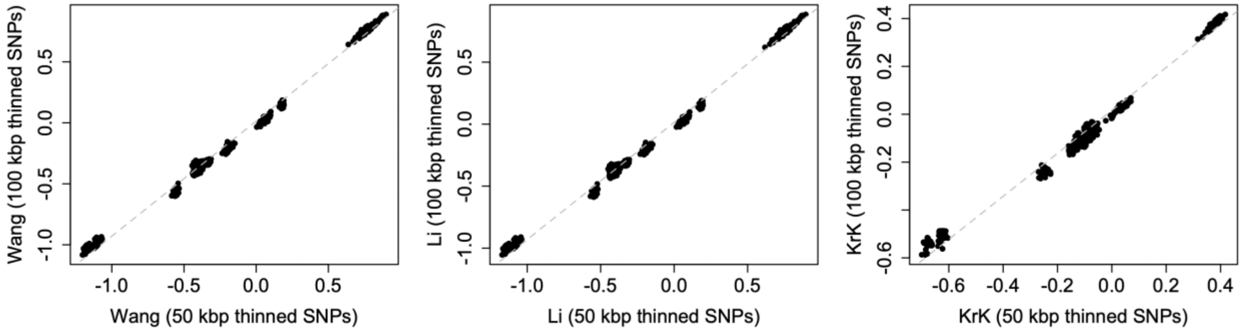

Table S1. Sequencing statistics per sample.

Colony ID	Species	Sample ID	Raw sequence data (bp)	Filtered sequence data (bp)	Alignment mean coverage
C-095	*C. herculeanus*	C-095	6,884,113,333	6,289,614,831	16.25
C-095	*C. herculeanus*	C-095a	5,344,017,062	4,867,019,761	10.42
C-095	*C. herculeanus*	C-095b	6,813,163,404	6,209,974,203	15.70
C-095	*C. herculeanus*	C-095c	5,984,530,885	5,441,695,623	13.36
C-095	*C. herculeanus*	C-095d	10,096,288,436	9,182,885,465	19.84
C-095	*C. herculeanus*	C-095e	9,172,078,656	8,368,135,999	21.08
C-106	*C. herculeanus*	C-106	8,687,618,088	7,922,501,236	18.68
C-106	*C. herculeanus*	C-106a	9,497,098,316	8,677,970,369	20.33
C-106	*C. herculeanus*	C-106b	7,299,825,046	6,641,355,479	16.60
C-106	*C. herculeanus*	C-106c	8,619,653,770	7,833,284,173	18.79
C-106	*C. herculeanus*	C-106d	6,607,632,043	6,010,847,688	14.73
C-106	*C. herculeanus*	C-106e	Sequencing failed		
C-116	*C. herculeanus*	C-116	9,423,248,439	8,596,132,014	24.01
C-116	*C. herculeanus*	C-116a	3,181,714,943	2,951,470,727	8.36
C-116	*C. herculeanus*	C-116b	4,850,845,534	4,479,574,519	13.33
C-116	*C. herculeanus*	C-116c	5,918,138,516	5,432,153,146	12.49
C-116	*C. herculeanus*	C-116d	6,187,512,196	5,734,529,419	15.46
C-116	*C. herculeanus*	C-116e	3,980,655,088	3,735,319,242	10.99
C-088	*C. laevissimus*	C-088	6,584,943,680	6,004,692,294	12.93
C-088	*C. laevissimus*	C-088a	9,424,621,193	8,572,019,325	20.87
C-088	*C. laevissimus*	C-088b	9,350,617,342	8,509,902,581	17.50
C-088	*C. laevissimus*	C-088c	9,904,710,527	9,033,912,200	21.67
C-088	*C. laevissimus*	C-088d	12,783,729,092	11,648,916,842	26.39
C-088	*C. laevissimus*	C-088e	10,624,473,784	9,666,437,636	22.93
C-092	*C. laevissimus*	C-092	5,116,756,831	4,594,063,722	12.21
C-092	*C. laevissimus*	C-092a	5,019,481,301	4,548,939,153	10.86
C-092	*C. laevissimus*	C-092b	5,691,738,472	5,124,410,369	12.73
C-092	*C. laevissimus*	C-092c	5,484,193,249	4,980,860,124	12.87
C-092	*C. laevissimus*	C-092d	6,538,601,012	5,912,815,535	14.99
C-092	*C. laevissimus*	C-092e	6,123,363,638	5,545,179,453	13.06
C-101	*C. laevissimus*	C-101	8,735,030,990	7,945,820,696	18.47
C-101	*C. laevissimus*	C-101a	7,462,639,389	6,808,880,802	14.51
C-101	*C. laevissimus*	C-101b	8,011,113,415	7,231,701,738	14.61
C-101	*C. laevissimus*	C-101c	10,302,510,623	9,305,741,985	16.86
C-101	*C. laevissimus*	C-101d	7,693,626,535	7,025,130,258	17.02
C-101	*C. laevissimus*	C-101e	6,108,382,699	5,562,432,646	11.02

C-097	*C. modoc*	C-097	10,565,548,393	9,656,064,137	23.93
C-097	*C. modoc*	C-097a	7,702,785,822	7,013,756,336	16.66
C-097	*C. modoc*	C-097b	9,378,487,743	8,555,174,594	20.93
C-097	*C. modoc*	C-097c	6,764,312,919	6,164,745,321	14.96
C-097	*C. modoc*	C-097d	4,441,991,610	4,051,010,711	9.14
C-097	*C. modoc*	C-097e	7,499,517,385	6,831,441,669	15.99
C-109	*C. modoc*	C-109	9,580,081,261	8,737,342,219	22.41
C-109	*C. modoc*	C-109a	6,342,771,909	5,745,212,275	11.56
C-109	*C. modoc*	C-109b	7,835,246,228	7,152,151,315	16.41
C-109	*C. modoc*	C-109c	8,193,488,427	7,468,695,618	15.29
C-109	*C. modoc*	C-109d	7,598,334,411	6,924,598,228	16.96
C-109	*C. modoc*	C-109e	7,915,383,182	7,217,992,060	18.16
C-122	*C. modoc*	C-122	9,868,337,227	8,995,488,735	22.54
C-122	*C. modoc*	C-122a	12,148,093,213	10,809,515,553	26.36
C-122	*C. modoc*	C-122b	6,975,068,618	6,354,731,693	14.27
C-122	*C. modoc*	C-122c	8,622,956,121	7,855,038,060	20.53
C-122	*C. modoc*	C-122d	7,502,300,355	6,851,899,827	15.68
C-122	*C. modoc*	C-122e	8,591,283,854	7,867,382,368	18.42

Table S2. Number of variants used in each of the species' datasets for estimating relatedness and kinship.

Species	# SNPs 50 kbp thinned dataset	# SNPs 100 kbp thinned dataset
C. herculeanus	5207	2787
C. laevissimus	5053	2698
C. modoc	5542	2880

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