AN INTEGRATIVE APPROACH TO IDENTIFY RETICULITERMES (RHINOTERMITIDAE) IN GEORGIA, USA

by

ALLISON FAYE JOHNSON

(Under the Direction of BRIAN T. FORSCHLER)

ABSTRACT

Reticulitermes subterranean termites are recognized globally as important economic pests as well as ecosystem engineers that are notoriously challenging to identify to species. A reorganization of this genus would greatly benefit *Reticulitermes* taxonomy, but in the interim, it has been recommended to identify specimens by combining molecular, morphological, and behavioral data to increase confidence in species designations. This dissertation examines methods used in an integrative taxonomic approach [ITA] to identify *Reticulitermes* including subjective morphological characters associated with soldier mandibles, dichotomous keys to the adult stage, flight phenology, and molecular markers to highlight the distribution and abundance of these important pests in their endemic range. The first section of the dissertation examines the feasibility and cost savings of identifying three *Reticulitermes* species using subjective morphological characters associated with the soldier caste. The second section examines the abundance, biodiversity, and distribution of *Reticulitermes* in the southeastern USA using integrative phylogenetic analyses based on mitochondrial DNA gene sequence and geospatial information systems (GIS). While the third examines sequence data from two mtDNA genes to

identify basic biological attributes of these maternally inherited molecular markers in this genus of ubiquitous ecosystem engineers.

INDEX WORDS: *Reticulitermes*, subterranean termite, integrative taxonomy, species distribution

AN INTEGRATIVE APPROACH TO IDENTIFY RETICULITERMES (RHINOTERMITIDAE) IN GEORGIA, USA

by

ALLISON FAYE JOHNSON

B.S., University of Georgia, 2017

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2022

AN INTEGRATIVE APPROACH TO IDENTIFY RETICULITERMES (RHINOTERMITIDAE) IN GEORGIA, USA

by

ALLISON FAYE JOHNSON

Major Professor:	Brian T. Forschler
Committee:	Joseph V. McHugh
	Kevin J. Vogel

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
August 2022

DEDICATION

This dissertation is dedicated to my grandmother and grandfather, Faye, and Bob Johnson. With all my love. A bushel and a peck and a hug around the neck.

ACKNOWLEDGEMENTS

I would like to thank faculty members Dr. Joe McHugh and Dr. Kevin Vogel for their valued participation on my committee, including constructive feedback on my research, and most particularly my advisor Dr. Brian Forschler. Thank you for your constant encouragement and advisement for almost a decade. You inspired my dedication for research and teaching, starting with undergraduate research and continuing throughout my grauduate school career. The completion of my doctoral research and dissertation would not have been possible without your guidance. I am additionally grateful to the undergraduate and graduate lab members that I was able to work alongside while completing my degree.

Thank you to my friends, family, and mentors that have supported my pursuit of higher education through fellowship, moral support, and reassurance that I could finish this degree.

Finally, a special thanks to my parents, Ann Marie Johnson, and Dave Johnson, for their constant motivation and teaching me the value of a strong work ethic.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Termites are the oldest eusocial insects estimated to have diverged from a sister group of subsocial wood-feeding cockroaches, family Cryptocercidae, in the Middle-Late Jurassic period, ~170 to 160 MYA [1, 2, 3]. Termites are commonly described as having two groups, the 'higher' termites, family Termitidae, and the 'lower' termites, families Mastotermitidae, Stolotermitidae, Archotermopsidae, Hodotermitidae, Kalotermitidae, Serritermitidae, and Rhinotermitidae characterized by the presence of flagellate protozoans in the hindgut of lower termites that are acquired by trophallaxis [4, 5]. Rhinotermitidae (Froggat) is estimated to have diverged in the early Paleocene period ~66 MYA [2]. There are six recognized genera in Rhinotermitidae; the largest genus within this family, *Reticulitermes* Holmgren, is the primary focus of this dissertation. *Reticulitermes* contribute to the reintroduction of essential elements from cellulosic materials into the soil through frass deposition inside shelter tubes [6, 7], while also causing intense economic damage annually as a structural pest [8].

Reticulitermes development follows two major pathways: a worker line, and a nymphal/imaginal line commonly described as a sexual line [5]. Eggs develop into 1st and 2nd instar nymphs referred to as 'larvae', despite having hemimetabolous reproduction [9]. Past the 3rd instar immatures develop into workers or nymphs. Nymphs can develop into winged reproductives (alates). Workers and nymphs from both pathways, that do not develop into alates,

can become neotenic reproductives or presoldiers to soldiers [5,9]. Terminal stages in *Reticulitermes* includes soldiers, neotenics, delates and alates [9]. Alates are produced seasonally and disseminate during ephemeral flights, in which 4 out of 5 described species found in Georgia phenologically overlap during the month of May [10]. After a flight, alates shed their wings (delates) form nuptial groups of at least a pair and construct a nuptial chamber to begin a new colony as primary reproductives [9]. Trials of lab reared *Reticulitermes* incipient colonies established with one queen and king cannot reach the population sizes found in some field studies [11,12]. Colonies can be polyandrous and are supplemented by neotenic reproductives [9,12] to reach the population sizes of hundreds of thousands of termites [11].

Reticulitermes are traditionally identified to species using dichotomous keys for the terminal castes alates or soldiers [10, 13, 14, 15, 16, 17, 18, 19, 13, 21, 22] which are only found in seasonal, ephemeral flights or comprise only 1-2% of the colony [23]. Additionally, characters used to identify these castes to species often do not account for the morphological plasticity found in this genus [13, 14, 15, 16, 13, 24, 12]. Immatures and workers are the most abundant in a colony, and for certain termite subfamilies like Apicotermitinae, immature characters like enteric valve morphology have been employed in identification [26], but this has never been accepted for *Reticulitermes*.

Termites in Georgia

Termites from two families have been reported in Georgia: Kalotermitidae and Rhinotermitidae [10, 19, 21, 27]. The most commonly reported species within Kalotermitidae are *I. snyderi, I. minor, K. approximatus*, and *C. brevis* [27,28]. Within Rhinotermitidae, there are two genera: *Reticulitermes* and *Coptotermes*. The Formosan subterranean termite, *Coptotermes*

formosanus, was first recorded in DeKalb County [27] and has since been found in the built environment of metropolitan Atlanta, Savannah, Brunswick, and at a wildland site on Cumberland Island by the UGA Household and Structural Entomology Research Program. There are five species of *Reticulitermes* in Georgia: *R. flavipes*, *R. virginicus*, *R. hageni*, *R. malletei*, and *R. nelsonae*. R. flavipes (Kollar) was originally described from termites in a greenhouse from Vienna, Austria in 1837 [29], *R. virginicus* (Banks) and *R. hageni* Banks from Falls Church, VA in 1907and 1920, respectively [21,30]. Two additional species have been described since Banks and Snyder's (1920) taxonomic revision. *R. malletei* was described from chemical characters and behavioral characters from type specimens collected in Athens, GA [31], and *R. nelsonae* using morphology and genetic data from Sapelo Island Georgia, USA in 2012 [32].

Identification

Taxonomic keys have been the traditional technique for identifying *Reticulitermes* specimens to species (Table 1). Keys created before 2012 employ static measurements which do not account for the morphological plasticity recorded in this genus, don't include recently described species and therefore should be used with discretion [31, 32]. The keys created before 2012 provide descriptions for 3/5 described spp in the southeastern US: *R. flavipes*, *R. virginicus*, *R. hageni*, with some providing additional descriptions for *Reticulitermes* found in the western US (Table 1). A revision of the genus is overdue and *Reticulitermes* taxonomists are encouraged to use behavior and molecular markers in addition to morphological characters for more confidence in species identification [15, 33, 34, 1].

The most recent published taxonomic key for southeastern *Reticulitermes* includes *R. flavipes*, *R. virginicus*, *R. hageni*, *R. malletei*, and *R. nelsonae* [10]. An integrative taxonomic

approach was employed that acknowledged the morphometric variation in *Reticulitermes* by providing the range of selected quantifiable characters (n= 32-431) from species designations supported by flight phenology and molecular data. Including a measurement range can provide increased confidence that samples are being morphologically identified to the appropriate species, while still recognizing that the overlap in ranges between species and morphological plasticity within can lead to misidentifications [10].

Molecular and chemical markers have also been employed to identify *Reticulitermes*. Successful characterization of species-specific cuticular hydrocarbons supplement morphological identification, separating *Reticulitermes* by c-chain length, which presented evidence of an undescribed species in Georgia [36, 37] which led to investigations of undescribed taxa in the southeastern US by combining morphological and molecular approaches, predominantly mtDNA gene sequencing either exclusively or in combination with cuticular hydrocarbons [38, 39, 40]. Additional mtDNA (12S, 16S) gene sequence and nuclear internal transcribed spaces (ITS1, ITS2) have been used to identify *Reticulitermes* [20, 41, 42, 43].

The use of a single technique for identification, morphological or molecular, in a genus desperately requiring revision should be avoided if possible. Lim and Forschler employed an integrative taxonomic approach (ITA) to describe the species *R. nelsonae* in 2012 [32] combining molecular markers (COI and COII mtDNA), morphological measurements, and flight phenology to publish a species description in accord with ICZN rules. This same approach was used to create a taxonomic key for the five *Reticulitermes* species endemic to Georgia, USA [10]. Combining multiple techniques to identify *Reticulitermes* should be employed to increase confidence in species designations [10].

Additional Justifications for a Taxonomic Revision

There is a critical need for the reorganization and revision of *Reticulitermes*. Several researchers have called for a taxonomic revision since the latter half of the 20th century [9, 24, 33, 35, 37, 44]. The advancements in identification techniques leading to additional species descriptions and apparent taxonomic synonyms in the literature support the need for further revision.

There is one accepted junior synonym *Reticulitermes santonensis* described from termites collected in French forests in 1924 by Feytaud [44], that was later recognized as a synonym *R. flavipes* first from cuticular hydrocarbons [45], then overlapping morphological and phylogenetic relationships based on morphology and mtDNA data [39, 42, 46, 47]. *Reticulitermes arenincola* was described in 1931 by Goellner from the sand dunes along the shoreline of Lake Michigan [49]. An investigation presented genetic evidence that *Reticulitermes flavipes* and *R. arenincola* were conspecific [42], providing identical COII mtDNA sequences that also matched an *R. santonensis* accession from France. Authors have supported synonymy with *R. flavipes*, later listing this species *nomen dubium* [43, 51] but using an alternate spelling– *R. arenicola*, which has added to the confusion of this species' taxonomic status. GenBank accessions from this species are listed *R. arenicola*, but reference work with the original spelling [42]. The validity of *R. arenincola* as a species is still contested- it was not synonymized in Ye et al. (2004) the reasoning being that there was recorded morphological variation between soldier characters. Considering the known morphological plasticity of *Reticulitermes*, and the genetic similarity between the two taxa [10, 16, 42, 51], *R. arenincola* is most likely a synonym of *R. flavipes* rather than a *nomen dubium* [50].

It must also be recognized that reports of *Reticulitermes,* before the acceptance of *R. malletei* and description of *R. nelsonae,* identified with keys that were originally developed for a single state or region [13, 24] present a cautionary tale of accepting reported species distributions recorded in the literature. *R. malletei* has most likely been misidentified as *R. flavipes* for the better half of the century due to overlapping diagnostic characters [52]. The western distribution of this species should be examined using an ITA-verified approach to infer species designation. Additionally, overlapping morphological measurements for *R. hageni* and *R. nelsonae* [52] and phylogenetic work from Lim and Forschler (2012) [10] that recovered GenBank accessions attributed to *R. hageni* collected in Florida and southern Georgia within the R. nelsonae clade provide a strong argument for reexamining the southern distribution of *R. hageni*.

Distribution

Reticulitermes distribution in the continental United States can be compiled from surveys found in the published literature including area and statewide surveys using an array of techniques for collection and identification (Table 3). Most surveys in the eastern USA were completed before the acceptance and description of new *Reticulitermes* in the last 30 years, and therefore highlight the distribution of *R. flavipes*, *R. virginicus*, and *R. hageni*. In the most recent taxonomic revision of Nearctic termites in 1920, Banks and Snyder report *Reticulitermes tibialis* and *R. hesperus* [53] in the western US, *Reticulitermes flavipes* anywhere east of the Mississippi river with spillover into TX, AR, and MO. *R. hageni* and *R. virginicus* were found along the eastern coast but primarily in the southern states. Weesner (1965) reports *Reticulitermes* present in all areas of the continental United States: *R. flavipes* distributed in the midwest and east, *R. arenincola* around MI and IL, and *R. virginicus* and *R. hageni* found majorly in the southeast.

Two state surveys in Ohio and Mississippi collected over 400 samples of *Reticulitermes* from PMP submissions and harvested timber stands, respectively, however findings are reported to genus [54, 55]. *R. flavipes* was recorded in 13 surveys from ten southeastern states (Table 3). *R. hageni* was reported in every collection except the 2001 survey from MS, and *R. virginicus* was reported in every collection except the 2000 survey completed in South Carolina. Two surveys recorded R. *malletei* in Alabama and Georgia. The most recently described species, *R. nelsonae,* was recorded in Georgia North Carolina and Florida (Table 3).

Five species of *Reticulitermes* have been collected from 6 surveys including sampling locations in Georgia (Table 3). Banks and Snyder (1920), Weesner (1965), and Scheffrahn et al. (2001) all recorded *R. flavipes*, *R. virginicus*, and *R. hageni*. The work investigating a dominant yet undescribed species in southern Georgia from Sillam-Dussès and Forschler (2010) established the foundation for Lim and Forschler's [10] survey that identified the three aformentioned species of *Reticulitermes* and additionally *R. nelsonae* and *R. malletei*. Two surveys since 2015 have included sampling sites from northern Georgia and identified samples as Rf, Rv, and Rm (Table 3).

Geographic Features of Georgia

Georgia comprises 59,425 sq. miles in the Southeastern United States [69] and encompasses 7 soil provinces following USDA-NRCS delineations (Sand Mountain, Appalachian Ridges and Valleys, Blue Ridge, Piedmont, Sand Hills, Coastal Plain, and Atlantic Coast Flatwoods; [71) over a 1,400-meter elevation range [72]. The main geographic regions are the Piedmont and Coastal Plain.

The ‘natural’ landscape of the Piedmont was described as heavily forested oak-hickory-pine communities [73], but in the early nineteenth century the Piedmont was almost completely deforested and land use was converted for corn and cotton agriculture [74,75]. Most agricultural land was abandoned in the late nineteenth to early 20th century due to soil degradation and hilly landscapes unsuitable for mechanization [76]. Surveys from the Southeastern Forest Experiment Station in the 1950s found forests reestablished over half of the Piedmont [77], but the ecological communities had changed, with slash/loblolly pine and pine plantations making up the majority of forests [77].

The Coastal Plain is separated from the Piedmont by the Fall Line Sandhills, a prehistoric shoreline of the Atlantic Ocean that existed during the Cretaceous period, ~65MYA [78]. Geographic regions below the Fall Line, consisting of the Coastal Plain and Atlantic Flatwoods, were subject to several cycles of rising and falling sea levels over history that can be estimated by marine deposits of sand and shells in the soil from the tertiary (65-2.6 MYA) and quaternary (2.58-0.1 MYA) periods [79]. The Coastal Plain region is lower in elevation than the Piedmont and with a more even terrain [79]. Present land use consists of agricultural land, oak-hickory-pine forests, and southern floodplain forests in the Atlantic Coast Flatwoods [80]. The Fall Line will be used as a boundary to describe the ecological shift between the main geographical regions of Georgia in relation to *Reticulitermes* distributions.

Organization of the Dissertation

1. Assess the feasibility of subjective characters of soldier mandible pairs [SMPs] to identify 3/5 spp of *Reticulitermes* described in Georgia.
2. Investigate the biodiversity and distribution of *Reticulitermes* in southeastern USA.

3. Examine the abundance and phylogenetic relationships of *Reticulitermes* in Georgia using cytochrome oxidase I (COI) and cytochrome oxidase II (COII).

Chapter one discusses the results of an inhouse survey with 6 participants placing 2,229 SMPs into one of four categories that were compared to species designations obtained from an mtDNA (COII) marker using an integrative taxonomic approach (ITA). An additional survey examined participant designations from an online training developed with high-quality SMP photos in an attempt to assess PMP's ability to identify *Reticulitermes* using subjective characters is also introduced.

Chapter two examines the biodiversity and distribution of *Reticulitermes* identified from the first state-wide survey of termites from WoG in Georgia with samples from every county in relation to Georgia's major geographic regions above and below the Fall Line.

Chapter three examines phylogenetic relationships of *Reticulitermes* from the aforementioned survey using mtDNA genes (COII and COI) to describe diversity between and within species collected from the entirety of Georgia, USA.

References

1. Lo, N., Tokuda, G., Watanabe, H., Rose, H., Slaytor, M., Maekawa, K., Bandi, C., Noda, H. 2000. Evidence from multiple gene sequences indicates that termites evolved from wood-feeding cockroaches. *Curr Biol.* 10: 801–804.

2. Engel, M.S., Grimaldi, D.A., Krishna, K. 2009. Termites (Isoptera): their phylogeny, classification, and rise to ecological dominance. *Am Mus Novit. 3650*, 1–27.

3. Bourguignon, T. Lo, N. Cameron, S.L. Šobotník, J. Hayashi, Y. Shigenobu, S. Watanabe, D. Roisin, Y. Miura, T. Evans, T.A. 2015. The Evolutionary History of Termites as Inferred from 66 Mitochondrial Genomes, *Molecular Biology and Evolution*, *32*(2), 406–421.

4. Krishna, K. Biology of Termites. 1969.

5. Noirot, C. 1985 Pathways of caste development in the lowertermites. pp. 41–57 in Watson, J.A.L., Okoi-Kotber, B.M. &Noirot, C. (Eds) Caste differentiation in social insects. Oxford, Permagon Press

6. La Fage J, Nutting W. 1978. Nutrient dynamics of termites. In: Brian MV, Ed. Production ecology of ants and termites. Cambridge: Cambridge University Press. p 165–232

7. Myer, A., Forschler, B.T. Evidence for the Role of Subterranean Termites (*Reticulitermes* spp.) in Temperate Forest Soil Nutrient Cycling. *Ecosystems* **22,** 602–618 2019. https://doi.org/10.1007/s10021-018-0291-8

8. Peterson, C. Wagner, T. L. Mulrooney J. E., Shelton, T. G. 2006. "Subterranean Termites— Their Prevention and Control in Buildings" United States Department of Agriculture Forest Service, *Home and Garden Bulletin* (*64*).

9. Thorne, B.L. 1996. Termite Terminology. Sociobiology 28 (3) 253-261.

10. Lim S.Y.; Forschler B.T. Reticulitermes nelsonae, a New Species of Subterranean Termite (Rhinotermitidae) from the South-eastern United States. Insects 2012, 3, 62-90, doi: 10.3390/insects3010062.

11. Howard, R.W., Jones, S.C., Mauldin, J.K., Beal, R.H. 1982. Abundance, Distribution, and Colony Size Estimates for Reticulitermes spp. (Isoptera: Rhinotermitidae) in Southern Mississippi, Environmental Entomology, 11(6), 1290–1293.

12. Grube, S., Forschler, B.T. 2004. Census of Monogyne and Polygyne Laboratory Colonies Illuminates Dynamics of Population Growth in *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). *Annals of the Entomological Society of America*, *97*(3), 466–475.

13. Miller, E. M. 1949. Florida Termites: a handbook, pp. 1-30. University of Miami Press, Coral Gables, FL, USA.

14. Weesner, F. M. 1965. The termites of the United States: a handbook. *National Pest Control Association*, Elizabeth, NJ.

15. Nutting, W. L. 1990. Insecta: Isoptera, pp 997-1032. In D.L. Dindal's, Soil biology guide, *Wiley,* NY, USA.

16. Hostettler, N., D. Hall, and R. Scheffrahn. 1995. Intracolony Morphometric Variation and Labral Shape in Florida Reticulitermes (Isoptera: Rhinotermitidae) Soldiers: Significance for Identification. The Florida Entomologist 78: 119-129.

17. Brown, K. S., Kard, B. M., & Doss, M. P. 2004. 2002 Oklahoma Termite Survey (Isoptera). *Journal of the Kansas Entomological Society, 77*(1), 1-9.

18. Gleason, R.W., Koehler, P.G. 1980 Termites of the Eastern and Southeastern United States: Pictorial Keys to Soldier and Winged Reproductives. Florida Cooperative Extension Service. University of Florida. Bulletin 192.

19. Snyder, T.E. 1954 Order Isoptera: The Termites of the United States and Canada. National Pest Control Association, New York, NY. 64 pp.

20. Wang, C., X. Zhou, S. Li, M. Schwinghammer, M. Scharf, G. Buczkowski, and G. Bennett. 2009. Survey and identification of termites (Isoptera: Rhinotermitidae) in Indiana. Annals of the Entomological Society of America 102: 1029-1036.

21. Banks, N., and T.E. Snyder. 1920. A revision of the nearctic termites. bull. no 108. U.S. National Museum Government Printing Office, Washington D. C., USA.

22. Banks, F. A. 1946. Species distinction in Reticulitermes (Isoptera: Rhinotermitidae), *Masters Thesis,* University of Chicago, Chicago, IL.

23. Haverty, M.I.. 1977. The proportion of soldiers in termite colonies: A list and a bibliography (Isoptera). *Sociobiology*, (*2*), 199-216.

24. Scheffrahn, R. and N.Y. Su. 1994. Keys to soldier and winged adult termites (Isoptera) of Florida. *Florida Entomologist*. *77*: 460-474.

25. King, S.W., J.W. Austin, and A. L. Szalanski. 2007. Use of soldier pronotal width and mitochondrial DNA sequencing to distinguishing the subterranean termites, Reticulitermes flavipes (Kollar) and R. virginicus (Banks) (ISOPTERA: RHINOTERMITIDAE), on the Delmarva Peninsula: Delaware Maryland, AND Virginia U.S.A. Entomological News 118: 41-48.

26. Scheffrahn, R. H., Carrijo, T., Postle, A., Tonini, F. 2017. Disjunctitermes insularis, a new soldierless termite genus and species (Isoptera, Termitidae, Apicotermitinae) from Guadeloupe and Peru. *ZooKeys*. *665*, 71-84.

27. Scheffrahn, R. H., N.-Y. Su, J. A. Chase, and B. T. Forschler. 2001. New termite (Isoptera: Kalotermitidae, Rhinotermitidae) records from *Georgia. J. Entomol. Sci. 36*: 109- 113.

28. Arbat S., A.M. Mondi, A. Sharma, B.T. Forschler 2021. The Case History of an Insect Infestation Revealed Using X-ray Computed Tomography and Implications for Museum Collections Management Decisions. *Heritage*, *4*, 1016–1025.

29. Kollar, V. 1837. Der gelbfüssige Termit. Termes flavipes. Kllr. Verschiedenem Holzwerke in den Treibhäusern schädlich. Verh. folio Landwirtsch.=Ges. *Wien Aufs. vermischten ökonomischen Inh.*, N. F. *5*: 411-413.

30. Banks, N. 1907. A new species of termes. pp. 392–393. In Entomological News and Proceedings of the Entomological Section Academy of Natural Sciences of Philadelphia, vol 13. Academy of Natural Sciences of Philadelphia, Philadelphia, PA, USA

31. Clément, J.L., Lange, C., Blum, M.S., Howard, R.W., and Lloyd, H. 1985. Chimosystematique du genre Reticulitermes (Isoptères) aux U.S.A. et en Europe. Actes Coll. Ins. Soc. 2:123–131.

32. Lim S.Y.; Forschler, B.T. A new species of Reticulitermes Holmgren, 1913 (Isoptera: Rhinotermitidae) from the Southeastern United States. Insecta Mundi 2012, O252:1-4.

33. Weesner, F. M. 1970. Termites of the Nearctic region, pp. 477-525. *In* K. Krishna and F. M. Weesner [eds.], *Biology of termites*. Academic Press, New York.

34. Kofoid, C. A. 1946. Termites and Termite Control (2nd ed., pp. 127–135). University of California Press.

35. Nelson, L., L. Cool, C. Solek, and M. Haverty. 2008. Cuticular Hydrocarbons and Soldier Defense Secretions of Reticulitermes in Southern California: A Critical Analysis of the Taxonomy of the Genus in North America. Journal of Chemical Ecology 34: 1452–1475.

36. Haverty, M.I., B.T. Forschler & L.J. Nelson. 1996. An assessment of the taxonomy of *Reticulitermes* (Isoptera: rhinotermitidae) from the Southeastern United States based on cuticular hydrocarbons. Sociobiology 28:287-318.

37. Haverty, Michael & Nelson, Lori. 1997. Cuticular Hydrocarbons of Reticulitermes (Isoptera: Rhinotermitidae) from Northern California Indicate Undescribed Species. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 118. 869-880. 10.

38. Jenkins, T.M., Haverty, M.I., Basten, C.J., Nelson, L.J., Page, M., and Forschler, B.T. 2000. Correlation of mitochondrial haplotypes with cuticular hydrocarbon phenotypes of sympatric Reticulitermes species (Isoptera: Rhinotermitidae) from the southeastern United States. *J. Chem. Ecol. 26*: 1525-1542.

39. Jenkins, T. M., Dean. R. E., Verkerk, R., and Forschler, B. T. 2001. Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate european subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy and introduction dynamics. *Mol Phylogenet Evol* 20: 286–293.

40. Sillam-Dussès, D, and Forschler, B. T. 2010. A Dominant and Undescribed Species of Reticulitermes in Sapelo Island (Georgia, USA). *Sociobiology*. *56*. 137-147

41. Uva, P., Clément, J. L., Austin, J. W., Aubert, J., Zaffagnini, V., Quintana, A., & Bagnères, A. G. 2004. Origin of a new Reticulitermes termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. *Molecular phylogenetics and evolution*, *30*(2), 344–353.

42. Ye, W., Lee, C., Scheffrahn, R. H., Aleong, J. M., Su, N., Bennett, G. W., & Scharf, M. E. 2004. Phylogenetic relationships of nearctic Reticulitermes species (Isoptera: Rhinotermitidae) with particular reference to Reticulitermes arenincola Goellner. *Molecular Phylogenetics and Evolution, 30*(3), 815-822.

43. Austin, J. W., A.-G. Bagnères, A. L. Szalanski, R. H. Scheffrahn, B. P. Heintschel, M. T. Messenger, J.-L. Clément, and R. E. Gold. 2007. Reticulitermes malletei (Isoptera: Rhinotermitidae): a valid Nearctic subterranean termite from Eastern North America. *Zootaxa 1554*: 1-26.

44. Feytaud, J. 1924. Le termite de Saintonge. C.R. Acad. Sc. Paris 178: 241-244.

45. Bagneres AG, Clément JL, Blum MS, Severson RF, Joulie C, Lange C 1990. Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonensis* (Freytaud): polymorphism and chemotaxonomy. *J Chem Ecol* 16: 3213–3244.

46. Clément, J. L., Bagnères, A. G., Uva, P., Wilfert, L., Quintana, A., and Reinhard, J. 2001. Biosystematics of *Reticulitermes* termites in Europe: morphological, chemical and molecular data. *Insect Soc* 48: 202–215.

47. Austin, J. W., A. L. Szalanski, R. H. Scheffrahn, M. T. Messenger, S. Dronnet, and A.-G. Bagneres. 2005b. Genetic Evidence for the Synonymy of Two Reticulitermes Species: Reticulitermes flavipes and Reticulitermes santonensis. Annals of the Entomological Society of America 98: 395-401.

48. Wang C., Zhou X., Li S., Schwinghammer M., Scharf M., Buczkowski G., Bennett G. Survey and Identification of Termites (Isoptera: Rhinotermitidae) in Indiana. *Ann. Entomol. Soc. Am.* 2009;102:1029–1036. doi: 10.1603/008.102.0611.

49. Goellner, E.J. 1931. A New Species of Termite, Reticulitermes arenincola, from the Sand Dunes of Indiana and Michigan along the Shores of Lake Michigan. *Pro. Ent. Soc. Wash*. *33*(*9*), 227-234.

50. Austin, J. W.; Szalanski, A. L.; Messenger, M. T. 2004. Mitochondrial DNA variation and distribution of the subterranean termite genus Reticulitermes (Isoptera: Rhinotermitidae) in Arkansas and Louisiana. Florida Entomologist, Gainesville, v. 87, n. 4, p. 473 – 480.

51. ICZN. 1999. International Code of Zoological Nomenclature, 4th ed. The International Trust for Zoological Nomenclature 1999, The Natural History Museum, London.

52. Messenger, M. T., 2002. The Termite Species of Louisiana: An Identification Guide. *New Orleans Mosquito & Termite Control Board Bulletin, 1*(1), 5-7.

53. Snyder, T.E. 1949. Catalog of the Termites (Isoptera) of the World. *Smithsonian Misc. Collections, 112*.

54. Jones, S., and A. Nuss. 2002. Ohio termite (Isoptera) survey: methodology and pest management industry involvement, pp. 476-478. In S. C. Jone, J. Zhai, and W. H. Robinson [eds.], *Proceedings of the 4th International Conference on Urban Pests*, 7-10 July 2002, Charleston, SC. Pocahontas Press, Inc., Blacksburg, VA.

55. Little, N. S., Blount, N. A., Caprio, M. A., & Riggins, J. J. 2014. Survey of Subterranean Termite (Isoptera: Rhinotermitidae) Utilization of Temperate Forests. *Sociobiology (Chico, CA), 61*(2), 198-206.

56. Kofoid, C. A., Light, S. F., & Pickens, A. L. 1946. American Subterranean Termites, Their Classification and Distribution. In *Termites and Termite Control* (2nd ed., pp. 150–156). essay

57. Kofoid, C. A., & Snyder, T. E. 1946. American Subterranean Termites Other Than Those of the Pacific Coast. In Termites and Termite Control (2nd , pp. 187–195). essay, University of California Press.

58. Garrick, Ryan C., Benjamin D. Collins, Rachel N. Yi, Rodney J. Dyer, and Chaz Hyseni. 2015. "Identification of Eastern United States Reticulitermes Termite Species via PCR-RFLP, Assessed Using Training and Test Data" Insects 6, no. 2: 524-537.

59. Hyseni, Chaz & Garrick, Ryan. 2019. Ecological Drivers of Species Distributions and Niche Overlap for Three Subterranean Termite Species in the Southern Appalachian Mountains, USA. Insects. 10. 33.

60. Stephens, C. 2012. Alabama Rhinotermitidae: Nomenclature, Identification, Survey and Phenology. Ph.D. Dissertation, Auburn University, Alabama.

61. Scheffrahn, R. H., Mangold, J. R., & Su, N.-Y. 1988. A Survey of Structure-Infesting Termites of Peninsular Florida. *The Fla. Entomol. 71*(4), 615–630.

62. Scheffrahn, R.H., Su N.-Y., Chase, J.A., Forschler, B.T., 2001. New Termite (Isoptera: Kalotermitidae, Rhinotermitidae) Records from Georgia. *J Entomol* Sci 36 (2): 109-113.

63. Messenger, M. T., N.-Y. Su, and R. H. Scheffrahn. 2002. Current distribution of the Formosan subterranean termite and other termite species (Isoptera: Rhinotermitidae, Kalotermitidae) in Louisiana. *Fla. Entomol. 85*, 580-587.

64. Wang, C., & Powell, J. 2001. Survey of Termites in the Delta Experimental Forest of Mississippi. *The Florida Entomologist, 84*(2), 222-226.

65. Pinzon, O. P., & Houseman, R. M. 2009. Species Diversity and Intraspecific Genetic Variation of Reticulitermes (Isoptera: Rhinotermitidae) Subterranean Termites in Woodland and Urban Environments of Missouri. *Annals of the Entomological Society of America, 102*(5), 868-880.

66. Botch, P. S., & Houseman, R. M. 2016. Landscape Patterns of Colonization by Subterranean Termites (Isoptera: Rhinotermitidae) in Missouri Neighborhoods. Oxford University Press (OUP).

67. Smith, A. L., Smith, M. P., & Kard, B. M. 2010. Oklahoma Formosan Subterranean Termite Surveillance Program and Termite Survey (Isoptera: Rhinotermitidae, Termitidae). *Journal of the Kansas Entomological Society, 83*(3), 248-259.

68. Hathorne, K. T., P. A. Zungoli, E. P. Benson, and W. C. Bridges. 2000. The termite (Isoptera) fauna of South Carolina. *J. Agric. Urban Entomol. 17*: 219-229.

69. Howell, H. N., P. J. Haman, and T. A. Granovsky. 1987. The geographical distribution of the termite genera Reticulitermes, Coptotermes, and Incisitermes in Texas. *Southwest. Entomol. 12*: 119-125

70. McCallie, S.W.. "Introduction: The State as a Whole." In LaForge's *Physical Geography of Georgia*, Stein Print. Co., Atlanta, 1925, p. 2.

71. United States Department of Agriculture, Natural Resources Conservation Service. Land Resource Regions and Major Land Resource Areas of the United States, the Caribbean, and the Pacific Basin. U.S. Department of Agriculture Handbook 296 2006.

72. Musser, Jonathan W. 1996. Digital Atlas of State of Georgia, Elevation, US Geological Survey, Digital Data Series, Atlanta, GA.

73. Nelson, T. C. 1957. The Original Forests of the Georgia Piedmont. *Ecology*, *38*(3), 390–397.

74. Harper, R. M. 1922. Development of Agriculture in Upper Georgia From 1890 to 1920. The Georgia Historical Quarterly, *6*(*3*), 211-232.

75. Cowell, C. M. 1993. Environmental Gradients in Secondary Forests of the Georgia Piedmont, U. S. A. *Journal of Biogeography*, *20*(2), 199–207.

76. Hart, J. F. 1968. Loss and Abandonment of Cleared Farm Land in the Eastern United States. *Annals of the Association of American Geographers*, *58*(*3*), 417-440.

77. Southeastern Forest Experiment Station. 1952. Annual report 1952. Asheville, N.C. : Southeastern Forest Experiment Station, U.S. Dept. of Agriculture, Forest Service, [1953], *22:3-4.*

78. Gore, P. J. W., Witherspoon, W. Introduction. In Roadside Geology of Georgia, 1st ed.; Mountain Press Publishing Company, Missoula, MT, USA, 2013; pp. 2–21.

79. McNab, H. W. and Avers, P. E. 1996. Ecological Subregions of the United States. Southern Region and Southeastern Forest Experiment Station. (*21*).

80. Griffith, G.E., Omernik, J.M., Comstock, J.A., Lawrence, S., Martin, G., Goddard, A., Hulcher, V.J., and Foster, T., 2001, Ecoregions of Alabama and Georgia, (color poster with map, descriptive text, summary tables, and photographs): Reston, Virginia, U.S. Geological Survey (map scale 1:1,700,000).

Table 1.1. A list of taxonomic keys to *Reticulitermes* including species described and citation. Species abbreviations include Rf= *R. flavipes*, Rv = *R. virginicus*, Rh = *R. hageni*, Ra = *R. arenincola*, Rm = *R. malletei*, Rt = *R. tibialis*, Rhp = *R. hesperus)*.

***Reticulitermes* spp described**	**Citation**
Rf, Rv, Rh, Rt, Rhp	Banks, N., and T.E. Snyder. (1920). A revision of the nearctic termites. bull. no 108. U.S. National Museum Government Printing Office, Washington D. C., USA.
Rv, Ra	Banks, F. A. (1946). Species distinction in Reticulitermes (Isoptera: Rhinotermitidae), *Master's Thesis,* University of Chicago, Chicago, IL.
Rf, Rv, Rh	Miller, E. M. (1949). Florida Termites: a handbook, pp. 1-30. University of Miami Press, Coral Gables, FL, USA
Rf, Rv, Rh, Ra, Rt, Rhp	Snyder, T.E. (1954) Order Isoptera: The Termites of the United States and Canada. National Pest Control Association, New York, NY. 64 pp.
Rf, Rv, Rh, Ra, Rt, Rhp	Weesner, F. M. (1965). The termites of the United States: a handbook. *National Pest Control Association*, Elizabeth, NJ.
Rf, Rv, Rh, Ra, Rt, Rhp	Nutting, W. L. (1990). Insecta: Isoptera, pp 997-1032. In D.L. Dindal's, Soil biology guide, *Wiley,* NY, USA.
Rf, Rv, Rh	Scheffrahn, R. and N.Y. Su. (1994). Keys to soldier and winged adult termites (Isoptera) of Florida. *Florida Entomologist*. *77*: 460-474.
Rf, Rv, Rh, Rm, Rn	Lim S.Y.; Forschler B.T. Reticulitermes nelsonae, a New Species of Subterranean Termite (Rhinotermitidae) from the South-eastern United States. Insects 2012, 3, 62-90, doi: 10.3390/insects3010062.

Table 1.2. Area and statewide surveys of subterranean termites in the United States including state, reported species, the number of samples collected (if reported), collection methods including direct sample submissions from pest control operators (PCOs), museum collections, field collection sites from structures (S) or wildlands (WL), and citation. If a publication did not report collection methods or samples size, it is listed NM (not mentioned).

Area Surveys of Subterranean Termites				
State	*Reticulitermes spp* reported	Samples reported (n)	Collection methods	Citation
GA FL SC NC VA WV KY TN AL MS	Rf	NM	S	[21]
TX FL SC VA	Rh			
GA LA FL NC VA	Rv			
TX LA AK MS AL GA FL SC NC TN KY VA WV	Rf	NM	PCOs	[56]
LA AK MS AL GA TN KY SC NC FL VA WV	Rh			
LA AK MS AL GA TN KY SC NC FL VA WV	Rv			
TX LA MS AK TN GA FL SC NC KY VA WV IL WI MI IN OH PA NY DW MD VT MA CT ME	Rf	NM	NM	[57]
LA MS GA FL SC NC VA WV KY MD DE IL IN OH	Rh			
LA MS GA FL SC NC VA WV KY MD DE IL IN OH	Rv			
TX LA MS AK TN GA FL SC NC KY VA WV IL WI MI IN OH PA NY DW MD VT MA CT MO	Rf	NM	NM	[15]
LA AK MS AL GA FL SC NC TN KY VA WV	Rh			
LA AK MS AL GA FL SC NC TN KY VA WV	Rv			

FL LA TX	Rf	n=785	S	[14]
LA	Rh			
FL LA	Rv			
GA SC NC AL TN MS VA WV	Rf	n=55	WL	[31]
GA TN	Rv			
GA AL	Rm			
AL	Rn			
GA SC NC AL TN MS WV VA KY	Rf	n=138	WL	[59]
GA KY VA AL SC NC	Rv			
AL SC	Rm			

State Surveys of Subterranean Termites

State	*Reticulitermes spp* reported	Samples reported (n)	Collection methods	Citation
Alabama	Rf, Rh, Rv, Rm	n=38	WL MC	[35]
Florida	Rf, Rh, Rv	n=785	PCOs	[61]
Georgia	Rf, Rv, Rh	n=50	S, WL	[62]
	Rf, Rv, Rh, Rm, Rn	n=237	WL	[10]
Indiana	Rf, Rv, Ra, Rt, Rh	n=289	PCOs, S, WL	[13]
Louisiana	Rf, Rv, Rh	n=426	PCOs	[63]
	Rf, Rv, Rh	n=386	PCOs	[63]
Mississippi	Rf, Rv	n=114	WL	[64]

Missouri	Rf, Rt, Rv, Rh Rf, Rv, Rh	n=611 n=224	PCOs, WL WL	[65] [66]
Oklahoma	Rf, Rt, Rv, Rh Rf, Rt, Rv, Rh	n=27 n=65	PCOs, S, WL PCOs, WL	[17] [67]
South Carolina	Rf, Rh	n=158	PCOs WL	[68]
Texas	Rf, Rt, Rv, Rh	n=380	PCOs MC	[69]

CHAPTER 2

COMPARING AN INTEGRATIVE TAXONOMIC ANALYSIS TO CATEGORIZATION OF SOLDIER MANDIBLE MORPHOLOGY FOR IDENTIFYING *RETICULITERMES* SPECIES FROM THE SOUTHEASTERN UNITED STATES.[1]

[1]Allison Johnson and B.T. Forschler. To be submitted to American Entomologist.

Abstract

Phenotypic plasticity challenges anyone using a dichotomous key to identify *Reticulitermes* subterranean termite species. The reliability of molecular markers is likewise complicated because many accessions do not account for species descriptions published in the last 15 years. An Integrative Taxonomic Approach (ITA) is generally considered superior to the aforementioned yet expense can be a barrier with large sample sets. We assessed the feasibility of using subjective characters of soldier mandible pairs [SMPs] to identify three *Reticulitermes* species and compared those classifications with ITA species designations from 2,229 field-collected samples. We developed trainings for identify 3 Reticulitermes species using SMPs and compared participant categorizations to ITA designations (termed success rates, SR) with in-house and online surveys. Participants with experience identifying SMPs provided SRs of >98.82% while less experienced provided 88.99-95.40%. The subjective nature of recognizing morphological characters was evident in survey results because no two participants categorized all samples the same. Combining participant SMP categorizations improved SR rates but reduced cost savings by lowering the number of samples destined for the more expensive ITA. The online survey showed participant SRs were disparately divided into two groups; participants able to (>89% SR) and those that struggled (<80% SR) with classifying images into morphological categorizes. The data are discussed in regard to reducing ITA costs for large-sample field studies using morphological species identification while highlighting training needs and screening efforts for citizen science data to support reasonably accurate insect identifications.

Keywords: *Reticulitermes,* subterranean termite, Integrative Taxonomic Approach (ITA), survey, soldier mandible morphology, field-collection, mtDNA

Introduction

Identifying an organism to species is crucial to delineating their biology, evolutionary history, and ecological role. The economically and ecologically important subterranean termites within the genus *Reticulitermes* have long represented a species identification conundrum [1]. There are 5 species described from the southeastern USA; *Reticulitermes flavipes* (Kollar) 1837 [Rf], *Reticulitermes virginicus* (Banks) 1907 [Rv], *Reticulitermes hageni* (Banks and Snyder) 1920 [Rh], *Reticulitermes malletei* (Howard and Clément) 1986 [2] [Rm], and *Reticulitermes nelsonae* (Lim and Forschler) 2012 [Rn] with the last two species added since the last revision of the genus in 1920. Entomologists traditionally identify specimens using published, morphology-based taxonomic descriptions, yet *Reticulitermes* developmental plasticity [2, 4, 5, 6, 7] exasperates those using dichotomous keys that provide a single break-point measurement, subjective terms like "narrower", or color characters that can fade [5, 8, 9, 10, 11, 12, 13]. Employing molecular techniques such as mtDNA markers and DNA barcoding, has expanded opportunities for species identification and illumination of evolutionary trends within the genus [14]. Yet, using a single method for species identification in a genus that desperately needs a taxonomic revision has caused some authors to question species designations in the literature [13, 15, 16, 17].

Reticulitermes identification based solely on molecular techniques has added to the morphological muddle with GenBank accessions listed to the wrong species designation, claims of new species in opposition to ICZN rules or simply ignoring the full complement of a species complex [18, 19, 20]. Confidence in *Reticulitermes* species identification, in lieu of a genus revision, can be increased using an Integrated Taxonomic Approach (ITA) employing molecular

reference sequences obtained from specimens identified using published dichotomous keys [1, 6,7, 22, 23, 24, 25, 26].

Researchers interested in studying *Reticulitermes* by collecting large numbers of samples would directly benefit from reliable species markers. The limitations of dichotomous keys have provoked some authors to report results at the Genus or Family level [27, 28, 29] while employment of molecular markers restricts others because of cost and access to equipment or expertise [1, 30, 31, 32]. Our experience with *Reticulitermes* dichotomous keys has involved examination of soldier-caste head capsule features, including width, length, and proportions of the labra and gula, as well as mandible size and curvature as diagnostic species characters [2, 5, 6, 8, 10, 11, 12, 13, 28, 33, 34, 35]. Anecdotally, we recognized qualitative characters (Figure 1) that appeared to consistently align for 3 of the 5 species from the eastern United States; the soldier mandibles of Rf were the longest (length), Rn the shortest, and Rv displayed a unique curvature of the right mandible. The objective of this study was to compare *Reticulitermes* taxonomic designations obtained using a visual examination of qualitative soldier mandible characters to a mtDNA marker confirmed by an Integrative Taxonomic Approach (ITA). Morphological designations were secured from 8 participants with different levels of familiarity with the genus as well as an online survey of images of soldier mandibles. Results are discussed in relation to the utility of these characters in *Reticulitermes* species identification as well as the reliability of using citizen science data that requires some level of taxonomic certainty.

Materials and Methods

Termite Samples

A single completist collected over 4,000 termite samples between January 2015 and May 2017 from wood on ground (WoG) within 100-m of state highways from all 159 counties in Georgia, USA. Samples were stored in 100% EtOH and labeled by site and date of collection. We examined 2,229 samples that had more than one soldier for this study.

Processing Termite mtDNA Samples

Whole-body DNA extractions from one worker per sample were conducted and processed utilizing adapted instructions (Supplemental Materials Methods) from the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Published primers for the entire length of the 685 bp COII mtDNA gene [36, 37] were used for the PCR protocol (QuantaBio, MA, USA) (Supplemental Materials). Impurities were removed using an EXO I and CIP enzymatic digestion (New England Biolabs, Ipswich, MA, USA) before purified PCR product was sent to Eurofins Genomics (Eurofins, Louisville, KY, USA) for 96-Well Microplate Sanger sequencing. Returned sequences were curated in Geneious Prime v2020.2 (http://www.geneious.com, Kearse, et al., 2012), and aligned using MUSCLE v3.8.31 [38]. Species designations were inferred from an alignment with at least 8 reference sequences, identified by alate morphology, per *Reticulitermes* species using a Maximum-Likelihood phylogenetic tree with an ITA verified *Coptotermes formosanus* sequence (AY683218) as the extant group. We employed the model of best fit (HKY + F + I+ G4) using ModelFinder [39] with 2,000 UF Bootstrap replicates [40] in IQ-Tree v2.6.12 [41]. The tree was annotated in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Processing Termite Soldier Mandible Samples

Soldier mandible pairs [SMPs] were excised from one soldier per sample under a stereoscope (Meiji, Japan) with insect pins (Size 2, Bioquip Products, CA, USA) then positioned with the base of the mandibles touching and parallel to the bottom line inside a 1-mm square printed on paper in a 4X5 grid in the center of a 9-cm diameter Petri dish (Thermo Fisher Scientific, Waltham, MA) (Supplemental Materials Figure S1). The rows of 1-mm squares were covered with a length of double-sided (Scotch, MN, USA) tape to hold SMPs in position. The 1-mm squares with each SMP were numbered and cataloged by site, totaling 189 Petri dishes labeled by county.

Categorizing Termite Mandible Samples

SMPs were viewed under a stereoscope at 2X magnification and classified into one of four categories; Longest (L) –distance between base and tip of mandible 0.8-1mm in length; Shortest (S) - length of SMPs < 0.6-mm; Curved Right Mandible (CRM) – angle of the dorsal condyle to the tip of the right mandible 12-15° characterized by Lim and Forschler, 2012; and Not Identified (NI) - SMPs that did not explicitly fall in the aforementioned categories (Figure 1). All characterizations into a category were conducted without measurements (of length or angle) and left to the observational discretion of individual participants.

Termite Soldier Mandible In-House Survey

Participants included two researchers defined as ‘experienced’ by having examined thousands (>5,000) of *Reticulitermes* SMPs in the system described above. Six participants had no prior experience in termite morphology and included two entomology graduate students, two undergraduate students that were not entomology majors, and two junior high school students (Table 1). Six participants examined 2,229 SMPs while the two junior high students provided

input for 343 samples. All less experienced participants received a ½ hour tutorial on termite mandible characters including examination of five SMPs from each of the four categories (n=20) termed the category-training grid [C-T grid]. Participants were allowed to view the C-T grid, ask questions about each category, and then complete a 'pre-survey test' [P-S test]. The P-S test involved categorizing 120 mandible pairs randomly arranged in the previously described grids (50 L, 29 S, 37 CRM, and 4 NI). Samples used in the P-S tests were identified to a category by both experienced participants (A & B, Table 1) and supported by ITA species designations. If the participant's categorizations on their P-S test agreed >90% (n=108/120) with the ITA determinations, they proceeded to the 2,229-sample survey. If a score of 90% was not achieved, that participant retook the P-S test after further discussion/training (Table 1). Survey participants were encouraged to review the C-T grid as needed while scoring the P-S test or entire SMP survey.

Integrating mtDNA and Morphological Data

The Integrated Taxonomic Approach (ITA) employed COII mtDNA sequences obtained from 35 alate specimens morphologically identified to a species herein termed 'reference sequences'. Samples were designated to a species based on association with a reference sequence inferred from a maximum likelihood analysis. We assumed the SMP categories L, S, CRM would align with Rf, Rn, and Rv, respectively, and the NI category with Rm or Rh. SMPs identified to a morphological category that did not match the respective ITA species were termed "not-matching". SMPs categorized as NI were destined for identification using ITA and therefore not included in the number not-matching.

The proportion of SMP categorizations that matched ITA species designations was termed the Success Rate [SR]. SR was calculated by dividing the number of SMPs in a morphological

category that matched the ITA species-designation (L=Rf, S=Rn, CRM=Rv) by the total number of SMPs in that morphological category, (Table 2, Column 4 ÷ Column 2) expressed as a percentage. SRs were calculated for each survey participant by SR category and overall, across all categories, (Tables 2-3). The same technique was used to calculate SRs for combinations of participants except that when combining participant data only those samples all participants identified to the same category were considered. (Table 5).

Success Rate Analysis

Analysis for evidence of significant differences in SRs between participants or groups (ALL, AB, C-F, D-F) and categories (L, S, CRM) within participant was conducted in R 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria). SRs between participants were analyzed using a Generalized Linear Model assuming quasi-Poisson distribution and tested post-hoc with Tukey's HSD. Comparisons of SRs between participant groups (Table 5), and category SRs (L, S, CRM) within a participant (Table 4) were conducted with Pearson's Chi-squared test analyses with Yates' continuity correction.

Examining Sampling Error

We processed one soldier (SMP) and one worker (ITA) from each sample under the assumption that a sample represented individuals from a single colony of termites. A test of that assumption involved 36 samples categorized by participants that did not match the ITA species designation. The 36 samples (1.6% of the data set) included 21 that provided additional soldiers. There were up to four SMPs excised and mounted, depending on availability by sample, in addition to sequencing 4-11 workers from 34 samples while 2 samples did not provide more workers (Supplemental Materials Table S1). The additional excised SMPs were examined by participants A and B as blind samples and placed in a category. The category designations were

compared, by sample, to the previous SMP characterization as well as the additional ITA designations obtained from the workers (Supplemental Materials Table S1).

Cost Evaluations

The cost of ITA was based on labor for sample preparation, data analysis, average price of reagents and supplies, and sequencing invoices, but excluded in-house equipment costs. SMP dollar expenditure was based on labor costs for time spent for sample preparation, survey completion, and data analysis plus average price of supplies (Table 4). Details for calculation of time spent, labor costs, and data analysis are in the Supplemental Materials (Table S2). Cost per sample was calculated for each method by dividing the gross dollar total by the number of samples. Estimated cost savings by participant and groups was calculated by subtracting the Cost Per Sample for ITA multiplied by the number of samples placed in the NI category plus the cost of the SMP from the ITA cost for all samples (Tables 2, 5).

Electronic Test

An electronic test [ET] modeled after the P-S Test was built using Google Forms (Google, Mountain View, CA, USA) by organizing 120 SMP images that matched an ITA species designation randomly arranged in groups of 3 per row and included 48 L, 21 S, 31 CRM, and 20 NI. Images were taken with a Keyence VHX-7000 Digital Microscope (Keyence Corporation of America, Itasca, IL, USA 60143) at 100X magnification. A reference key [RK] was produced identifying 24 SMPs to the appropriate category (6 SMPs per category). The ET was posted online with a dedicated link and participants were asked to identify SMP images to a category using their best judgment while referencing the RK. Analysis of SRs for the ET was conducted in R 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

ITA Species Designations

The model aligned the 2,229 samples into species clades with 1,245 associating with Rf (55.8%) reference sequence followed by 537 Rn (24%), 337 Rv (15.1%), 97 Rm (4.4%), and 13 Rh (0.6%) (Figure 2).

In-House P-S Test Results

The two most-experienced participants (A & B) did not take the P-S test but all remaining survey participants (C-H) achieved >90% success rate after two attempts (Table 1). Three participants C, G, H did not achieve a >90% score on the first attempt. The junior high school students scored the lowest (78.4 & 86.7%) on the first round, and despite improving to >90% on the second P-S test, their success rates were <70% after 300+ SMP categorizations (Table 3) and therefore did not complete the survey nor were those data included in compiling or discussing results.

In-House Participant Individual Results

Participant A categorized 1,274 SMPs (Table 2) into a defined morphology category (822 L, 139 S, 313 CRM) and 955 (43%) as NI (Table 3). Participant B categorized 1,341 SMPs into a defined category (851 L, 176 S, 314 CRM) with 888 (40%) NI. Participant C placed 1,553 SMPs into a category (872 L, 409 S, 272 CRM) with 676 (30%) NI. Participant D placed 1,435 SMPs into a category (912 L, 235 S, 288 CRM) and 794 (36%) NI. Participant E categorized 1,676 SMPs (987 L, 386 S, 303 CRM) leaving 553 (25%) NI. Participant F categorized 1,681 SMPs (Table 2) (1013 L, 344 S, 324 CRM) with 548 (24%) NI (Table 3).

Participant A had a 98.82% overall SR (Table 2) and category SRs of 99.88% for L, 98.56% S, and 97.17% CRM (Table 3). Participant B had a 98.88% SR with category SRs of

99.88%, 100.00%, and 95.54% for L, S, CRM respectively. Participant C had an 88.99% overall and category SRs of 97.48%, 77.02%, and 79.78% L, S, CRM respectively. Participant D had a 95.40% SR with category SRs of 97.04%, 92.34%, and 92.71%, L, S, CRM respectively. Participant E had a 94.09% SR with category SRs of 98.7%, 82.90%, and 93.07%, L, S, CRM respectively. Participant F had a 95.12% SR (Table 2) and category SRs of 97.93%, 93.02%, and 88.58%, L, S, CRM respectively (Table 3).

In-House Participant Grouped Results

All participants combined placed 904 SMPs (40%) to a defined category with a 99.45% SR and respective category SRs of 100.00%, 100.00%, and 97.76% L, S, CRM respectively. The combined data left 1,330 (60%) NI (Table 5).

The combined data for the two most experienced participants, A&B, categorized 1027 SMPs (46%) to a category for a 99.03% total SR including category SRs of 100.00%, 100.00%, and 95.86% L, S, CRM respectively with 1,202 (54%) NI (Table 5).

The combined data for the less experienced participants, C-F, placed 985 SMPs (44%) to a category for a 98.07% SR with category SRs of 100.00%, 95.59%, and 94.17% L, S, CRM respectively leaving 1,244 (56%) NI (Table 5).

The combined data for less experienced participants that passed the P-S test on the first attempt, D-F, placed 1,184 SMPs (53%) to a category for a 98.23% overall SR with category SRs of 100.00%, 96.70%, and 94.33% L, S, CRM respectively leaving 1,045 (47%) NI (Table 5).

In-House Success Rate Comparisons

There was no significant difference comparing SRs between participant groups, (ALL, AB, C-F, D-F) using Pearson's Chi-squared (Supplemental Materials Table S3). Pearson's Chi-

squared, of categories (L, S, CRM) within participants showed only C had a significant difference (p=0.005) between S (77.0) and L (97.5) (Supplemental Materials Table S6). Comparing SRs by participant using a Generalized Linear Model with a 'Quasi-Poisson' distribution, C and E were not different but C was significantly different from A, B, D, and F (Supplemental Materials Tables S4, S5).

Examining Sampling Error

Nineteen of 36 samples (19/36, 53%) provided evidence of at least 2 species in the same sample (Supplemental Materials Table S1). Seventeen samples were moved from not-matching to matching for SR calculations and attributed to the appropriate individual/group/category for analysis. Two samples provided 2 ITA species designations representing 2 species in a single sample but did not match SMP categories (Supplemental Materials Results) and were included as not-matching when calculating SRs.

Time and Cost Analysis

The cost of labor and materials for ITA was estimated at 6.95 USD per sample or 15,497.00 USD for all 2,229 samples (Table 4, Supplemental Materials Table S2). The cost of labor and materials for the SMP identifications was estimated at 1.08 USD per sample or 2,402.50 USD for one participant. Cost to identify SMPs with 6 participants totaled 2,890.00 USD or 1.30 per sample (Table 4, Supplemental Materials Table S2).

Electronic Test [ET]

Twenty-five anonymous participants completed the ET with 15 scoring SRs >89% (range 100-89%) while 10 scored <80% (range 79-53%) (Supplemental Materials Table S8). Analysis using GLM with a 'Quasi-Poisson' distribution showed 6 participants had significantly different SRs (Supplemental Materials Table S9). Participants that scored >89%, when combined, placed

54 SMPS (45%) to a category (31 L, 10 S, 3 CRM) with 100% SRs leaving 66 (55%) SMPs not identified (Table 6). Participants that scored <80%, when grouped, placed 12 SMPs (10%) to a category for a 100% SR (7L, 3 S, 2 CRM) leaving 108 (90%) SMPs not identified (Table 6).

Discussion

These survey data addressed a range of topics related to insect identification. The admonition attributed to an "Anonymous Crank" that 'keys are made by those who don't need them for those who can't use them' [32] has some validity if the metric of complete (100%) agreement between SMP and ITA designations was the expectation (Table 3). The most experienced participant categorized 1,341 SMPs with 15 samples that did not match the ITA for a SR of 98.88% (Table 2). The number of samples placed in the NI category was higher (≈10%) comparing more experienced observers (A&B) with the others (CDEF) but the samples not matching increased by at least a factor of 10 with experience level (Table 2). The SR for the combined data from the less experienced observers (98.07%, 19 not matching) (Table 5) was improved over the individual overall SRs (range 88.99-95.40%, 66-171 not matching) (Table 2). The in-house survey showed participants did not agree on the same SMP category for all samples illustrating the intuitive, subjective nature of categorizing morphometric descriptions. The level of agreement illustrated by SR values generated in this work was greater than reported in the literature for other taxonomic comparisons of molecular markers and morphometric designations that range from [42,43].

The lack of agreement on SMP category, by sample, between observers was not surprising given the subjective, qualitative nature of our category definitions and the morphological plasticity of *Reticulitermes*. Categories that provided the lowest agreement with

ITA also varied by participant. The L category exemplifies the aforementioned subjectivity along with high ITA agreement (Table 3). None of the 6 participants provided an individual SR of 100% for this category but individually they all had SRs >97% and combining either the 2 experienced or 4 less experienced data sets, the SR was 100% for L (Table 5). Category L accounted for over 50% of the collection using ITA and the high SR for that category indicates that this character can be used by others with confidence to identify *R. flavipes* samples (Figure 2). Observer C, who did not pass the P-S test on the first attempt (Table 1), had the lowest SRs among all participants for S (77.02%) and CRM (79.78%) while E had the next lowest SR for S (82.90%) and F had the next lowest SR for CRM at 88.58% (Table 3). The SMP category S provided the most variability among all participants (77.02-100.00%) but 4 of 6 observers had SRs >92% for category S (Table 3). The high proportion of agreement between SMP categories and ITA with the combined participant data was encouraging that a simple-to-explain morphological categorization could be used to identify 3 of the 5 *Reticulitermes* species common in the eastern USA.

Employment of simple morphological characters to identify selected *Reticulitermes* species can be justified when time and cost are considered, especially for large data sets (Table 4). Cost savings varied by participant, influenced by the number of SMPs categorized and ranged from >6,000 to >9,000 USD for individual participants (Table 2). Costs savings decreased with increasing numbers of observers and SRs increased when combining data from different participants (Table 5). The tradeoff between cost savings entails the number of participants, the samples removed from the ITA sample-pool and willingness to accept a certain lack-of-agreement between SMP categorizations and ITA. This point is illustrated by data from the most experienced participants that alone would have saved ≈$6,000 (Table 2) while their combined

data reduced saving to ≈$4,000 (Table 5). The SR data varied by participant and SMP category but interestingly the only participant (C) that scored <90% on their first P-S test (Table 1) provided the lowest individual overall SR (88.99%) (Table 2). Yet, not including the participant C data provided an increased savings of $1,400 because of the cost of an additional participant that offset the increase of ≈200 samples removed from the ITA pool with little improvement in the SR (Tables 7,8). The results of the P-S test in regard to predicting SR is a point worth noting in light of the ET survey results. Our results support the value of experience and the long-standing extension recommendation to send insect specimens to an 'expert' for identification. The data also suggests training and combining categorizations from multiple observers can provide a high degree of agreement with ITA for subterranean termite species identification. The idea that these characters could be used to identify *Reticulitermes* species prompted the ET survey.

The ET survey illuminated a disparity in the ability of citizen science participants, with an undefined range of taxonomic experience, to appropriately identify broadly defined morphological categories (Table 6). The ET participants fell into two groups - those that were able (SR>89%) and those that were unable (SR<80%) (Table 6) to reliably identify morphological characters in agreement with ITA despite encouragement to use and availability of a visual guide throughout the survey. There is still debate on the biological and psychological inputs and outcomes related to the ability of an individual to consistently recognize shapes, but our results suggest that some individuals have more difficulty than others consistently matching images with categories [44]. A surprising outcome of the ET was that the combined data from both groups were in complete agreement (100% SR) with ITA although the 'could-not' group classified far fewer samples (10% compared to 45%) (Table 6). The reliability of citizens to

appropriately identify insect pests by silhouette [45] supports our call to screen citizen science data for reliability. The results from both the in-house and ET surveys support screening either pre-or post-data collection to gauge the reliability of citizen science submissions prior to including data for analysis and is in agreement with Kosmala et al. (2016) along with their suggestions for designing citizen science projects.

It is interesting to note that all participants categorized one sample as CRM that did not match the ITA designation and despite the disparity observed between participants that one sample was what prevented the overall data from 100% agreement with ITA designations (Table 5). That point raises a second caution to carefully approach the assumption that termites collected from a single feeding site, log, or piece of wood-on-ground represent a single colony. *Reticulitermes* populations have 1-2% soldiers intuitively illustrating the potential for sharing a resource in the field rather than claiming territorial exclusivity compared to subterranean termites from the genus *Coptotermes* with 10-20% soldiers [47]. Examining one specimen per collection site, as we did, assuming a single colony/collection site was sometimes not valid (n=19/36, 53% of samples examined from the not matching category). Interestingly all 36 samples were collected in 2015 and 2016 before a discussion between the completist and authors on the need to place collections obtained from disparate locations on the same log into different vials (samples). Designing experiments to delineate the likelihood of mixed species occupying the same resource in the field should be an exciting opportunity for expanding our ecological understanding of *Reticulitermes*. Our recommendation is to consider designing sampling protocols that accept the potential for species sharing a single resource in the field.

Acknowledgments

We sincerely thank Jim Chase (Retired, Terminix) for collecting and donating all voucher specimens used in this study, Sabine Grube for developing the SMP grid used in these comparisons, Travis Redmond for assistance in dissecting and mounting SMPs, and all survey participants. Funding was provided by the Georgia Structural Pest Control Commission through the Georgia Department of Agriculture.

References

1. Nelson, L., L. Cool, C. Solek, and M. Haverty. 2008. Cuticular Hydrocarbons and Soldier Defense Secretions of Reticulitermes in Southern California: A Critical Analysis of the Taxonomy of the Genus in North America. Journal of Chemical Ecology 34: 1452–1475.

2. Clément, J.-L.; Howard, R.W.; Blum, M.S.; Lloyd, H. L'isolement spécifique des termites du genre *Reticulitermes* (Isoptera) du sudest des Etats-Unis. Mise en èvidence grâce à la chimie et au comportement d'une espèce jumelle de R. virginicus = R. malletei sp. nov. et d'une semi-species de R. flavipes. *C R Acad Sci Paris* 1986, 302:67–70.

3. Banks, N., and T.E. Snyder. 1920. A revision of the nearctic termites. bull. no 108. U.S. National Museum Government Printing Office, Washington D. C., USA.

4. Banks, F. A. 1946. Species Distinction in Reticulitermes (Isoptera: Rhinotermitidae). M.S. Thesis, University of Chicago, IL.

5. Nutting, W.L. 1990. Insecta: Isoptera, pp 997-1032. In D.L. Dindal, Soil biology guide, Wiley, NY, USA.

6. Brown, K.S., B. Kard, M. Payton. 2005. Comparative Morphology of Reticulitermes Species (Isoptera: Rhinotermitidae) of Oklahoma. Journal of the Kansas Entomological Society 78: 277.

7. Lim S.Y., and B.T. Forschler. 2012. Reticulitermes nelsonae, a New Species of Subterranean Termite (Rhinotermitidae) from the Southeastern United States. Insects 3: 62-90.

8. Miller, E. M. 1949. Florida termites: a handbook, pp. 1-30. University of Miami Press, Coral Gables, FL, USA.

9. Weesner F.M. 1965. The termites of the United States: a handbook. The National Pest Control Association. Elizabeth, N.J., USA.

10. Scheffrahn, R. and N.Y. Su. 1994. Keys to soldier and winged adult termites (Isoptera) of Florida. Florida Entomologist 77: 460-474.

11. Hostettler, N., D. Hall, and R. Scheffrahn. 1995. Intracolony Morphometric Variation and Labral Shape in Florida Reticulitermes (Isoptera: Rhinotermitidae) Soldiers: Significance for Identification. The Florida Entomologist 78: 119-129.

12. King, S.W., J.W. Austin, and A. L. Szalanski. 2007. Use of soldier pronotal width and mitochondrial DNA sequencing to distinguishing the subterranean termites, Reticulitermes flavipes (Kollar) and R. virginicus (Banks) (ISOPTERA: RHINOTERMITIDAE), on the Delmarva Peninsula: Delaware Maryland, AND Virginia U.S.A. Entomological News 118: 41-48.

13. Wang, C., X. Zhou, S. Li, M. Schwinghammer, M. Scharf, G. Buczkowski, and G. Bennett. 2009. Survey and identification of termites (Isoptera: Rhinotermitidae) in Indiana. Annals of the Entomological Society of America 102: 1029-1036.

14. DeSalle, R., B. Schierwater, H. Hadrys. 2017. MtDNA: The small workhorse of evolutionary studies. Frontiers in Bioscience 22: 873-887.

15. Haverty, M.I. and L.J. Nelson. 1997. Cuticular hydrocarbons of Reticulitermes (Isoptera: Rhinotermitidae) from northern California indicate undescribed species. Comparative Biochemistry and Physiology 118: 869-880.

16. Copren, K.A., L.J. Nelson, E.L.Vargo, and M.I. Haverty. 2005. Phylogenetic analyses of mtDNA sequences corroborate taxonomic designations based on cuticular hydrocarbons in subterranean termites. Molecular Phylogenetics and Evolution 35: 689-700.

17. Will, K., B. Mishler, and Q. Wheeler. 2005. The Perils of DNA Barcoding and the Need for Integrative Taxonomy. Systematic Biology 54: 844-851.

18. Austin, J., A. Szalanski, P. Uva, A-G. Bagnères, and A. Kence. 2002. A Comparative Genetic Analysis of the Subterranean Termite Genus Reticulitermes (Isoptera: Rhinotermitidae). Annals of the Entomological Society of America 95(6): 753-760.

19. Austin, J. W.; Szalanski, A. L.; Messenger, M. T. 2004. Mitochondrial DNA variation and distribution of the subterranean termite genus Reticulitermes (Isoptera: Rhinotermitidae) in Arkansas and Louisiana. Florida Entomologist, Gainesville, v. 87, n. 4, p. 473 – 480.

20. Su, N.Y., W. Weimin, R. Ripa, R. Scheffrahn, and R. Giblin-Davis. 2009. Identification of Chilean Reticulitermes (Isoptera: Rhinotermitidae) Inferred from Three Mitochondrial Gene DNA Sequences and Soldier Morphology. Annals of the Entomological Society of America 99: 352-363.

21. Janowiecki, M.; Vargo, E.L. Seasonal Activity, Spatial Distribution, and Physiological Limits of Subterranean Termites (Reticulitermes Species) in an East Texas Forest. Insects 2021, 12, 86.

22. Dayrat, B. 2005. Towards integrative taxonomy. Biological Journal of the Linnean Society 85: 407-415.

23. Austin, J., A.G. Bagnères, A. Szalanski, R. Scheffrahn, B.P. Heintschel, M. Messenger, J-L. Clément, and R. Gold. 2007. Reticulitermes malletei (Isoptera: Rhinotermitidae): A valid Nearctic subterranean termite from Eastern North America. Zootaxa 1554: 1-26.

24. Smith, A., M. Smith, and B. Kard. 2010. Oklahoma Formosan Subterranean Termite Surveillance Program and Termite Survey (Isoptera: Rhinotermitidae, Termitidae). Journal of the Kansas Entomological Society 83: 248-259.

25. Lumely, L., and F.A.H. Sperling. 2010. Integrating morphology and mitochondrial DNA for species delimitation within the spruce budworm (Choristoneura fumiferana) cryptic species complex (Lepidoptera: Tortricidae). Systematic Entomology 35: 416-428.

26. Martinet, B., T. Lecocq, N. Brasero, M. Gerard, K. Urbanová, I. Valterová, J.O. Gjershaug, D. Michez, and P. Rasmont. 2019. Integrative taxonomy of an arctic bumblebee species complex highlights a new cryptic species (Apidae: Bombus). Zoological Journal of the Linnean Society 187: 599–621.

27. Scheffrahn, R., J. Mangold, and N.Y. Su. 1988. A Survey of Structure-Infesting Termites of Peninsular Florida. The Florida Entomologist 71: 615-630.

28. Donovan, S.E., D.T. Jones, W.A. Sands, and P. Eggleton. 2000. Morphological phylogenetics of termites (Isoptera). Biological Journal of the Linnean Society 70: 467-513.

29. Little, Nathan & Blount, Nathan & Caprio, Michael & Riggins, John. (2014). Survey of Subterranean Termite (Isoptera: Rhinotermitidae) Utilization of Temperate Forests. Sociobiology. 61. 198-206. 10.13102/sociobiology.v61i2.198-206.

30. Szalanski, A., J. Austin, and C. Owens. 2003. Identification of Reticulitermes spp. (Isoptera: Reticulitermatidae) from South Central United States by PCR-RFLP. Journal of Economic Entomology 96(5): 1514-1519.

31. Garrick, Ryan C., Benjamin D. Collins, Rachel N. Yi, Rodney J. Dyer, and Chaz Hyseni. 2015. "Identification of Eastern United States Reticulitermes Termite Species via PCR-RFLP, Assessed Using Training and Test Data" Insects 6, no. 2: 524-537.

32. Smith Jr., J.P. 2017. Dichotomous Keys - Their Structure and Use. Botanical Studies 58. https://digitalcommons.humboldt.edu/botany_jps/58

33. Emerson, A. E. and E.M. Miller. 1943. A key to the termites of Florida. Flordia Entomologist 54: 184-187.

34. Heintschel, B., J.W. Austin, and R. Gold. 2006. Soldier Labral Morphology and Genetic Comparisons of Reticulitermes (Isoptera:Rhinotermitidae) from Texas. Sociobiology 48: 63-84.

35. Stephens, C. 2012. Alabama Rhinotermitidae: Nomenclature, Identification, Survey and Phenology. Ph.D. Dissertation, Auburn University, Alabama.

36. Liu, H., and A.T. Beckenbach 1992. Evolution of the Mitochondrial Cytochrome Oxidase II Gene among 10 Orders of Insects. Molecular Phylogenetics and Evolution 41: 41-52.

37. Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. Annals of the Entomological Society of America 87: 651–701.

38. Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acid Research 5: 1792.

39. Kalyaanamoorthy, S., B.Q. Minh, T.K.F. Wong, A. von Haeseler, and L.S. Jermiin. 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods, 14: 587-589.

40. Hoang, D.T., O. Chernomor, A. von Haeseler, B.Q. Minh, and L.S. Vinh. 2018. UFBoot2: Improving the ultrafast bootstrap approximation. Molecular Biology and Evolution 35: 518–522.

41. Nguyen, L-T. H.A. Schmidt, A. von Haeseler, and B.Q. Minh. 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Molecular Biology and Evolution 32: 268-274.

42. Wang, Y., Nansen, C., & Zhang, Y. 2016. Integrative insect taxonomy based on morphology, mitochondrial DNA, and hyperspectral reflectance profiling. Zoological Journal of the Linnean Society, 177(2), 378-394. 10.1111/zoj.12367

43. Wagner, I., Koch, N. I., Sarsby, J., White, N., Price, T. A. R., Jones, S., Hurst, J. L., & Beynon, R. J. 2020. The application of rapid evaporative ionization mass spectrometry in the analysis of Drosophila species-a potential new tool in entomology10.1098/rsob.20.0196

44. Wagemans, J., J.H. Elder, M. Kubovy, S. E. Palmer, M.A. Peterson, M. Singh, R. v.d. Heydt. 2012. A century of gestalt psychology in visual preception 1. Perceptual grouping and figure-ground organization. Psychol Bull. 138(6): 1172-1217. doi:10.1037/a0029333

45. Penn, H., Penn, J., Potter, M., & Hu, W. 2017. *BED BUGS AND HOTELS* Traveler Insights and Implications for the Industry. American Entomologist. 63(2): 79-88.

46. Kosmala, M., Wiggins, A., Swanson, A., & Simmons, B. 2016. Assessing data quality in citizen science. Front Ecol Environ 2016; 14(10): 551–560, doi:10.1002/fee.1436

47. Haverty, Michael. 1977. The proportion of soldiers in termite colonies: A list and a bibliography (Isoptera). Sociobiology. 2. 199-216.

Tables

Table 2.1. Survey participants insect taxonomy experience, number of P-S tests and P-S success rate [SR].

Survey Participant	Insect Taxonomy Experience /Educational Level	Pre-survey test SR ($1^{st}/2^{nd}$ test)
A	Examined thousands of termite soldier mandible pairs/ Ph.D. Student, Entomology	NA
B	Examined thousands of termite soldier mandible pairs/ P.I., Entomology	NA
C	Classroom and research experience with insects/ Ph.D. Student, Entomology	89.2% / 94.2%
D	Classroom and research experience with insects/ Ph.D. Student, Entomology	96.7%
E	Classroom experience with insects/ Journalism/Ecology Undergraduate Student	97.5%
F	Classroom experience with insects/ Agricultural Science Undergraduate Student	100%
G	No classroom or research experience with insects/ Junior High School Student	78.4 / 90%
H	No classroom or research experience with insects/ Junior High School Student	86.7 / 95%

Table 2.2. The number, by survey participant, of SMPs placed in a category (L, S, CRM), not identified to a category (NI), that matched and did not match an ITA species designation along with success rate [SR] and estimated cost savings (ITA *Gross* total (15,497) − ((number of samples not identified (column 3) × 6.95) + SMP *Gross* total (2,305 + 97.50 per participant))).

Participant	Identified to 1 of 3 morphology categories	Not identified by SMP	Morphology and ITA designations matched	Morphology and ITA designations not matching	Overall SR	Estimated Cost Savings
A	1,274	955	1,259	15	98.82%	≈11,262 USD
B	1,341	888	1,326	15	98.88%	≈11,727 USD
C	1,553	676	1,382	171	88.99%	≈13,201USD
D	1,435	794	1,369	66	95.40%	≈12,381 USD
E	1,676	553	1,577	99	94.09%	≈14,056 USD
F	1,681	548	1,599	82	95.12%	≈14,091 USD

Table 2.3. The number of SMPs designated to a category by survey participant, samples that did not match (NM) ITA species designations, corresponding category success rates, and proportion of samples NI requiring additional identification.

Part	L	L and ITA NM	L SR	S	S and ITA NM	S SR	CRM	CRM and ITA NM	CRM SR	Not Identified/ % of entire collection
A	822	1	99.88%	139	2	98.56%	313	12	97.17%	955/43%
B	851	1	99.88%	176	0	100.00%	314	14	95.54%	888/40%
C	872	22	97.48%	409	94	77.02%	272	55	79.78%	676/30%
D	912	27	97.04%	235	18	92.34%	288	21	92.71%	794/36%
E	987	12	98.78%	386	66	82.90%	303	21	93.07%	553/25%
F	1013	21	97.93%	344	24	93.02%	324	37	88.58%	548/24%
G*	124	15	87.90%	54	26	51.90%	26	7	73.10%	NA
H*	59	6	89.80%	34	16	52.90%	21	8	61.90%	NA

Table 2.4. Time and cost variables used to compare to two techniques for identification of subterranean, termite species. Soldier mandible pair [SMP] morphology and mtDNA haplotypes using an ITA approach. Details can be found in the Time and Cost Appendix (Supplemental Materials Table 2).

Technique	**ITA**		**Mandible Morphology**	
	Time	Cost in USD	Time	Cost in USD
Preparation	384 hours	9,272	149 hours	2,305
Analysis	15 hours	6,225	5 hr (survey) + 1.5 hr (analysis) per participant	97.50 per participant
Gross Total		15,497.00		2,402.50
Per Sample Total		6.95 USD		1.08 per participant 1.30 for 6 participants

Table 2.5. The number of SMPs identified by all participants combined, 2 more experienced participants (AB) combined, and 4 participants that completed the P-S test combined (D-F). Results organized by individual category (L, S, CRM), and combined category data, including the number that did and did not match ITA with corresponding success rates [SR] for each, and estimated cost savings (ITA *Gross* total (15,497) − ((number of samples not identified × 6.95) + SMP *Gross* total (2,305 + 97.50 per participant))).

SMP Category/ITA Species Designation	Number of SMPs by category or combined data	Number of SMPs that matched ITA	Number of categorized SMPs and ITA that were 'not-matching'	SR	Estimated Cost Savings
ALL Participants (n=6)					
Longest/Rf	613	613	0 NM	100.00%	
Shortest/Rn	68	68	0 NM	100.00%	
Curved/Rv	223	218	7 NM	97.76%	
Combined category data	904	899	7 NM	99.45%	≈9,178 USD
Participants AB (n=2)					
Longest/Rf	667	667	0 NM	100.00%	
Shortest/Rn	70	70	0 NM	100.00%	
Curved/Rv	290	278	10 NM	95.86%	
Combined category data	1027	1,017	10 NM	99.03%	≈9,643 USD
Participants CDEF (n=4)					
Longest/Rf	626	626	0 NM	100.00%	
Shortest/Rn	136	130	6 NM	95.59%	
Curved/*Rv*	223	210	13 NM	94.17%	
Combined category data	985	966	19 NM	98.07%	≈9,546 USD
Participants DEF (n=3)					
Large/*Rf*	755	754	1 NM	99.87%	
Small/*Rn*	182	176	6 NM	96.70%	
Curved/*Rv*	247	233	14 NM	94.33%	
Combined category data	1,184	1,163	21 NM	98.23%	≈10,831 USD

Table 2.6. The number of SMPs identified by ET participants with an overall SR >89% (range 100-89%) and <80% (range 79-53%) by individual category (L, S, CRM) and combined category data, including the number scored by category, and the number categorized that did and did not match ITA with corresponding success rates [SR] and proportion of the collection identified.

SMP Category/ITA Species Designation	Number of SMPs ET participants scored to same category	Number of categorized SMPs that matched ITA	Number of categorized SMPs that did not match ITA 'not matching'	Total SR/% of collection ID
ET Participants with **>89% SR** (n=15)				
L/Rf	31	31	0	100.0%/25.8%
S/Rn	10	10	0	100.0%/8.3%
CRM/Rv	13	13	0	100.0%/10.8%
Combined category data	54	54	0	100.0%/45%
ET Participants with **<80% SR** (n=10)				
L/Rf	7	7	0	100.0%/5.8%
S/Rn	3	3	0	100.0%/2.5%
CRM/Rv	2	2	0	100.0%/1.7%
Combined category data	12	12	0	100.0%/10.0%

Figure Captions

Figure 1.1. SMPs representing five species of *Reticulitermes* from Georgia, USA. In the top row SMPs representing R. flavipes - Longest (L), R. nelsonae - Shortest (S), and R. virginicus - Curved Right Mandible (CRM), the second row are species in the not-identified category (NI), R. hageni and R. malletei. (Photographs using Keyence VHX-7000 Digital Microscope (Keyence Corporation of America, Itasca, IL, USA 60143) at 100X magnification).

Figure 1.2. Collapsed Maximum-Likelihood HKY + F + I+ G4 tree topology assembled using the model of best fit program ModelFinder (Kalyaanamoorthy et al., 2017) in IQ-Tree v 2.6.12 (Nyugen et al. 2015) and annotated in FigTree v. 1.4 (http://tree.bio.ed.ac.uk/software/figtree/) utilized for inferring species identification utilizing 2,229 COII mtDNA sequences paired with 35 ITA identified reference sequences from alates and 18 published references accessioned from GenBank (Supplemental Materials Table S10). Reference sequences (Rf n=17, Rn n=9 Rv n=8, Rm n=10, Rh n=8, C.f. n=1) were not reported in the total species count for each clade listed above. A *Coptotermes formosanus* COII sequence (AY683218) was used as the extant group. Branch numbers represent percentage of posterior probability from 2,000 UF Bootstrap iterations with support of ≥50% (Hoang et al. 2018).

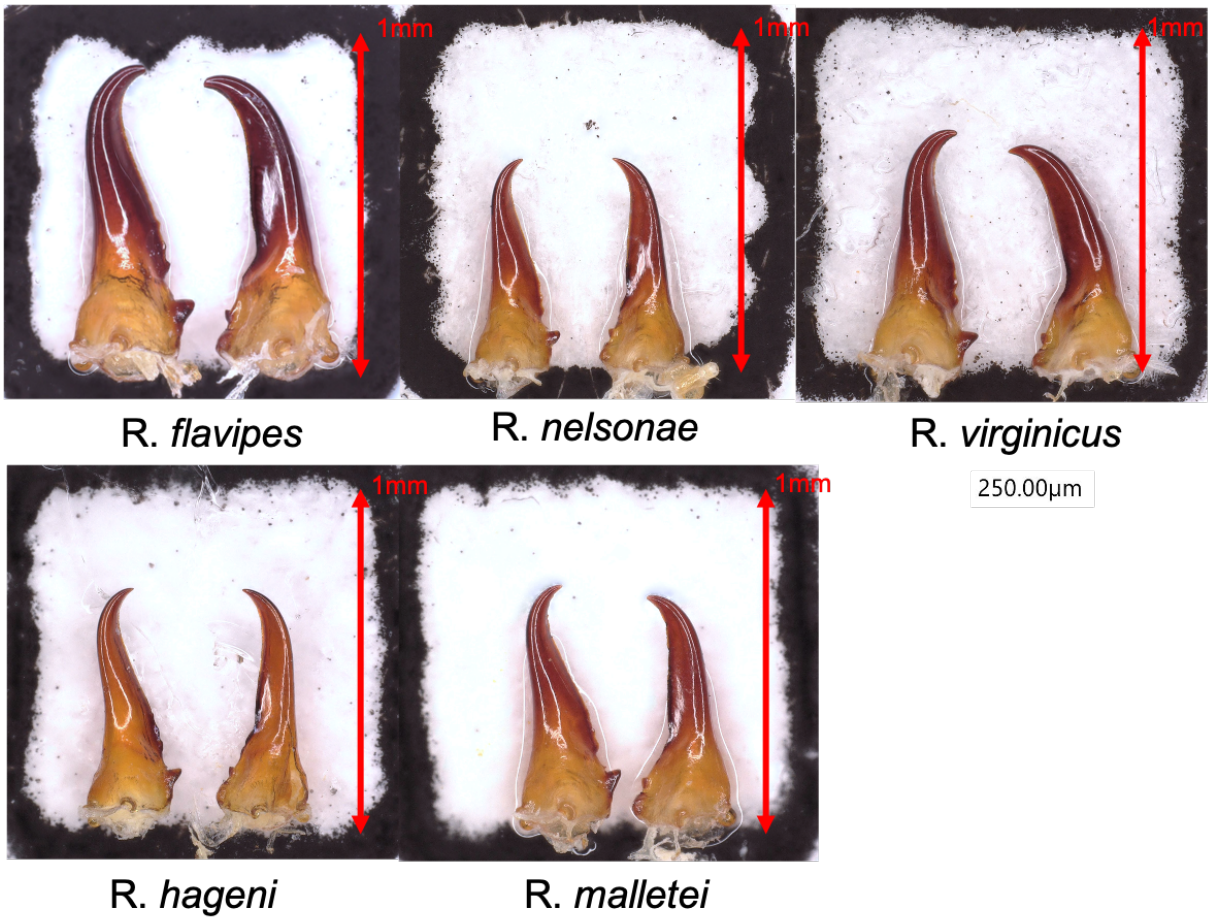
1mm
1mm
1mm
R. flavipes
R. nelsonae
R. virginicus
250.00µm
1mm
1mm
R. hageni
R. malletei

Figure 1.1

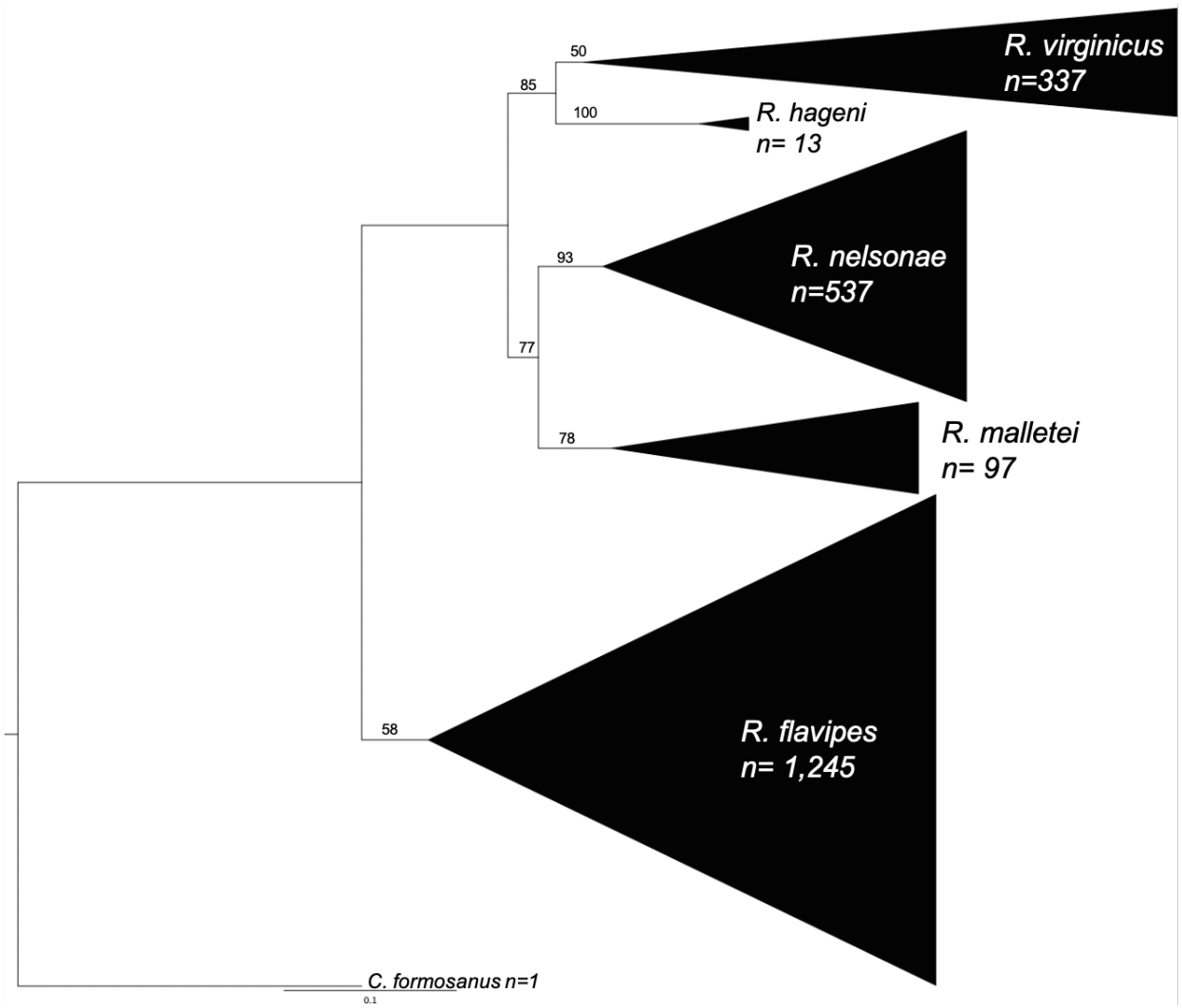
50
85
100
93
77
78
58
R. virginicus
n=337
R. hageni
n= 13
R. nelsonae
n=537
R. malletei
n= 97
R. flavipes
n= 1,245
C. formosanus n=1
0.1

Figure 1.2

APPENDIX A: SUPPLEMENTARY MATERIALS

Supplemental Methods for Adapted Wizard Genomic DNA Purification Protocol

One termite worker, air-dried for 1 hr, was added to 150 µL chilled (4°C) Nuclei Lysis solution (Promega, WI) in a 1.7 mL microcentrifuge tube (Thermo Fisher Scientific, Waltham, MA), crushed with a sterilized pestle, combined with 17 µL of 20mg/mL Proteinase K (New England Biolabs), and incubated for 2 hr at 65°C. RNAse A solution (5 µL of 4 mg/mL) (Promega, WI) was added to the mixture and incubated for 30 mins at 37°C. Promega protein precipitation solution (200 uL) was added to the mixture, chilled at 4°C for 5 mins, and centrifuged for 4 mins at a maximum speed of 12,400 RPM (Thermo Fisher Scientific, Waltham, MA). The supernatant (700 uL) was removed and added to a new 1.7 mL microcentrifuge tube. Molecular grade isopropanol (600 uL) (Thermo Fisher Scientific, Waltham, MA) was added to the 1.7 mL tube and centrifuged at maximum speed. The resulting supernatant was removed (1100 uL), and a second precipitation was performed with 600uL of 70% ethanol (Decon Laboratories Inc, King of Prussia, PA) with a 20 min centrifuge cycle at maximum speed. The ethanol supernatant was removed, and the pellet was air-dried for 1 hr and resuspended in 40 uL of DNA rehydration solution (Promega, WI) for an hour at 65°C.

Supplemental Results for Examining Sources of Error due to mixed species in a single sample.

Thirty-six samples where category placements from participants did not match ITA were examined for mixed species within a single sample. Thirty-four out of 36 samples provided additional workers for updated ITAs and 21/36 provided additional SMPs.

Twenty of 36 samples provided evidence of at least 2 species in one sample (vial). Eighteen out of the mixed 20 samples were moved from not matching to matching during this examination. Eleven samples provided evidence of mixed populations that matched the initial

ITA, but not SMP category (2_1-4, 1_7-1, 116_8-2, 72_5-1, 1_3-3, 48_7-1, 9_2-2, 65_5-3B, 86_5-2, 2_8-3, 94_10-1) termed ‘mixed by ITA but not category’ in Supplemental Table S1. Five samples provided evidence of mixing from additional SMPs that matched the original category, but not initial ITA (134_9-1, 26_2-3, 13_8-3, 48_6-3, 134_7-3) termed ‘mixed by category but not ITA’ (Table S1). Two samples provided evidence of mixed species matching both original ITA and SMP categories after additional examination (103_9-5, 1_5-2) termed ‘mixed by category and ITA’ (Table S1).

Eighteen of the 36 samples categorized by participants still did not match initial ITAs and are included when calculating SR’s. Two samples (2_1-5 and 134_3-1) were originally categorized as CRM by group CDEF, and the initial ITAs were Rn. The updated ITAs provided evidence of 2 species not matching participant category (Rf, Rn) and are included when discussing proportions of mixed samples, but still considered errors when calculating SRs (termed ‘mixed by ITA but not matching participant’, Table S1). Six samples provided additional SMPs and workers for identification; (48_9-2; 99_2-2; 18_6-3; 97_1-1; 45_5-2; 34_2-4) that did not match the initial ITA. Two samples (38_7-5, 19_3-2) provided additional SMPs that did not match the initial ITA and did not provide additional workers for sequencing. Six samples provided additional workers that did not match the initial ITA (22_9-2, 41_4-4, 85_2-5, 41_6-3, 116_5-3, 88_6-4), and did not provide additional SMPs for excision and mounting. These 14 samples are termed ‘no evidence of mixed population and not matching participant’ in Supplemental Table S1.

Table S2.1. Samples (n=36) examined for sources of error due to mixed populations within a sample and with initial not matching ITA designation from one worker per sample, updated ITA designations from 4-5 workers per sample, collection date of sample, sample local id, and initial SMP category listed by participant. "N/A" indicates no additional workers or soldiers were available for those samples.

Legend	
	No evidence of mixed population and not matching participant
	Mixed by ITA but not Category
	Mixed by Category but not ITA
	Mixed by ITA and Category
	Mixed by ITA or category and not matching participant

Initial ITA	Updated ITA	Updated SMP Category	Date	Sample	**A**	**B**	**C**	**D**	**E**	**F**
Rm	Rm	N/A	6/3/15	22_9-2	CRM	CRM	CRM	CRM	CRM	CRM
Rh	N/A	S	6/1/15	38_7-5	S					
Rf	Rf	S	6/9/15	48_9-2	S					
Rn	Rn	?	3/3/15	101_7-1	S	CRM	S			CRM
Rm	N/A	L	3/2/16	19_3-2		L				
Rh	Rh	N/A	7/31/16	41_4-4			S	S	S	S
Rh	Rh	N/A	8/2/16	85_2-5			S	S	S	S
Rm	Rm	N/A	7/31/16	41_6-3			S	S	S	S
Rm	Rm	S	4/15/15	99_2-2			S	S	S	S
Rm	Rm	S, ?	6/24/16	18_6-3			S	S	S	S
Rm	Rm	S	5/23/16	97_1-1			S	S	S	S
Rn	Rn	N/A	4/26/16	116_5-3			CRM	CRM	CRM	CRM
Rn	Rn	N/A	3/9/16	88_6-4			CRM	CRM	CRM	CRM
Rn	Rn	CRM, ?	3/23/16	45_5-2			CRM	CRM	CRM	CRM
Rn	Rn	CRM, ?	4/19/15	34_2-4			CRM	CRM	CRM	CRM
Rf	Rf	?	3/22/16	134_5-1			CRM	CRM	CRM	CRM
Rn	Rn	CRM	4/26/16	116_5_2	CRM	CRM		CRM		CRM
Rn	Rn	N/A	4/26/16	116_5_3	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn	N/A	1/26/16	142_8_1	CRM	CRM	CRM	CRM	CRM	CRM

Rn	Rn	N/A	3/2/16	19_8_2	CRM	CRM	S		CRM	
Rn	Rn	N/A	6/1/15	38_7_3		CRM			CRM	
Rn	Rn	N/A	3/9/16	88_8_1	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rf, Rn	N/A	4/20/15	2_1-5			CRM	CRM	CRM	CRM
Rn	Rf, Rn	S	3/22/16	134_3-1			CRM	CRM	CRM	CRM
Rn	Rn, Rf	CRM	4/26/16	116_5_5	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rf	N/A	4/26/16	116_6_1	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rf	L, ?	5/9/15	33_4_2	CRM	CRM	S		CRM	CRM
Rn	Rn, Rf	S, ?	4/24/16	46_6_3			CRM	S	S	
Rn	Rn, Rv	N/A	4/20/15	2_1-4	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rf	N/A	6/18/15	1_7-1	L	L	L	L	L	L
Rn	Rn, Rf	N/A	4/26/16	116_8-2	L	L	L	L	L	L
Rm	Rm, Rv	CRM	1/26/15	72_5-1	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rv, Rf	N/A	6/18/15	1_3-3	CRM	CRM	CRM	CRM	CRM	CRM
Rf	Rf, Rm	N/A	6/9/15	48_7-1	S					
Rn	Rn, Rv	CRM	3/23/15	86_5-2			CRM	CRM	CRM	CRM
Rf	Rf, Rv	N/A	4/20/15	2_8-3			CRM	CRM	CRM	CRM
Rf	Rf, Rn	N/A	6/6/16	94_10-1			S	S	S	S
Rv	Rv, Rn	N/A	2/2/16	9_2-2		S				
Rf	Rf, Rv	CRM	2/8/15	65_5-3B		CRM				
Rn	Rn, Rv	N/A	6/20/16	102_2_3	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rv, Rf	N/A	6/20/16	103_4_3	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rv	N/A	3/17/15	126_2_1	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rv	CRM	3/17/15	126_8_2	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rv	CRM	1/29/15	128_6_1	CRM	CRM	S			CRM
Rn	Rn, Rv	S, CRM	2/9/15	43_4_1	CRM	CRM	S			CRM
Rn	Rn, Rv	CRM	4/15/15	99_6_3	CRM	CRM	CRM	CRM	CRM	CRM
Rf	Rf	L, S, ?	3/22/16	134_9-1	S	S	S	S	S	S
Rn	Rn	S	7/28/16	26_2-3	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn	?, S	4/21/15	13_8-3	CRM	CRM	CRM	CRM	CRM	CRM
Rm	Rm	?	6/9/15	48_6-3	S					
Rf	Rf	L, S	3/22/16	134_7-3		S	S	S	S	S
Rn	Rn	S, CRM	4/15/15	99_7_1	CRM	CRM	S		CRM	CRM
Rn	Rf, Rn	?, L	3/20/16	103_9-5	L		L	L	L	L
Rv	Rf, Rv	L, CRM	6/18/15	1_5-2	L					
Rn	Rn, Rv	S, CRM	6/20/16	102_2_1	CRM	CRM	CRM	CRM	CRM	CRM
Rn	Rn, Rv	CRM, ?	1/29/15	126_8_1	CRM	CRM	CRM	CRM	CRM	CRM

Rn	Rn, Rv, Rf	?, CRM, L	1/29/15	128_3_2	CRM	CRM	S		CRM	CRM

Table S2.2. Appendix for time and cost analysis of ITA versus SMP Morphology method containing information about average available prices of reagents (as of December 2021) and supplies including labor.

Time and Cost Analysis Appendix
ITA Preparation (Time): Sample selection, loading, dry time, sample grinding, Proteinase K digestion, Rnase A digestion, protein precipitation, isopropanol elution, ethanol elution, drying time, DNA rehydration ≅16 hrs x 24 plates = 384 hours of labor @ 15.00 USD an hour (*adapted from* Wizard Genomic DNA Purification Kit (Promega, Madison WI)) Total: 5,760.00 USD
ITA Preparation (Cost): Reagents: Taq Polymerase (Quantabio, MA, USA) ~428.10 DNTPS (New England Biolabs, MA, USA) ~79.28 Exonuclease I (New England Biolabs, MA, USA)~420.00 Quick CIP (New England Biolabs, MA, USA) ~450.00 Protein Precipitation Solution (Promega, WI, USA) ~727.20 Nuclei Lysis Solution (Promega, WI, USA)~786.24 Hardware: 1.7mL tubes) (VWR, PA, USA) ~ 180.84 Micropipette Tips (10 uL, 200 uL, 1000 uL) (VWR, PA, USA)~440.30 Total: 3,512.00 USD
ITA Analysis (Time) Data compilation and phylogenetic analysis (Geneious Prime, http://www.geneious.com, Kearse et al., 2012) ≅ 15 hrs @ 15 USD an hour = 225.00 USD Total: 225.00 USD
ITA Analysis (Cost) Direct Sanger Sequencing 96 Well Plates (Eurofins, KY, USA) = 250.00 x 24 = 6,000.00 USD Total: 6,000.00 USD
Gross Total: 15,496.96 USD/ 2,229 samples = 6.95 USD/sample
SMP Morphology Preparation (Time) Sample excision and mounting, 4 minutes/pair x 2,229 pairs ≅ 149 hrs of labor @ 15 USD per hour Total: 2,235.00 USD
SMP Morphology Preparation (Cost) Hardware Petri Dishes (VWR, PA, USA) ~60.00 Double Sided Tape (Scotch, MN, USA) ~10.00 Total: ~70.00 USD
SMP Morphology Analysis (Time) Per participant scoring ~5 hours @ 15 USD an hour + data compilation and analysis ~1.5 hours @ 15 USD an hour = 97.50 USD per participant

Total: ≅97.50 USD *for 1 participant;* ≅ 585.00 USD *for 6 participants*
SMP Morphology Analysis (Cost) There were no additional analysis costs directly associated with this method other labor for data compilation and analysis.
Gross Total *for 1 participant*: 2,402.50 USD/ 2,229 samples =1.08 $ USD/sample **Gross Total *for all 6 participants:*** 2,890.00 USD / 2,229 samples = 1.30 $ USD/sample

Table S2.3. P-values comparing total SRs between participant groups from Pearson's Chi-squared analyses with Yates' continuity correction conducted in R. 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

	ALL	AB	CDEF	DEF
ALL		>0.05	>0.05	>0.05
AB			>0.05	>0.05
CDEF				>0.05
DEF				

Table S2.4. P-values comparing total SRs between participants from a Generalized Linear Model with 'Quasi-Poisson' distribution conducted in R. 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

GLM p-value <2e-16***	
b	>0.05
c	<0.05
d	>0.05
e	>0.05
f	>0.05

Table S2.5. P-values from a post hoc Tukey's HSD test following the Generalized Linear Model conducted in R. 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

Tukey's HSD	
Comparison	p-value
b-a	>0.05
c-a	<0.01
d-a	>0.05
e-a	>0.05
f-a	>0.05
c-b	<0.01
d-b	>0.05
e-b	>0.05
f-b	>0.05
d-c	<0.05
e-c	>0.05
f-c	<0.05
e-d	>0.05
f-d	>0.05
f-e	>0.05

Table S2.6. P-values within individual SRs by category (L, S, CRM) from Pearson's Chi-squared analyses with Yates' continuity correction conducted in R. 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

Participant A	L	S	CRM
L		>0.05	>0.05
S			>0.05
Participant B	L	S	CRM
L		>0.05	>0.05
S			>0.05
Participant C	L	S	CRM
L		<0.01	>0.05
S			>0.05
Participant D	L	S	CRM
L		>0.05	>0.05
S			>0.05
Participant E	L	S	CRM
>0.05L		0.05145	>0.05
S			>0.05
Participant F	L	S	CRM
L		>0.05	>0.05
S			>0.05

Table S2.7. The number, by ET participant (1-25), of 120 SMPs placed in a category, not placed in a category, and that matched and did not match an ITA species designation along with the success rate [SR].

Part.	Identified to 1 of 3 morphology categories	Not identified by morphology category	Morphology and mtDNA designations matched	Morphology and ITA designations not matching	SR
1	97	23	93	3	95.88%
2	98	22	97	1	98.98%
3	101	19	99	2	98.02%
4	101	19	98	3	97.03%
5	112	8	66	46	58.93%
6	93	27	90	3	96.77%
7	114	6	91	23	79.82%
8	96	24	86	10	89.58%
9	106	14	69	37	65.09%
10	118	2	72	46	61.02%
11	103	17	76	27	73.79%
12	114	6	60	54	52.63%
13	100	20	99	1	99%
14	93	27	85	8	91.40%
15	106	14	101	5	95.28%
16	110	10	59	51	53.64%
17	103	17	96	7	93.20%
18	98	22	91	7	92.86%
19	96	24	69	27	71.88%
20	120	0	85	35	70.83%
21	91	29	72	19	79.12%
22	100	20	88	12	89.00%
23	97	23	97	0	100%
24	98	22	88	10	89.79%
25	91	29	88	3	96.70%

Table S2.8. The number of SMPs designated to a category by ET participant (1-21), samples that did not match ITA species designations, corresponding success rates, and proportion of samples requiring additional methods of identification.

Part.	L	L and mtDNA not matching	SR	S	S and mtDNA not matching	SR	CRM	CRM and mtDNA not matching	SR	NI/ % of entire collection
1	45	1	97.78%	20	1	95%	32	1	96.88%	23/19.17%
2	48	0	100%	22	1	95.45%	31	0	100%	22/18.33%
3	50	2	96%	21	0	100%	30	0	100%	19/15.83%
4	47	0	100%	22	1	95.45%	32	2	93.75%	19/15.83%
5	25	2	92%	42	19	54.76%	45	24	46.67%	8/6.67%
6	47	0	100%	24	3	87.5%	22	0	100%	27/22.5%
7	57	9	84.21%	34	13	61.76%	23	1	95.65%	6/5%
8	47	1	97.87%	25	6	76%	24	3	87.5%	24/20%
9	58	16	72.41%	37	17	54.05%	11	4	63.63%	14/11.67%
10	37	10	72.97%	27	8	70.37%	54	28	48.14%	2/1.67%
11	38	4	89.47%	28	9	67.86%	37	13	64.86%	17/14.17%
12	31	9	70.97%	6	0	100%	71	45	36.62%	6/5%
13	47	0	100%	23	1	95.65%	30	0	100%	20/16.67%
14	40	0	100%	21	1	95.24%	32	7	78.13%	27/22.5%
15	51	3	94.12%	24	2	91.67%	31	0	100%	14/11.67%
16	29	7	75.86%	27	10	62.96%	54	32	40.74%	10/8.33%
17	48	0	100%	24	5	79.17%	31	2	93.55%	17/14.17%
18	47	0	100%	26	6	76.92%	25	1	96%	22/18.33%
19	39	6	84.62%	26	9	65.38%	31	12	61.29%	24/20%
20	60	9	85%	46	25	45.65%	14	1	92.85%	0/0%
21	44	6	86.36%	16	2	87.5%	31	11	64.51%	29/24.17%
22	47	0	100%	21	3	85.71%	32	8	75.00%	20/16.67%
23	47	0	100%	22	0	100%	28	0	100%	23/19.17%
24	46	0	100%	21	2	90.47	31	8	74.19%	22/18.33%
25	42	0	100%	25	3	88%	24	0	100%	29/24.17%
26	35	0	100%	28	8	71.43%	22	0	100%	35/26.17%
27	47	1	97.87%	23	4	82.61%	29	1	96.55%	21/17.5%
28	47	0	100%	25	8	68.00%	38	12	68.42%	10/8.33%
29	37	4	89.19%	33	11	66.67%	20	4	80.00%	30/25%
30	47	0	100%	19	1	94.74%	30	2	93.33%	24/20%
31	46	3	93.48%	35	21	40.00%	18	12	33.33%	21/17.5%
32	40	0	100%	29	8	72.41%	20	9	55.00%	31/25.83%
33	45	0	100%	11	0	100.00%	41	14	65.85%	23/19.17%
34	51	9	82.35%	13	4	69.23%	36	14	61.11%	20/16.67%
35	46	0	100%	24	3	87.50%	30	0	100%	20/16.67%
36	36	0	100%	18	1	94.44%	22	7	68.18%	44/36.67%
37	43	5	88.37	16	0	100.00%	26	5	80.77%	35/29.17%
38	37	0	100%	19	2	89.47%	35	13	62.86%	15/12.5%

Table S2.9. P-values comparing total SRs between ET participants from a Generalized Linear model with 'Quasi-Poisson' distribution conducted in R. 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

GLM p-value < 2e-16 ***	
2	>0.05
3	>0.05
4	>0.05
5	<0.05*
6	>0.05
7	>0.05
8	>0.05
9	<0.05*
10	<0.05*
11	>0.05
12	<0.05*
13	>0.05
14	>0.05
15	>0.05
16	<0.01*
17	>0.05
18	>0.05
19	<0.05
20	>0.05
21	>0.05
22	>0.05
23	>0.05
24	>0.05
25	>0.05

CHAPTER 3

BIODIVERSITY AND DISTRIBUTION OF *RETICULITERMES* IN THE SOUTHEASTERN USA[1]

[1]Allison Johnson and B.T. Forschler. To be submitted to Insects.

Abstract

Reticulitermes subterranean termites are widely distributed ecosystem engineers and structural pests yet describing their species distribution world-wide or regionally has been hindered by taxonomic uncertainties. Morphological plasticity confounds use of taxonomic keys while recent species descriptions and molecular techniques lacking taxonomic support have caused a muddle for interpreting the literature on *Reticulitermes* species distributions. We employed an integrative taxonomic approach combining behavioral, morphological, and molecular techniques to identify 4,371 *Reticulitermes* samples to species. Five *Reticulitermes* species were collected from wood-on-ground at 1,570 sites covering 153,900 km^2 in the state of Georgia, USA. Three species were collected throughout Georgia with *R. flavipes* identified from every county (n=159). *R. nelsonae* was the second most frequently collected species found in 128 counties with *R. virginicus* third from 122. Two species had distributions confined to the northern part of the state. *R. malletei* was collected from 73 counties while the least collected species *R. hageni* was found in 16. Results show that the most recently described species (*R. nelsonae*, 2012) is widely distributed and the second-most frequently encountered termite representing 23% of all samples. The invasive species *R. flavipes* represented half of all the samples collected while *R. hageni* the least at less than 1%. A search of GenBank identified a number of accessions mis-matched to a species designation resulting in the literature under-reporting the biodiversity of the genus. We therefore outline a path to standardize methods for species identification using an integrated taxonomic approach with appropriate barcodes for consistent identification across research teams worldwide. The data also illuminate new opportunities to examine questions related to the ecology, evolution, dispersal, and resource partitioning behaviors of these sympatric species across distinct geographical regions.

Keywords: subterranean termite, mtDNA markers, integrative taxonomy, species distribution

Introduction

Biodiversity is a term used to describe the variety of living organisms on earth that is founded in the nomenclature used to identify an animal life form to a species designation. A number of broad issues constrain delineating species not the least of which are long-standing, global, concerns affecting all biological systems including the taxonomic impediment [1] taxonomic wrong-headiness [2], and a lack of consensus on appropriate integrative methods for species identification [3, 4, 5, 6, 7] all contingent on adherence to the internationally agree codes of rules for animals outlined in International Code of Zoological Nomenclature (ICZN) [8]. Measuring insect biodiversity is a critical component of understanding the impact of anthropogenic changes affecting life on earth as exemplified by discussions surrounding the so-called insect apocalypse [9, 10]. The acknowledged diversity of insects however requires using a common system for identifying species that, if not consistent across studies, often hinders comparing results on any number of topics including insect distributions [11].

The renowned ecological and economic impact of subterranean termites [12, 13, 14, 15], has not translated into a concerted effort to understand the global distribution of *Reticulitermes* species across their Holarctic range. Subterranean termite risk maps of the USA place Georgia, and surrounding states, in the middle of a 'very-high hazard' termite belt [13, 16, 17, 18]. Acknowledging the dense populations in the region has, however, translated into few concerted appraisals of *Reticulitermes* distributions in that same area. Impediments for synthesizing termite distribution data from the literature includes the variety of techniques used to collect

Reticulitermes such as examining wood on ground, in-ground surveillance/monitoring devices, light traps, collections from Pest Management Professionals (PMPs) or property owners, and museum collections (Tables S1, S2). Discerning *Reticulitermes* distributions in the southeastern USA from the literature is also complicated by two species descriptions in the last thirty years [19, 20] meaning surveys completed before 2012 should be interpreted with discretion. Nelson et al. [21] eloquently, more than a decade ago, detailed the main obstacles to *Reticulitermes* species identification while echoing the call for a taxonomic revision of the genus that goes back over 50 years [22].

The usefulness of taxonomic keys for *Reticulitermes* species identification is confounded by morphological plasticity and resulting range of phenotypes [21, 23, 24, 25, 26, 27]. Molecular markers have numerous advantages for obtaining consistent across-research program species identification, yet markers used for *Reticulitermes* identification in the southeastern USA have been published with questionable, if any, taxonomic support especially prior to 2012 rending those designations suspect [21, 28, 29, 30]. An integrative taxonomic approach [ITA] using molecular markers must include reference sequences associated with specimens identified by morphology, behavioral attributes, or chemical signatures to interpret phylogenies and increase the confidence in species designations [3, 31, 32].

The objective of this study was to identify specimens using an ITA-validated mtDNA marker applied to 4,371 samples collected from wood-on-ground (WoG) at 1,570 wildland sites over the entire state of Georgia, USA to illuminate species distributions and proportions. Results are reported for 5 described species, *Reticulitermes flavipes* (Kollar) 1837 (Rf), *R. virginicus* (Banks) 1907 (Rv), *R. hageni* Banks 1920 (Rh), *R. malletei* Clément et al. 1986 (Rm), *R.*

nelsonae Lim and Forschler 2012 (Rn) and discussed in relation to the literature on the taxonomy, ecology, evolution, management, and future research directions with these pestiferous ecosystem engineers.

Materials And Methods

Sample Area

This survey sampled over 153,900 km^2 or the entire state of Georgia, USA and with 1,570 sampling sites provided, on average, a sample for every 98 km^2 between 30-35°N, 85-81°W. Georgia includes five soil provinces following USDA-NRCS classification [33] with elevations ranging from 0-1,400 m [34]. The two largest geographical regions, Piedmont and Coastal Plain, are separated by the Fall Line, a prehistoric shoreline during the Cretaceous period (66-140 mya) (Figure 1). The Fall Line extends from Alabama to Maryland separating the Piedmont from Coastal Plains in those southeastern states [35]. The land south of the Fall Line was subject to cycles of rising and falling sea levels during the quaternary period as the shoreline receded to present day levels exposing the Coastal Plains and Atlantic Coastal Plains Soil Provinces between 20-5 million years ago [36].

Sample Collection

Samples were collected by a single collector from January 2015 to March 2017 from wood on ground (WoG) off of state highways and roads in Georgia, USA. Sampling routes were chosen based on access by roadways that provided coverage of accessible areas of every county (n=159) in the State. Sampling was conducted within a 100-m radius of a GPS-recorded point termed a site and there were 5-17 sites in each county. Sampling was conducted by locating all wood on ground (WOG) such as stumps, logs, or course woody debris and opening the WOG

using a hatchet and chisel to collect all termites into vials of 100% ETOH. The number of samples per site ranged from 1-7. The survey included 4,371 samples from 1,570 sites (1 site per 98 km^2) across all counties (n=159) in the State of Georgia, USA.

Sample Processing

One termite from each sample was processed by extracting Genomic DNA using an adapted protocol (Supplemental Materials, S1) from the Wizard® Genomic DNA Purification Kit (Promega, WI, USA). Resuspended DNA was amplified using a 10µL volume PCR protocol (S1 Table 3) with published primers Modified A-t Leu [37] and B-t Lys [38]. PCR conditions were set in an Eppendorf Mastercycler X50s (Hamburg, Germany) at 95°C for 2 mins (1 cycle), 95°C for 15s, 53°C for 15s, and 68°C for 45s (35 cycles), followed by an extension of 68°C for 5 mins (1 cycle). PCR product was purified using enzymatic digestion of .2 µL Exonuclease I (New England Biolabs, Ipswich, MA, USA) and .2 µL Calf Intestinal Alkaline Phosphatase (Quick CIP; New England Biolabs, Ipswich, MA, USA) per reaction. PCR purification conditions were set in an Eppendorf Mastercycler X50s (Hamburg, Germany) at 37°C for 15 mins (1 cycle) and 85°C for 15 mins (1 cycle). The product was diluted 1:1 with Type 1 Ultrapure Water (Direct-Q 3 UV; Millipore Sigma, Burlington, MA, USA). Purified PCR product was sent to Eurofins Genomics (Eurofins, Louisville, KY, USA) for 96-Well Microplate Sanger sequencing.

Sample Identification

A reference sequence (Table S2) was a mtDNA haplotype attributed to a species designation from a sample identified using morphology and flight phenology [27]. The reference

sequences were included in a GTR + G + I model maximum likelihood tree with 1000 bootstrap replicates in IQ-TREE v2.6.12 to identify species-specific clades [39]. A published *Coptotermes formosanus* sequence (Ref no. AY683218) from GenBank was utilized as the extant group. Species designations for all haplotypes were inferred based on alignment with the aforementioned reference sequences in trees annotated and visualized in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

GIS Visualization

All site coordinates were recorded with a GPS unit and compiled in an open-source geospatial information system (GIS) with identification attributes. *Reticulitermes* distribution across Georgia's geographic features was visualized in QGIS Geographic Information System v 3.22.3 (QGIS Association; http://www.qgis.org). Features included a base map from the Georgia Association of Regional Commissions and 2016 Major Land Resource Areas soil survey data.

Spatial Data Analysis

Clark and Evans aggregation indices and Kernel Density Estimation maps were analyzed and developed in R 4.1.2 (The R Foundation for Statistical Computing, Vienna, Austria) using maptools, rgdal v1.5-28, sf v1.0-7, sp, and spatstat v2.3-3 packages [40, 41, 42, 43, 44]. Edge effects for Clark and Evans aggregation indices were corrected using the state border of Georgia as a guard buffer. Shannon indices of diversity and richness and Simpson reciprocal indices (1/ D) were calculated for species at sites from the entire state as well as above and below the Fall Line. Simpson's 1/ D were calculated using

$$D = (N(N-1))/(\Sigma\, n(n-1))$$

where N is the total number of species collected from sites and n is the total for each species collected from sites [45]. Shannon indices were calculated using

$$H = -\Sigma pi \times ln(pi)$$

where *pi* is the proportion of species collected from sites [46].

Results

Survey

We collected termites from all 159 counties at 1,570 wildland sites illuminating the ubiquity of *Reticulitermes* as part of the soil macroarthropod fauna in Georgia. There were 78 sites (5%) where we collected 3 species, 643 sites (41%) had 2 species, 851 (54%) sites provided one species and there were no sites where we identified 4 or more species. On a broader scale, 100% of the counties (n=159) supplied at least 2 species including 5 species from 7 counties, 4 species from 32 counties, 3 species from 98 counties and 2 species in 22 counties (Figure S1). *R. flavipes*, *R. virginicus* and *R. nelsonae* were collected throughout the state while *R. malletei* and *R. hageni* were found only in counties north of the 32nd parallel roughly approximating the geologic featured called the Fall Line (Figure 1).

Reticulitermes flavipes was the most frequently encountered termite, identified from every county in Georgia (n=159), collected at 1131 sites (72%), and represented in 2,260 samples (52%) that were distributed almost equitably north (53%) and south (51%) of the Fall Line (Figure 2, Table 1). *Reticulitermes nelsonae* was the second most common species collected statewide at 128 counties (81%), 559 sites (36%), and 1,031 samples (23%) (Table 1). *R. nelsonae* was found in a higher proportion of counties below the Fall Line (99%) than above (60%) and represented 34% of samples south and 11% north of the Fall Line (Table 1).

Reticulitermes virginicus was identified statewide from 318 sites (20%) and 122 counties (78%) with a slightly higher proportion of counties below (84%) than above (70%) the Fall line (Table 1). Statewide *R. virginicus* was 11% of all samples (n=494) with the proportion north of the Fall line (7%) more than doubled (15%) south of that geologic feature (Table 1). *Reticulitermes malletei* was identified from 73 counties (46%), collected at 323 sites (21%) with a much higher proportion of counties above (95%) than below (5%) the Fall Line (Table 1). *R. malletei* was represented statewide in 557 samples (13%) divided into 28% above and >1% below the Fall Line (Table 1). *Reticulitermes hageni* was the least collected species identified from 18 counties (11%), 21 sites (1%) and 29 samples (>1%) with all collections (100%) north of the Fall Line (Figure 2, Table 1).

Spatial Data Analysis

Shannon diversity indices (H) support the aforementioned distributions because there were 5 species collected north the Fall Line (H=1.26) compared to 4 south (H=1.06) (Table 2). Simpson's Reciprocal index values likewise reflected the species distributions for above and below the Fall Line, 1/D=3 and 1/D=2.65, respectively (Table 2). Clark and Evans aggregation indices supported a regular distribution for Rf, Rn, and Rv across the state, while Rm and Rh had significantly clustered distributions in the northern part of the state (Table 3). Kernel Density Estimation [kde] maps illustrate the interplay between site and county distributions. Despite being distributed throughout the state Rn was more prevalent in the south (Figure 3). Rv despite its statewide distribution was, proportionally, more frequently collected in the southern part of the state while Rm and Rh clustered north of the Fall Line (Figure 3).

Discussion

The survey identified 5 *Reticulitermes* species across a landmass encompassing ecological sub regions representative of the southeastern United States east of the Appalachian Mountains into the mid-Atlantic states (Figure 1) [35]. The transition zone between the Piedmont and the Southern Coastal Plains, formed about 500 million years ago (the Fall Line), is a demarcation of an ancient shoreline bordering a shallow sea that started receded 20 mya before taking its current position about 5 mya [36]. An examination of species proportions across Georgia shows widespread, regular distributions for Rf, Rn and Rv while Rh and Rm were found predominantly north of the Fall Line. This biogeographic distribution, similar to *Loxoceles reclusa* in the same state [47], raises interesting questions about the evolution and dispersal abilities of these species that should inspire future research projects.

A literature search of termite field studies in the southeastern USA published between 2012-2022 turned up 6 of 17 that mention either *R. malletei* or *R. nelsonae* (Table S3). The prevalence of *R. nelsonae* in Georgia, collected in 81% (n=128) of counties statewide including 99% of counties and 34% of samples south of the Fall Line (Table 1), provides solid evidence it has been underrepresented in the literature. Strict deference to literature published before the descriptions of *R. malletei* and *R. nelsonae* can skew *Reticulitermes* distribution estimates and underestimate the biodiversity of this genus across a wider geographic area. The only published dichotomous key to all five eastern *Reticulitermes* species [27] states that Rn has a southerly distribution which the present data clearly disputes while highlighting the possibility that much of the Rh reported from the southeastern USA are Rn [30, 48, 49, 50, 51, 52, 53]. We present three lines of evidence morphological, behavioral (flight phenology), and genetic to rationalize a re-

examination of the species distributions in the United States and present a path toward a concerted global effort to identify *Reticulitermes* biodiversity.

Characteristic of the genus, a comparison of morphological measurements from descriptions of Rh and Rn reveal no quantifiable characters that clearly separate the diagnostic castes although Rn alates are generally smaller (7.08-mm +0.29) than Rh (7.81-mm +0.31) [27, 54]. The same 'phenotypic overlap' is revealed with qualitative characters where Rh "winged" forms were described as "Pale yellowish brown" similar to the Rn "alate" described as "Body pale brown" [27, 54]. There is, however, a significant behavioral character, flight phenology, that separates Rn from Rh. The description of Rh includes an alate Type Specimen collected, north of the Fall Line, in Falls Church, Virginia and the description states "In the vicinity of Washington (DC *sic*), hageni flies the later part of July or early in August" [54, 55]. The description also contains the following entry: "Occurs from Florida (Jacksonville, Apr. 29) to Maryland west to Illinois and Texas" which has generally assumed to reference an 'out-of-place' collection of alates from FL in late April [54]. It is understandable prior to the description of Rn that that passage in the original description provoked identifying 'light-colored' adult termites flying in Florida in springtime as Rh [24, 53]. The original description of Rn reports adults collected in February, March and May while the present survey provided 5 Rn alate samples dated March and April [54]. All the ITA verified Rh (n=17) in our archived assembly of alates (unpublished data) were collected in July-September. Rn flight phenology strongly suggests that the "R.h." collected from springtime swarms in the United States should, regardless of precedence and in light of the more recent species description, be considered Rn.

The molecular markers (COI, COII or 16S sequence) used to identify the 5 focal *Reticulitermes* species provide an equally compelling argument for the prevalence of Rn in the

southeastern USA. We searched GenBank and included sequence attributed to the 5 species with the ITA reference sequence (Table S2) to produce phylogenies (Figure 4). We found (186) COI GenBank accessions assigned to Rf, Rv, Rh, Rm, and Rn and 185 aligned with their respective ITA-reference sequence except one anomaly, collected in Florida, attributed to Rh that aligns with Rn (Figure S2, Table S4). COII accessions from GenBank attributed to all Rf (n=558), Rn (4) and Rm (8) agreed with the ITA references including an Rn sample collected in Louisiana and a Rm from Mississippi [56] which extends the western range of both species. The southern range of Rn was illuminated by 3 samples from Florida including one of our ITA references [27, 56]. There were 29 COII accessions in GenBank attributed to Rv of which 21 agreed with the reference sequence while 7 direct submissions grouped with Rn and 1 with Rm references (Figure 4, Table S4). There were 9 Rh Genbank accessions and 5 grouped with the reference sequences while anomalies included previously published incongruous Rh accessions that unfortunately have been used in studies published in the past decade (Table S4). There were 4 'Rh' COII accessions including one collected in Arkansas and seven 'Rh' 16S accessions with one collected in Brazos, TX that grouped with the Rn references further extending the potential western distribution of Rn (Table S4). We posit, given the aforementioned morphological, behavioral, and genetic evidence and because we collected Rh infrequently (<1% of >4,000 samples) and only from North Georgia that the historic range of Rh has been overestimated and that of Rn underestimated.

Reticulitermes malletei, originally described from specimens collected near Athens, Georgia in 1986 using chemical characters later clarified with morphological and genetic characters in 2007, has been reported from AL, MD, NC, SC, DE, GA, and MS [19, 27, 56, 58, 59, 60, 61]. The distribution of Rm from this survey (Figure 2), almost exclusively in north

Georgia (Figure 1), coincides with its' published range along the eastern coastal states yet when combined with a GenBank accession from Indiana, our reference sequence from Mississippi and a GenBank accession from the same state [58] (Table S4) begs further investigation of their western distribution. The qualitative phenotypic character "dark wings" used in dichotomous keys for both Rm and Rf likely provoked misidentification affecting historic range estimates for Rm [20, 62, 63]. Quantifiable morphological characters for Rm and Rf overlap for the soldier caste while for the adult ablw measurements show Rm to be, in general, smaller (8.23 +0.39) than Rf (8.97 +0.40) [20]. The adult Rm from this survey were collected in May-June and Rf from February-May indicating a temporal overlap that needs further clarification.

These data represent the largest survey of WOG over a contiguous land area, 153,900 km^2, ever conducted for subterranean termites in the United States, but we caveat it should not be considered definitive. This research program, for example, has been located in Clarke County, Georgia for 20 years and we have collected Rh and Rv on several occasions although the present survey did not collect either species in that county (Figure 2). This survey of wildland sites also failed to collect another invasive subterranean termite *Coptotermes formosanus* despite our program having identified *C. formosanus* from the built environment in Atlanta, Columbus, Savannah, Brunswick, Hinesville and Thomasville, Georgia in addition to a wildland site on Cumberland Island. Notwithstanding that proviso the collection data from this extensive survey when combined with examination of GenBank accessions and published literature illuminated how *Reticulitermes* biodiversity in the eastern United States has been underestimated. The *Reticulitermes* literature in the USA continues to reference any number of approaches to species identification without heeding the taxonomic inconsistencies discussed 15 years ago [21].

The difficulties associated with identifying biologically cryptic insect complexes that display phenotypic plasticity makes *Reticulitermes* an ideal candidate for species-specific molecular markers [65, 66, 67]. The monophyletic status of the genus makes it appropriate for a common set of ICZN-compliant, ITA-supported molecular marker(s) for species determination [68, 69, 70]. A common DNA barcode should interface with datasets of worldwide biodiversity and could set the foundation for broader discussions on a unified definition of what constitutes a species within this genus [71, 72]. We suggest *Reticulitermes* surveys employ an ITA-validated mitochondrial DNA marker, specifically COI and/or COII sequence as the species marker of choice given their widespread employment and reported advantages while acknowledging potential conflicts of linking phenotype with genotype [73, 74]. We conducted a simple proof-of-concept test using the 55 COII reference sequences from this survey (Table S2) with GenBank accessions attributed to *Reticulitermes* species from the Western USA, Europe, and Asia (Table S5). All published species separated into strongly supported species-specific clades suggesting the utility of that mtDNA marker on a global scale (Figure 5). It is likely that 16S sequence is too conserved to be useful across a global survey as there were 2 Rm 16S-haplotypes obtained from 22 samples collected across 5 states (estimated 1045-km linear distance) while we identified 15 Rm COII-haplotypes from 29 samples across 5 counties in north Georgia (estimated 60-km linear distance) [59, 75]. In addition, attempts to corroborate 16S GenBank accessions for Rf, Rh, Rv, Rm, and Rn were thwarted by entries containing premature stop codons and ambiguities leaving disproportionate gaps in the multiple sequence alignment needed for analysis [49, 50, 64, 76]. Microsatellite markers or other electromorphs would be least preferred because they exhibit a propensity toward size homoplasy that would require additional research to verify repeatability and corroborate with IZCN-validated species designations [77, 78, 79].

This survey illustrates that subterranean termite biodiversity has been underestimated in the eastern United States and adds voice to the need for a concerted effort to develop a world-wide data base suitable for assisting a generic taxonomic revision of *Reticulitermes*. That species complex would benefit from a formal reorganization following the ICZN code to provide an appropriate interpretation of the taxonomic literature and help correct the current muddle associated with inappropriate species attributions in the literature and datasets such as GenBank. In the meantime, the research community is encouraged to employ ITA-validated molecular markers to appropriately address biologically relevant research. Delineating species-level subterranean termite biodiversity should inspire future research in a broad array of topics including evolution, ecology, sympatry, resource partitioning and meaningful, identification-based pest-status monitoring of these important ecosystem engineers.

Acknowledgments

We sincerely thank Jim Chase (Retired, Terminix) for collecting and donating all termite specimens identified in this survey. We also thank Hadden Duling, Olivia Katz and Jake Wasdin for their assistance with sample maintenance and organization, as well as Jacob Winkles for data management, analysis, and figure creation. We dedicate this work to Kunio Tsunoda and Tsuyoshi Yoshimura for their support and inspiration due to their remarkable dedication researching wood destroying organisms while pursuing a relentless commitment to grooming the next generation of Isopterists.

References

1. Engel, M. S.; Ceríaco, L. M.; Daniel, G. M.; Dellapé, P. M.; Löbl, I.; Marinov, M.; Reis, R. E.; Young, M. T.; Dubois, A.; Agarwal, I.; Lehmann A., P.; Alvarado, M.; Alvarez, N.; Andreone, F.; Araujo-Vieira, K.; Ascher, J. S.; Baêta, D.; Baldo, D.; Bandeira, S. A.; Barden, P.; Barrasso, D. A.; Bendifallah, L.; Bockmann, F. A.; Böhme, W.; Borkent, A.; Brandão, C. R.; Busack, S. D.; Bybee, S. M.; Channing, A.; Chatzimanolis, S.; Christenhusz, M. J.; Crisci, J. V.; D'elía, G.; Da Costa, L. M.; Davis, S. R.; De Lucena, C. A.; Deuve, T.; Fernandes Elizalde, S.; Faivovich, J.; Farooq, H.; Ferguson, A. W.; Gippoliti, S.; Gonçalves, F. M.; Gonzalez, V. H.; Greenbaum, E.; Hinojosa-Díaz, I. A.; Ineich, I.; Jiang, J.; Kahono, S.; Kury, A. B.; Lucinda, P. H.; Lynch, J. D.; Malécot, V.; Marques, M. P.; Marris, J. W.; Mckellar, R. C.; Mendes, L. F.; Nihei, S. S.; Nishikawa, K.; Ohler, A.; Orrico, V. G.; Ota, H.; Paiva, J.; Parrinha, D.; Pauwels, O. S.; Pereyra, M. O.; Pestana, L. B.; Pinheiro, P. D.; Prendini, L.; Prokop, J.; Rasmussen, C.; Rödel, M.-O.; Rodrigues, M. T.; Rodríguez, S. M.; Salatnaya, H.; Sampaio, Í.; Sánchez-García, A.; Shebl, M. A.; Santos, B. S.; Solórzano-Kraemer, M. M.; Sousa, A. C.; Stoev, P.; Teta, P.; Trape, J.-F.; Dos Santos, C. V.-D.; Vasudevan, K.; Vink, C. J.; Vogel, G.; Wagner, P.; Wappler, T.; Ware, J. L.; Wedmann, S.; Zacharie, C. K. The Taxonomic Impediment: A Shortage of Taxonomists, Not the Lack of Technical Approaches. *Zool J Linn Soc-Lond* 2021, *193* (2), 381–387, doi: 10.1093/zoolinnean/zlab072.

2. Lobl, I. Assessing biotic diversity: the glorious past, present, and the uncertain future. *Bulletin of the Entomological Society of Malta* 2018, *10*, 5-15.

3. Sharkey, M. J.; Janzen, D. H.; Hallwachs, W.; Chapman, E. G.; Smith, M. A.; Dapkey, T.; Brown, A.; Ratnasingham, S.; Naik, S.; Manjunath, R.; Perez, K.; Milton, M.; Hebert, P.; Shaw, S. R.; Kittel, R. N.; Solis, M. A.; Metz, M. A.; Goldstein, P. Z.; Brown, J. W.; Quicke, D. L.; van Achterberg, C.; Brown, B. V.; Burns, J. M. Minimalist Revision and Description of 403 New Species in 11 Subfamilies of Costa Rican Braconid Parasitoid Wasps, Including Host Records for 219 Species. *ZooKeys* 2021, *1013*, 1–665, doi: 10.3897/zookeys.1013.55600.

4. Cardenas, C. R.; Luo, A. R.; Jones, T. H.; Schultz, T. R.; Adams, R. M. M. Using an Integrative Taxonomic Approach to Delimit a Sibling Species, *Mycetomoellerius Mikromelanos* Sp. Nov. (Formicidae: Attini: Attina). *PeerJ* 2021, *9*, doi: 10.7717/peerj.11622.

5. Kjærandsen, J. Current State of DNA Barcoding of Sciaroidea (Diptera)—Highlighting the Need to Build the Reference Library. *Insects* 2022, *13* (2), 147, doi: 10.3390/insects13020147.

6. Petrović, A. Sizing the Knowledge Gap in Taxonomy: The Last Dozen Years of Aphidiinae Research. *Insects* 2022, *13* (2), 170, doi: 10.3390/insects13020170.

7. Dubois A.; Ohler A.; Pyron R.A. New concepts and methods for phylogenetic taxonomy and nomenclature in zoology, exemplified by a new ranked cladonomy of recent amphibians (Lissamphibia). *Megataxa* 2021, *5*, 1–738, doi: 10.11646/megataxa.5.1.1

8. Ride W.D.L. International Commission on Zoological Nomenclature.; International Union of Biological Sciences. *London: International Trust for Zoological Nomenclature*, c/o Natural History Museum, 1999.

9. Wagner D.L.; Grames E.M.; Forister M.L.; Berenbaum M.R.; and Stopak D. Insect decline in the anthropocene: Death by a thousand cuts. *Proc Natl Acad Sci* 2021, *118*, doi: 10.1073/pnas.2023989118.

10. Saunders M.E.; Janes J.K.; O'Hanlon J.C. Moving On from the Insect Apocalypse Narrative: Engaging with Evidence-Based Insect Conservation, *BioScience* 2020, *70:1*, 80–89, doi: 10.1093/biosci/biz143.

11. Mallet J.; Willmott K. Taxonomy: renaissance or Tower of Babel? *Trends Ecol Evol.* 2003, *18*(2)*,* 57-59, doi: 10.1016/S0169-5347(02)00061-7.

12. Rust, M. K.; Su, N.-Y. Managing Social Insects of Urban Importance. *Annu Rev Entomol* 2012, *57* (1), 355–375, doi: 10.1146/annurev-ento-120710-100634.

13. Peterson C.; Wagner T.L.; Mulrooney J.E.; Shelton T.G. Subterranean Termites— Their Prevention and Control in Buildings. *United States Department of Agriculture Forest Service, Home and Garden Bulletin* 2006. 64.

14. Myer, A.; Forschler, B. T. Evidence for the role of subterranean termites (*Reticulitermes* spp.) in temperate forest soil nutrient cycling. *Ecosystems* 2018, *22* (3), 602–618 DOI: 10.1007/s10021-018-0291-8.

15. Jouquet, P.; Bottinelli, N.; Shanbhag, R. R.; Bourguignon, T.; Traoré, S.; Abbasi, S. A. Termites: The Neglected Soil Engineers of Tropical Soils. *Soil Sci* 2016, *181* (3/4), 157–165 DOI: 10.1097/ss.0000000000000119.

16. International Code Council. International building code. Falls Church, Va. *International Code Council*. 2000.

17. Orkin. Termite infestation probability zones map (tip). Available online: https://www.orkin.com/pests/termites/termite-infestation-probability-zones-map (accessed 30/5/22)

18. What are the worst U.S. states for termites? Available online: https://www.groundworks.com/resources/worst-us-states-for-termites/ (accessed 30/5/22)

19. Clément, J.-L.; Howard, R.W.; Blum, M.S.; Lloyd, H. L'isolement spécifique des termites du genre *Reticulitermes* (Isoptera) du sudest des Etats-Unis. Mise en èvidence grâce à la chimie et au comportement d'une espèce jumelle de R. virginicus = R. malletei sp. nov. et d'une semi-species de R. flavipes. *C R Acad Sci Paris* 1986, 302:67–70.

20. Lim S.Y.; Forschler, B.T. A new species of *Reticulitermes* Holmgren, 1913 (Isoptera: Rhinotermitidae) from the Southeastern United States. *Insecta Mundi* 2012, O252:1-4.

21. Nelson, L.J.; Cool, L.G.; Solek, C.W.; Haverty, M.I. Cuticular Hydrocarbons and Soldier Defense Secretions of *Reticulitermes* in Southern California: A Critical Analysis of the Taxonomy of the Genus in North America. *J Chem Ecol* 2008, *34*, 1452-1475.

22. Weesner, F.M. Termites of the Nearctic region. In: Krishna, K. editor, Weesner, F.M. editor. *Biology of Termites*, 1st ed.; Academic Press, New York, NY, USA, 1970; Volume 2, pp. 477-525.

23. Banks, F.A. Species Distinction in *Reticulitermes* (Isoptera: Rhinotermitidae). Master's Thesis, University of Chicago, Chicago, IL, 1946.

24. Miller, E.M. Florida termites: a handbook. University of Miami Press, Coral Gables, FL, USA, 1949; pp. 1-30.

25. Nutting, W.L. Insecta: Isotopera. In: Dindal D.L., editor. *Soil Biology Guide*, 1st ed.; John Wiley & Sons, New York, NY, USA, 1990; pp. 997-1032.

26. Hostettler, N. C.; Hall, D. W.; Scheffrahn, R. H. Intracolony Morphometric Variation and Labral Shape in Florida *Reticulitermes* (Isoptera: Rhinotermitidae) Soldiers: Significance for Identification. *Fla Entomol* 1995, *78* (1), 119, doi: 10.2307/3495675.

27. Lim S.Y.; Forschler B.T. *Reticulitermes nelsonae*, a New Species of Subterranean Termite (Rhinotermitidae) from the Southeastern United States. Insects 2012, *3,* 62-90, doi: 10.3390/insects3010062.

28. Copren, K. A.; Nelson, L. J.; Vargo, E. L.; Haverty, M. I. Phylogenetic Analyses of Mtdna Sequences Corroborate Taxonomic Designations Based on Cuticular Hydrocarbons in Subterranean Termites. *Mol Phylogenet Evol* 2005, *35* (3), 689–700, doi: 10.1016/j.ympev.2005.03.002.

29. Cameron, S. L.; Whiting, M. F. Mitochondrial Genomic Comparisons of the Subterranean Termites from the Genus *Reticulitermes* (Insecta: Isoptera: Rhinotermitidae). *Genome* 2007, *50* (2), 188–202, doi: 10.1139/g06-148.

30. Austin, J.W.; Szalanski, A.L.; Uva, P.; Kence, A. A comparative genetic analysis of the subterranean termite genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Ann Entomol Soc Amer* 2002, *95*, 753-760, doi: 10.1603/0013-8746(2002)095[0753:ACGAOT]2.0.CO;2.

31. Will, K. W.; Mishler, B. D.; Wheeler, Q. D. The Perils of DNA Barcoding and the Need for Integrative Taxonomy. *Syst Biol* 2005, *54* (5), 844–851, doi: 10.1080/10635150500354878.

32. Lumely, L.; Sperling. . Integrating morphology and mitochondrial DNA for species delimitation within the spruce budworm (Choristoneura fumiferana) cryptic species complex (Lepidoptera: Tortricidae). *Syst Entomol* 2010, *35*, 416-428, doi: 10.1111/j.1365-3113.2009.00514.x.

33. United States Department of Agriculture, Natural Resources Conservation Service. Land Resource Regions and Major Land Resource Areas of the United States, the Caribbean, and the Pacific Basin. *U.S. Department of Agriculture Handbook 296* 2006.

34. Musser, Jonathan W. Digital Atlas of State of Georgia, Elevation, US Geological Survey, Digital Data Series, Atlanta, GA. 1996.

35. McNab, H. W.; Avers, P. E. Ecological Subregions of the United States. Southern Region and Southeastern Forest Experiment Station. 1996, *21*.

36. Gore, P. J. W., Witherspoon, W. Introduction. In *Roadside Geology of Georgia*, 1st ed.; Mountain Press Publishing Company, Missoula, MT, USA, 2013; pp. 2–21.

37. Liu, H.; Beckenbach A.T. Evolution of the Mitochondrial Cytochrome Oxidase II Gene among 10 Orders of Insects. *Mol Phylogenet Evol* 1992, *41*, 41-52.

38. Simon, C.; Frati, F.; Beckenbach, A.; Crespi, B.; Liu, H.; Flook. P. Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. *Ann Entomol Soc Am* 1994, *87*, 651–701.

39. Nguyen, L.-T.; Schmidt, H. A.; von Haeseler, A.; Minh, B. Q. IQ-Tree: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol Biol Evol* 2014, *32* (1), 268–274, doi: 10.1093/molbev/msu300.

40. Lewin-Koh, N.; Bivand, R.; Pebesma, E.; Archer, E.; Baddeley, A.; Bibiko, H-J.; & Dray, S.; Forrest, D.; Friendly, M.; Giraudoux, P. maptools: Tools for reading and handling spatial objects. R package version 0.8-27 2011.

41. Keitt T.H.; Bivand R.; Pebesma E.; Rowlingson B. rgdal: bindings for the Geospatial Data Abstraction Library 2022.

42. Pebesma, E. Simple Features for R: Standardized Support for Spatial Vector Data. *The R Journal* 2018. 10:1, 439-446

43. Pebesma E.J.; Bivand R.S. Classes and methods for spatial data in R. *R News* 2005, *5*(2), 9–13.

44. Baddeley A.; Rubak E.; Turner R. *Spatial Point Patterns: Methodology and Applications with R,* 1st ed.; Chapman and Hall/CRC Press: London, UK, 2015.

45. Simpson, E. Measurement of Diversity. *Nature* 1949, *163*, 688.

46. Shannon, C.E.; Weaver, W. *The mathematical theory of communication*. The University of Illinois Press, Urbana, IL, USA, 1949; 117.

47. Vetter R.S.; Hinkle N.C.; Ames, L.M. Distribution of the Brown Recluse Spider (Araneae: Sicariidae) in Georgia with Comparison to poison Center Reports of Envenomations. *J Med Entomol* 2009, *46,* 15-20, doi: 10.1603/033.046.0103.

48. Vargo E.L., Juba T.R., Deheer C.J. Relative Abundance and Comparative Breeding Structure of Subterranean Termite Colonies (*Reticulitermes flavipes*, *Reticulitermes hageni*, *Reticulitermes virginicus*, and *Coptotermes formosanus*) in a South Carolina Lowcountry Site as Revealed by Molecular Markers. *Ann Entomol Soc Am 2006. 99*(6), 1101–1109, doi: 10.1603/0013-8746(2006)99[1101:RAACBS]2.0.CO;2.

49. Janowiecki, M.A.; Austin, J.W.; Szalanski, A.W.; Vargo, E. Identification of *Reticulitermes* Subterranean Termites (Blattodea: Rhinotermitidae) in the Eastern United States Using Inter-Simple Sequence Repeats. *J Econ Entomol* 2021, 114(3), 1242-1248, doi: 10.1093/jee/toab028.

50. Su N.-Y.; Ye W.; Ripa R.; Scheffrahn R.H.; Giblin-davis R.M. Identification of Chilean *Reticulitermes* (Isoptera: Rhinotermitidae) Inferred from Three Mitochondrial Gene DNA Sequences and Soldier Morphology *Ann Entomol Soc Am* 2006, *99*(2), 352-363, doi: 10.1603/0013-8746(2006)099[0352:IOCRIR]2.0.CO;2.

51. Chouvenc, T.; Scheffrahn, R. H.; Buss, L. Termite Species Distribution in Florida and UF Termite Identification Services. *EDIS* 2022, *2022* (3), doi: 10.32473/edis-IN1360-2022.

52. Su, N.-Y.; Scheffrahn, R. H.; Cabrera, B. Native Subterranean Termites: *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), *Reticulitermes hageni* Banks (Insecta: Isoptera: Rhinotermitidae). *EDIS* 1969, *2004* (5), doi: 10.32473/edis-in369-2001

53. Termite Distribution - Fort Lauderdale Research and Education Center - University of Florida, Institute of Food and Agricultural Sciences - UF/IFAS. Available online: https://flrec.ifas.ufl.edu/termites-in-florida/termite-distribution/ (accessed on 30/5/22)

54. Banks N.; Snyder T.E. A revision of the nearctic termites. *Bull. U.S. Natl. Mus*. 1920, *108*.

55. Smithsonian National Museum of Natural history. Entomology Collections Search. Available Online: http://n2t.net/ark:/65665/3e999984f-7dbc-4a21-bc9b-348732688df1 (accesed 31/5/22)

56. Dedeine F.; Dupont S.; Guyot S.; Matsuura K.; Wang C.; Habibpour B.; Bagnères A.G.; Mantovani B.; Luchetti A. Historical biogeography of *Reticulitermes* termites (Isoptera: Rhinotermitidae) inferred from analyses of mitochondrial and nuclear loci. *Mol Phylogenet Evol* 2016, *94*, 778-790, doi: 10.1016/j.ympev.2015.10.020.

57. Hoang, D. T.; Chernomor, O.; von Haeseler, A.; Minh, B. Q.; Vinh, L. S. Ufboot2: Improving the Ultrafast Bootstrap Approximation. *Mol Biol Evol* 2017, *35* (2), 518–522.

58. Garrick R.C.; Collins B.D.; Yi R.N.; Dyer R.J.; Hyseni C. Identification of Eastern United States *Reticulitermes* Termite Species via PCR-RFLP, Assessed Using Training and Test Data. *Insects* 2015, *6*(2), 524-537, doi: 10.3390/insects6020524

59. Austin, J.; Bagnères, A.G.; Szalanski, A.; Scheffrahn, R.; Heintschel, B.P.; Messenger, M.; Clément, J-L.; Gold, R. *Reticulitermes malletei* (Isoptera: Rhinotermitidae): A valid Nearctic

subterranean termite from Eastern North America. *Zootaxa* 2007, *1554,* 1-26, doi: 10.11646/zootaxa.1554.1.1.

60. Whitney-King, S.; Austin, J. W.; Szalanski, A.L. Use of soldier pronotal width and mitochondrial DNA sequencing to distinguish the subterranean termites, *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) (Isoptera: Rhinotermitidae) on the Delmarva peninsula Delaware, Maryland, and Virginia, U.S.A. *Entomol News* 2007, *118*, 41–48, doi: 10.3157/0013-872X(2007)118[41:UOSPWA]2.0.CO;2.

61. Stephens, C. Alabama Rhinotermitidae: Nomenclature, Identification, Survey and Phenology. Master's Thesis. Auburn University, Auburn, AL, 2012.

62. Scheffrahn, R.; Su, N.Y. Keys to soldier and winged adult termites (Isoptera) of Florida. *Fla Entomol* 1994, *77*, 460-474, doi: 10.1016/j.ympev.2015.10.020.

63. Messenger, M.T. New Orleans Mosquito & Termite Control Board Bulletin No. 01-01 2nd Edition. *New Orleans Mosquito and Termite Control Board*. 2002, *2*:1-12.

64. Szalanski, A.L.; Austin, J.W.; Owens, C.B.; Identification of *Reticulitermes* spp. (Isoptera: Reticulitermatidae) from South Central United States by PCR-RFLP. *J Econ Entomol* 2003, *96*(5), 1514–1519, doi: 10.1093/jee/96.5.1514

65. Lv, J.; Wu, S.; Zhang, Y.; Chen, Y.; Feng, C.; Yuan, X.; Jia, G.; Deng, J.; Wang, C.; Wang, Q.; et al. Assessment of four DNA fragments (COI, 16S rdna, ITS2, 12s rdna) for species identification of the IXODIDA (Acari: Ixodida). *Parasites & Vectors* 2014, *7* (1), doi: 10.1186/1756-3305-7-93.

66. Ruihua Z.; Ping J.; Chuanbo S.; Deyong S.; Feng Z.; Chaochao H. The analysis of genetic variation in the mitochondrial genome and its application for the identification of *Papilio* species. *Mitochondr DNA Part B* 2018, *3*(2)*,* 687-690, doi: 10.1080/23802359.2018.1481776.

67. Jörger, K. M.; Schrödl, M. How to describe a cryptic species? practical challenges of molecular taxonomy. *Frontiers in Zoology* 2013, *10* (1), 59, doi: 10.1186/1742-9994-10-59.

68. Goulding, T.C.; Dayrat, B. Integrative Taxonomy: Ten Years of Practice and Looking into the Future. *Archives of Zoological Museum of Lomonosov Moscow State University* 2016, *54*, 116-133.

69. Yeates, D.; Seago, A.; Nelson, L.; Cameron, S.; Joseph, L.; Trueman, J. Integrative taxonomy, or iterative taxonomy? *Syst Entomol* 2010, *36* (2), 209–217 DOI: 10.1111/j.1365-3113.2010.00558.x.

70. Bourguignon, T.; Lo, N.; Šobotník, J.; Sillam-Dussès, D.; Roisin, Y.; Evans, T. A. Oceanic Dispersal, Vicariance and Human Introduction Shaped the Modern Distribution of the Termites *Reticulitermes*, *Heterotermes* and *Coptotermes*. *P Roy Soc B-Biol Sci* 2016, *283* (1827), 20160179, doi: 10.1098/rspb.2016.0179.

71. De Queiroz, K., Species Concepts and Species Delimitation, *Syst Biol* 2007. *56*:*6*, 879–886, doi:10.1080/10635150701701083.

72. Zachos, F.E. (New) Species concepts, species delimitation and the inherent limitations of taxonomy. *J Genet*. 2018, *97*: 811–815, doi:10.1007/s12041-018-0965-1.

73. Gross, M. Barcoding Biodiversity. *Curr Biol* 2012, *22*(3)*,* R-73-R76.

74. Ghiselli, F.; Milani, L. Linking the mitochondrial genotype to phenotype: a complex endeavour. *Phil Trans R Soc B* 2019, *375*: 20190169, doi:10.1098/rstb.2019.0169.

75. Chan, A. H.; Chaisiri, K.; Saralamba, S.; Morand, S.; Thaenkham, U. Assessing the Suitability of Mitochondrial and Nuclear DNA Genetic Markers for Molecular Systematics and Species Identification of Helminths. *Parasites & Vectors* 2021, *14* (1), doi: 10.1186/s13071-021-04737-y.

76. Austin, J.W.; Szalanski, A.L.; Messenger, M.T. Mitochondrial DNA Variation and Distribution of the Subterranean Termite Genus *Reticulitermes* (Isoptera: Rhinotermitidae) in Arkansas and Louisiana. *Fla Entomol* 2004, *87*(4), 473-480, doi: 10.1653/0015-4040(2004)087[0473:MDVADO]2.0.CO;2.

77. Estoup A.; Jarne P.; Cornuet J.M. Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Mol Ecol* 2002, *11* (9),1591-604, doi: 10.1046/j.1365-294X.2002.01576.x.

78. Hausdorf, B.; Hennig, C. Species delimitation and geography. *Mol Ecol Resour* 2020, *20*(4), 950–960.

79. Nagaraju J.; Reddy K.D.; Nagaraja G.M.; Sethuraman B.N. Comparison of multilocus RFLPs and PCR-based marker systems for genetic analysis of the silkworm, *Bombyx mori*. *Heredity* 2001, *86,* 588-597.

Tables

Table 3.1. The number and proportion of counties, sites, and samples by *Reticulitermes* species collected from the entire state as well as north and south of the Fall Line.

Counties						
	Statewide		**North of Fall Line**		**South of Fall Line**	
Species	# of counties (n=159)	% of counties	# of counties (n=73)	% of counties	# of counties (n=86)	% of counties
Rf	159	100%	73	100%	86	100%
Rn	128	80.5%	43	58.9%	85	98.8%
Rm	73	45.9%	69	94.5%	4	4.7%
Rv	122	76.7%	50	68.5%	72	83.7%
Rh	18	14.1%	18	24.7%	0	0%
Sites						
	Statewide		**North of Fall Line**		**South of Fall Line**	
Species	# of sites (n=1,570)	% of sites	# of sites (n=688)	% of sites	# of sites (n=882)	% of sites
Rf	1131	72.0%	504	73.3%	627	71.1%
Rn	559	35.6%	130	18.9%	429	48.6%
Rm	323	20.6%	313	45.5%	10	1.1%
Rv	318	20.3%	97	14.1%	221	25.1%
Rh	21	1.3%	21	3.0%	0	0%
Samples						
	Statewide		**North of Fall Line**		**South of Fall Line**	
Species	# of samples (n=4,371)	% of samples	# of samples (n=1,975)	% of samples	# of samples (n=2,396)	% of samples
Rf	2260	51.7%	1036	52.5%	1224	51.1%
Rn	1031	23.6%	219	11.1%	812	33.9%
Rm	557	12.7%	546	27.7%	11	.5%
Rv	494	11.3%	145	7.3%	349	14.6%
Rh	29	.7%	29	1.5%	0	0%

Table 3.2. Shannon and Simpson diversity indices for *Reticulitermes* species collected by site from the entire state as well as north and south of the Fall Line.

	Shannon Diversity Indices	Simpson Diversity Indices
Entire State	H: 1.279 EH: 0.795	1-D: 0.68 1/D: 3.08
North of Fall Line	H: 1.266 EH: 0.787	1-D: 0.67 1/D: 3
South of Fall Line	H: 1.057 EH: 0.762	1-D: 0.62 1/D: 2.65

Table 3.3. Clark and Evans Aggregation Index scores for *Reticulitermes* species distribution point patterns by site (R. 4.0.5, The R Foundation for Statistical Computing, Vienna, Austria). Asterisks denote a significant p-value.

Species	**Clark and Evans R Score**	**p-value**
Rf	R = 1.1899	< .001*
Rn	R = 1.0526	<.05*
Rm	R = 0.71012	< .001*
Rv	R = 0.99239	.7941
Rh	R= 0.57601	<.001*

Figure Captions

Figure 3.1. Map of the eastern United States outlining the 5 major soil provinces in Georgia that extend through other states in southeastern USA. Two soil provinces, the Piedmont and Southern Coastal Plain, occupy 90% of the landmass of Georgia and are demarked by the Fall Line that extends from Alabama to New Jersey.

Figure 3.2. Distribution of four *Reticulitermes* species, by county, in Georgia: upper left; *R. nelsonae* (n=128); upper right; *R. virginicus* (n=122); lower left; *R. malletei* (N=73) and lower right; *R. hageni* (n=18). A map of *R. flavipes* distribution was not included because it was collected in every county (n=159). The dark line running east to west represents the position of the fall line separating the Piedmont and Southern Coastal Plains soil provinces.

Figure 3.3. Kernel Density Estimation maps of *Reticulitermes* species distribution using site, by species created in R. 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria).

Figure 3.4. Collapsed Maximum-Likelihood GTR+G+I tree topology used to identify the survey samples to species including 1,186 full length (685bp) COII reads from the survey, highlighting the 55 full length ITA-Verified references by species clade (Table S2). A *Coptotermes formosanus* COII sequence (AY683221) was used as the extant group. Branch numbers represent percentage of posterior probability from 1,000 UF Bootstrap iterations [57]. The tree also included 3 full and 1 aligned partial reads from GenBank COII accessions shown by collection site and accession number reported as Rh that should be considered anomalous because they grouped with Rn (Table S4). Full data available upon request from authors.

Figure 3.5. Collapsed Maximum-Likelihood GTR+G+I tree topology assembled using 55 full length (685bp) COII ITA-verified references for southeastern *Reticulitermes* (Table S2) and 22 full or partial reads of the same gene retrieved from GenBank for 10 additional species across Europe, Asia, and the western United States (Table S5). A *Coptotermes formosanus* sequence (AY683221) was used as the extant group. Strong node support from 1,000 UF bootstrap iterations [57] illustrates the global utility of this marker. Full data available upon request from authors.

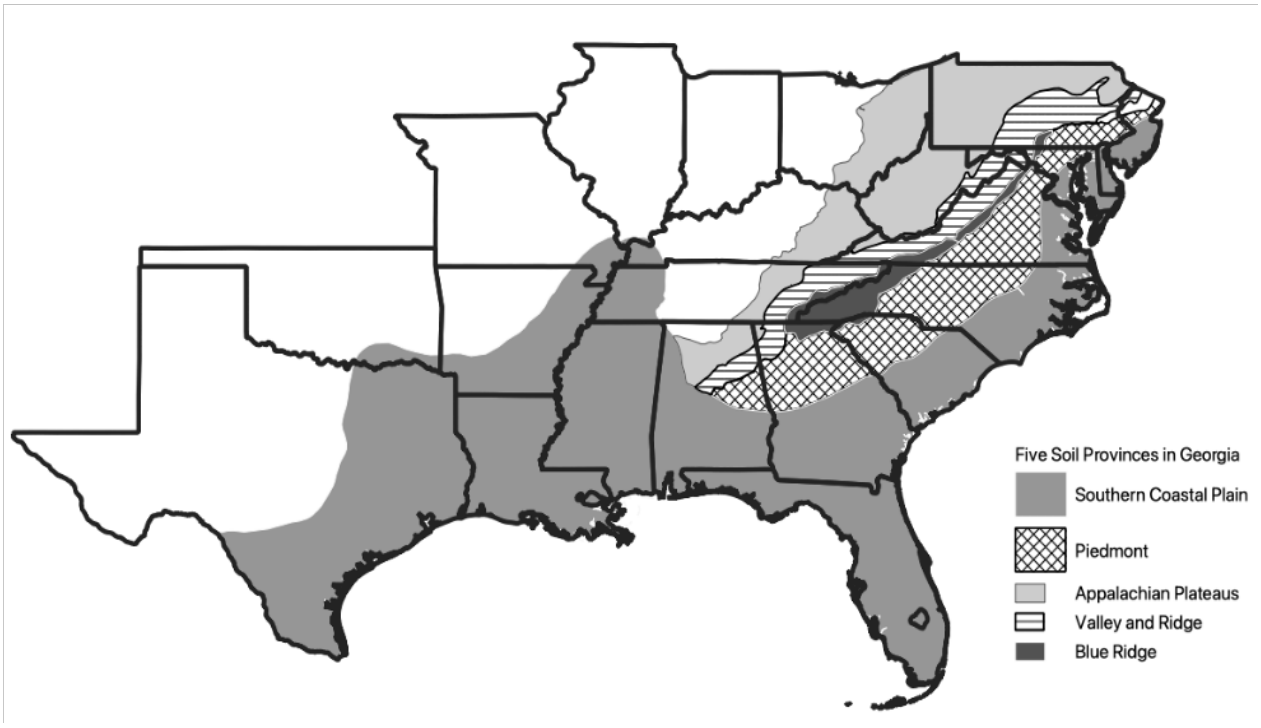
Five Soil Provinces in Georgia
Southern Coastal Plain
Piedmont
Appalachian Plateaus
Valley and Ridge
Blue Ridge

Figure 3.1

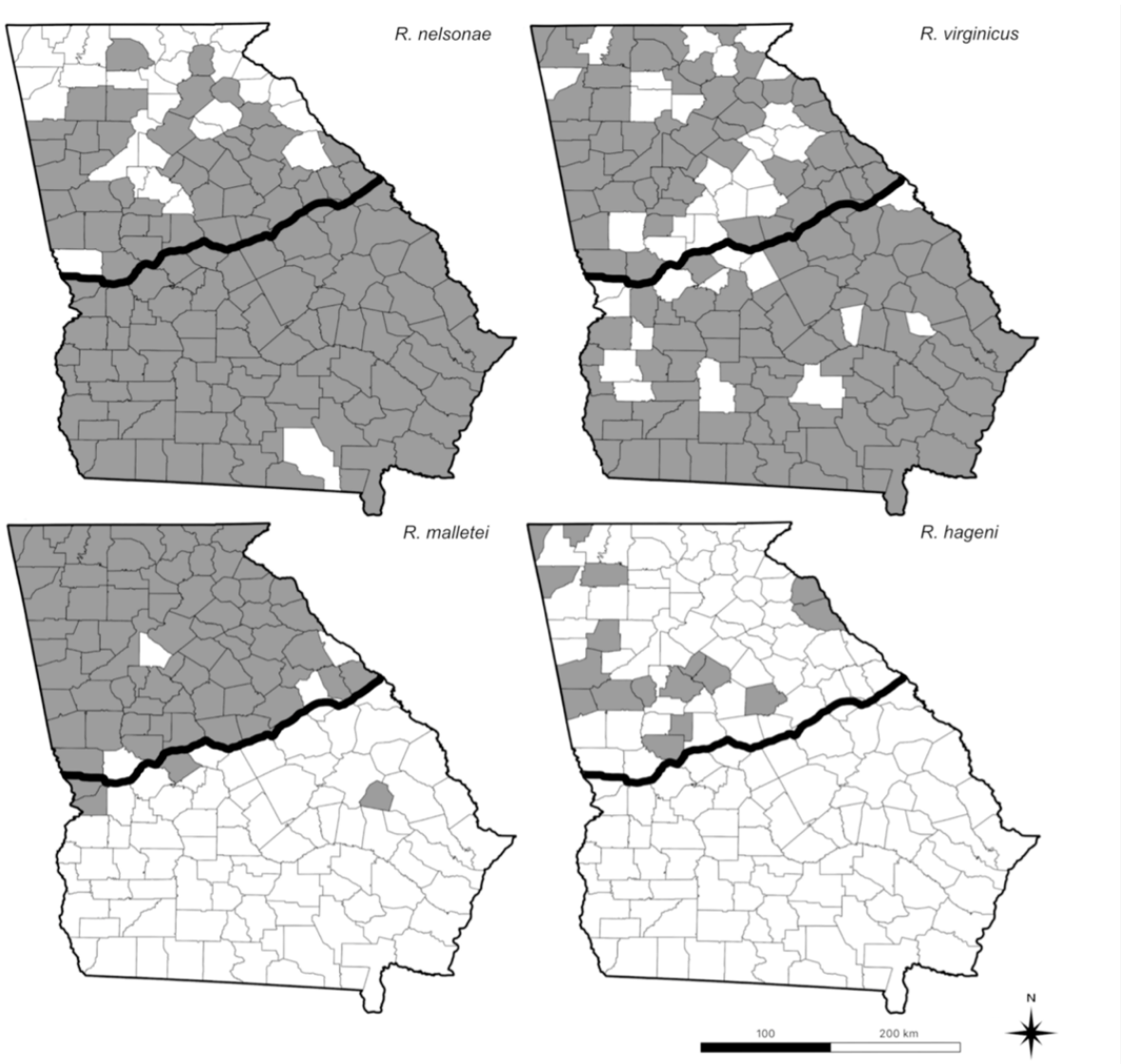

Figure 3.2

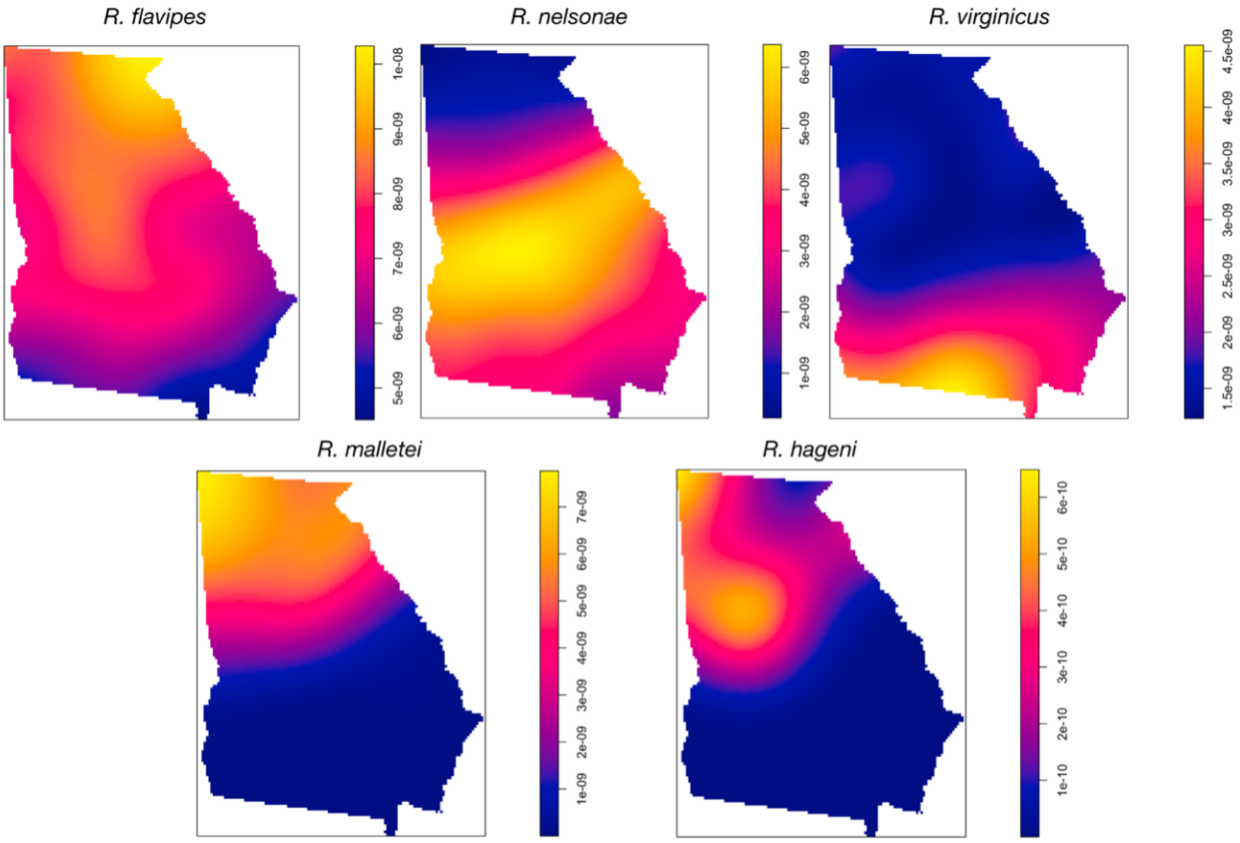

Figure 3.3

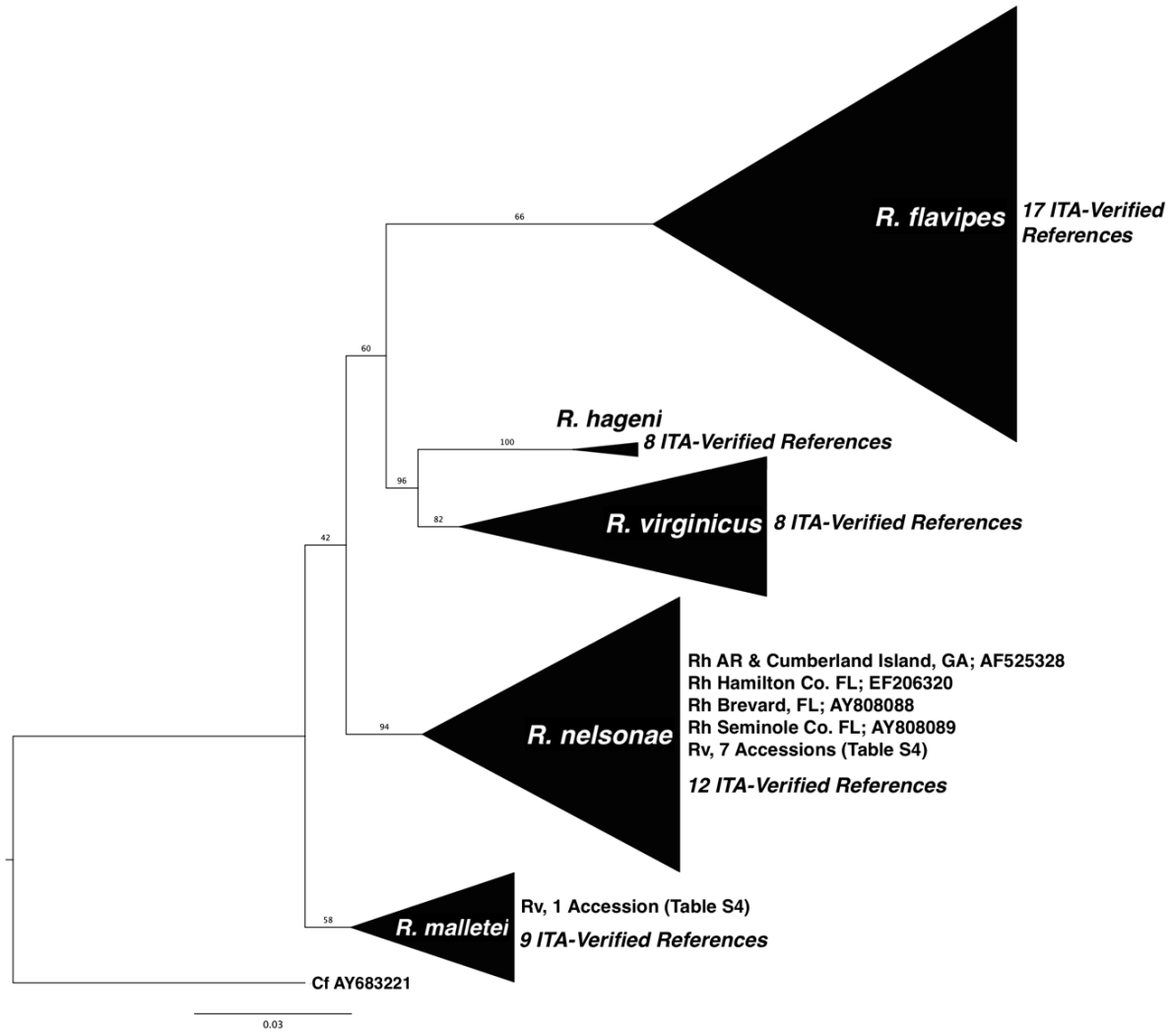
R. flavipes
17 ITA-Verified References
R. hageni
8 ITA-Verified References
R. virginicus
8 ITA-Verified References
R. nelsonae
Rh AR & Cumberland Island, GA; AF525328
Rh Hamilton Co. FL; EF206320
Rh Brevard, FL; AY808088
Rh Seminole Co. FL; AY808089
Rv, 7 Accessions (Table S4)
12 ITA-Verified References
R. malletei
Rv, 1 Accession (Table S4)
9 ITA-Verified References
Cf AY683221
66
60
100
96
82
42
94
58
0.03

Figure 3.4

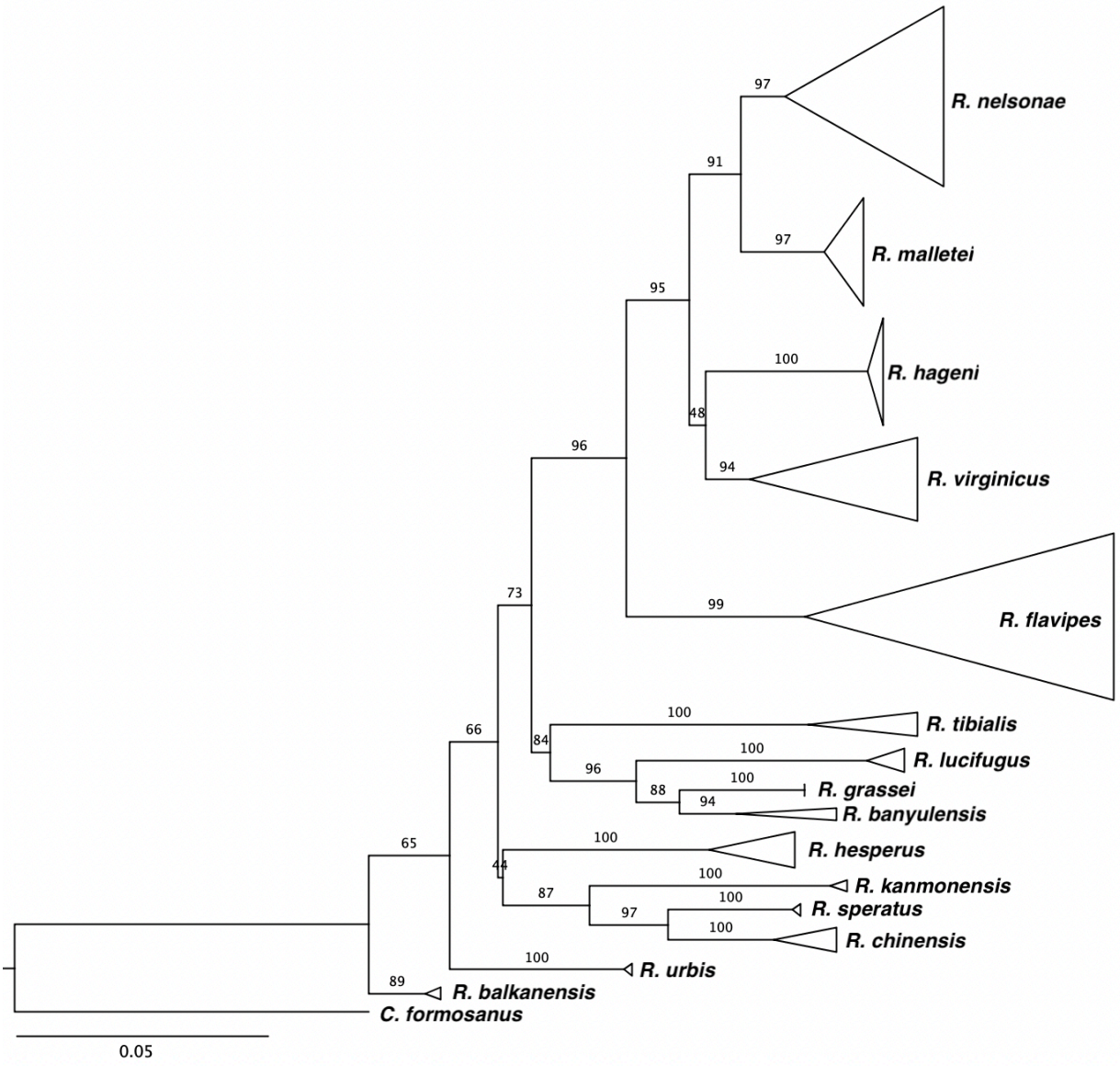
R. nelsonae
R. malletei
R. hageni
R. virginicus
R. flavipes
R. tibialis
R. lucifugus
R. grassei
R. banyulensis
R. hesperus
R. kanmonensis
R. speratus
R. chinensis
R. urbis
R. balkanensis
C. formosanus
0.05

Figure 3.5

APPENDIX B: SUPPLEMENTARY MATERIALS

Sample Preparation

Samples were air-dried on filter paper (MilliporeSigma, Burlington, MA, USA) for 1 hr and added to 150 µL chilled (4°C) Nuclei Lysis (NL) solution (Promega, WI, USA) in a 1.7 mL microcentrifuge tube (Thermo Fisher Scientific, Waltham, MA). Samples were pulverized in NL solution with a sterilized pestle, combined with 17 µL of 20 mg/mL water suspended solution of Proteinase K (New England Biolabs), and incubated for 2 hr at 65°C in a dry bath incubator (Thermo Fisher Scientific, Waltham, MA). TE buffer suspended RNAse A solution (5 µL of 4 mg/mL) (Promega, WI) was added to the mixture and incubated for 30 mins at 37°C. Protein Precipitation solution (200 µL; Promega, WI, USA) was added to the mixture, chilled at 4°C for 5 mins, and centrifuged for 4 mins at a maximum speed of 12,400 RPM (Thermo Fisher Scientific, Waltham, MA). The supernatant (700 µL) was removed and added to a new 1.7 mL microcentrifuge tube. Molecular grade isopropanol (600 µL) (Thermo Fisher Scientific, Waltham, MA) was added to the 1.7 mL tube and centrifuged at maximum speed. The resulting supernatant was removed (1100 µL), and a second precipitation was performed with 600 µL of 70% ethanol (Decon Laboratories Inc, King of Prussia, PA) with a 20 min centrifuge cycle at maximum speed. The ethanol supernatant was removed, and the pellet was air-dried for 1 hr and resuspended in 40 µL of DNA rehydration solution (Promega, WI) for an hour at 65°C.

Table S3.1. List of publications that provide information on subterranean termite species distributions in the Eastern United States including the following 5 species; Rf= *R. flavipes*, Rv= *R. virginicus*, Rh= *R. hageni*, Rm= *R. malletei*, Rn= *R. nelsonae*, reported by state (standard abbreviations), sample collection method and corresponding citation. WL= samples from wildlands wood on ground or research plots; S = samples from pest management professionals; T= samples from light trap surveys; MC= samples from museum collections; Li= literature search; NM= Not mentioned.

Regional Publications			
State	***Reticulitermes spp* reported**	**Collection methods**	**Citation**
GA FL SC NC VA WV KY TN AL MS	Rf	WL; S	[54]
TX FL SC VA	Rh		
GA LA FL NC VA	Rv		
TX LA AK MS AL GA FL SC NC TN KY VA WV	Rf	Li	Light and Pickens, 1946
LA AK MS AL GA TN KY SC NC FL VA WV	Rh		
LA AK MS AL GA TN KY SC NC FL VA WV	Rv		
TX LA MS AK TN GA FL SC NC KY VA WV IL WI MI IN OH PA NY DW MD VT MA CT ME	Rf	NM	Snyder, 1946
LA MS GA FL SC NC VA WV KY MD DE IL IN OH	Rh		
LA MS GA FL SC NC VA WV KY MD DE IL IN OH	Rv		
TX LA MS AK TN GA FL SC NC KY VA WV IL WI MI IN OH PA NY DW MD VT MA CT MO	Rf	NM	[25]
LA AK MS AL GA FL SC NC TN KY VA WV	Rh		
LA AK MS AL GA FL SC NC TN KY VA WV	Rv		
FL LA TX	Rf	S	[22]
LA	Rh		
FL LA	Rv		
GA SC NC AL TN MS VA WV	Rf	WL	[58]

GA TN	Rv		
GA AL	Rm		
AL	Rn		
GA SC NC AL TN MS WV VA KY	Rf	WL	Hyseni and Garrick, 2019
GA KY VA AL SC NC	Rv		
AL SC	Rm		
State Surveys			
State	***Reticulitermes spp* reported**	**Collection methods**	**Citation**
Alabama	Rf, Rh, Rv, Rm	WL MC	[61]
Florida	Rf, Rh, Rv	S, Li	[24]
	Rf, Rh, Rv	S	Scheffrahn et al., 1988
	Rf, Rh, Rv	S	[53]
Georgia	Rf, Rv	N/A	(Light, 1946)
	Rf, Rv, Rh	WL MC	Scheffrahn et al., 2001
	Rf, Rv, Rh, Rm, Rn	WL	[27]
Louisiana	Rf, Rv, Rh	S	[63]
Mississippi	Rf, Rv	WL	Wang and Powell, 2001
South Carolina	Rf, Rh, Rv	WL S MC T	Hathorne et al., 2000
Texas*	Rf, Rv, Rh	S MC	Howell et al., 1987

* Rt= *R. tibialis* also reported in Howell et al., 1987.

Table S3.2. GenBank Accession numbers for the ITA-Verified COI and COII sequences termed Reference Sequence along with collection date and site. The reference sequences were used to designate survey samples to a species designation using a GTR + G + I ML phylogeny.

COI			
Species	**Accession No.**	**Date Collected**	**Location**
C. formosanus	AY027472	2001	USA
R. flavipes	JN207470	2/6/07	Sapelo Island, GA, USA
	JN207471	2007	Sapelo Island, GA, USA
	JN207472	11/13/07	Sapelo Island, GA, USA
	JN207473	11/23/07	Sapelo Island, GA, USA
	JN207474	10/23/07	Athens, GA, USA
	JN207475	4/13/09	Athens, GA, USA
	JN207476	4/25/09	Athens, GA, USA
	JN207477	4/21/09	Athens, GA, USA
	F182	3/12/14	Athens, GA, USA
	F192	3/20/19	Athens, GA, USA
R. hageni	JN207483	2007	Athens, GA, USA
	H13	8/20/13	Clarke, Co. USA
R. virginicus	JN207478	11/13/07	Sapelo Island, GA, USA
	JN207479	1/30/08	Sapelo Island, GA, USA
	JN207480	2007	Athens, GA, USA
	JN207481	10/23/07	Athens, GA, USA
	JN207482	10/23/07	Athens, GA, USA
	V41	5/10/16	Athens, GA, USA
	V44	5/13/17	Athens, GA, USA
R. nelsonae	JN207486	2/6/07	Sapelo Island, GA, USA
	JN207487	2007	Sapelo Island, GA, USA
	JN207488	1/30/08	Sapelo Island, GA, USA
	JN207489	2/6/07	Sapelo Island, GA, USA

	JN207490	2/6/07	Sapelo Island, GA, USA
	JN207491	2/6/07	Sapelo Island, GA, USA
	N5	2/6/07	McIntosh Co, GA, USA
	N9	3/31/11	Branford, FL, USA
	N10	4/18/13	McIntosh, GA, USA
R. malletei	JN207484	2007	Athens, GA, USA
	JN207485	10/23/07	Athens, GA, USA
	M47	5/14/12	Athens, GA, USA
COII			
Species	**Accession #**	**Date Collected**	**Location**
C. formosanus	AY683218	6/12/03	Savannah, GA, USA
R. flavipes	JF796216	2009	Athens, GA, USA
	JF796217	8/25/09	Thomasville, GA, USA
	JF796218	7/6/09	USA
	JF796219	3/31/10	Branford, FL, USA
	JF796220	11/5/10	Branford, FL, USA
	F2	4/19/96	Pike Co., GA, USA
	F24	3/28/02	Clarke Co., GA, USA
	F42	4/19/12	Clarke Co., GA, USA
	F50	2/13/13	Clarke Co., GA, USA
	F51	2/20/13	Clarke Co., GA, USA
	F52	2/28/13	Athens, GA, USA
	F58	4/13/13	Athens, GA, USA
	F174	3/10/13	Athens, GA, USA
	F182	3/12/14	Athens, GA, USA
	F192	3/20/19	Athens, GA, USA
	F193	4/27/19	Athens, GA, USA
	FJB	3/4/21	Athens, GA, USA
R. hageni	AF107486	8/97	Barnesville, GA, USA
	JF796224	2007	Athens, GA, USA
	JF796225	2007	Athens, GA, USA
	EU689026	2008	USA

	H11	8/5/07	Athens, GA, USA
	H13	8/20/13	Clarke Co., GA, USA
	H18	8/15/16	Athens, GA, USA
	H19	8/15/16	Athens, GA, USA
R. virginicus	JF796234	5/26/05	Madison, FL, USA
	JF796223	2009	Thomasville, GA, USA
	JF796222	7/5/09	Sapelo Island, GA, USA
	JF796221	5/30/08	Athens, GA, USA
	V41	5/10/16	Athens, GA, USA
	V44	5/13/17	Athens, GA, USA
	V46	5/14/17	Athens, GA, USA
	V47	5/14/17	Athens, GA, USA
R. nelsonae	JF796235	N/A	Sapelo Island, GA, USA
	JF796229	2009	Croatan NF, NC, USA
	JF796233	12/20/06	Apopka, FL, USA
	JF796232	6/18/09	GA, USA
	JF796231	3/31/10	Branford, FL, USA
	JF796230	2009	Thomasville, GA, USA
	EU689013	2008	GA, USA
	N5	2/6/07	McIntosh Co, GA, USA
	N9	3/31/11	Branford, FL, USA
	N10	4/18/13	McIntosh, GA, USA
	N11	4/8/13	McIntosh Co, GA, USA
	N12	5/29/10	Athens, GA, USA
R. malletei	JF796226	10/23/07	Athens, GA, USA
	JF796228	2009	NC, USA
	GU550074	N/A	USA
	JF796227	5/21/10	Athens, GA, USA
	M11	4/26/00	Winston, MS, USA
	M47	5/14/12	Athens, GA, USA
	M49	6/24/12	Athens, GA, USA
	M62	5/26/14	Athens, GA, USA

	M44	4/22/12	Athens, GA, USA

Table S3.3. Citations for the 17 *Reticulitermes* field surveys in the SE USA published between 2012-2022 including species mentioned, year published, and index to the citation.

Species Mentioned	**Year**	**Citation**
Rf, Rm	2012	Fredericks, 2012
Rf, Rv, Rh	2012	Blount, 2012
Genus only	2013	Little et al., 2013
Genus only	2014	Ulyshen, 2014
Genus only	2014	Little et al., 2014
Genus only	2014	Riggins et al., 2014
Rf, Rv, Rh, Rm, Rn	2015	Janowiecki, 2015
Rf, Rv, Rh, Rm	2015	Janowiecki and Szalanski, 2015
Rf	2016	Su et al., 2016
Genus only	2016	Clay et al., 2016
Rf, Rv, Rm	2019	Hyseni and Garrick, 2019
Rf	2019	Hyseni and Garrick, 2019
Rf, Rv, Rm	2020	Hyseni, 2020
Rf, Rv, Rh, Rm, Rn	2021	Janowiecki and Vargo, 2021
Rf, Rv, Rh	2021	[49]
Rf	2021	Aguero et al., 2021
Rf, Rv	2021	Shults et al., 2021

Table S3.4. COI, COII, and 16S accessions included in Maximum-Likelihood phylogenies with ITA-verified references that did not align in a clade matching the GenBank species designation. Table includes accession, collection location, GenBank species designation and updated based- on-grouping-with-ITA species designations, and publications referencing those aberrant accessions.

COI				
Accession(s)	**Collection Site**	**GenBank Species Designation**	**ITA Species Designation**	**Publications**
EF206320*	Hamilton, Co. FL	*R. hageni*	*R. nelsonae*	[29, 70] Bourguignon et al., 2014
COII				
Accession(s)	**Collection Site**	**GenBank Species Designation**	**ITA Species Designation**	**Publications**
EF206320*	Hamilton, Co. FL	*R. hageni*	*R. nelsonae*	[29, 70] Bourguignon et al., 2014; Wang et al., 2016; Lee et al., 2017; Li et al., 2018
AF525328*	Cumberland Island, GA & AR[t]	*R. hageni*	*R. nelsonae*	[30, 64] Austin et al., 2004; Nobre et al., 2006
AY808089*	Seminole Co. FL	*R. hageni*	*R. nelsonae*	[50]
AY808088*	Brevard, FL	*R. hageni*	*R. nelsonae*	[50]
AY536419	Jasper Pulaski IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536418	Jasper Pulaski IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536424	Indiana Dunes, Paradise Valley Ridge, IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536422	Jasper Pulaski, IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536423	Jasper Pulaski, IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536425	Indiana Dunes, Paradise Valley Ridge, IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536417	Jasper Pulaski, IN	*R. virginicus*	*R. nelsonae*	Direct GenBank Submission
AY536421	Jasper Pulaski, IN	*R. virginicus*	*R. malletei*	Direct GenBank Submission
16S**				

Accession(s)	Collection Site	GenBank Species Designation	ITA Species Designation	Publications
AY808122	Brevard, FL	*R. hageni*	*R. nelsonae*	[50]
AY808123	Seminole Co. FL	*R. hageni*	*R. nelsonae*	[50]
AY257230-AY257235	Brazos, TX Cumberland Island, GA	*R. hageni*	*R. nelsonae*	[64, 49] McKern et al., 2006

* Rh accessions identified to Rn mentioned in the published description of Rn in 2012 [27].

**All 16S accessions were only those we could cross list with published COII sequence because the aforementioned accessions could not be aligned with multiple sequences using Muscle *3.8.425* for phylogenetic analysis.

[t] This same haplotype was also attributed to samples collected in Arkansas (Austin et al., 2004).

Table S3.5. Accession numbers for the 16 COII sequences used to construct the global comparison phylogeny in Figure 5 along with collection date and site. These sequences were used alongside ITA-Verified reference sequence (Table S2) to designate accessions to a species designation using a GTR + G + I ML phylogeny.

COII			
Species	**Accession No.**	**Submission Date**	**Location**
R. balkanensis	KM245783.1	7/14/14	Schinias, Greece
	KM245784.1	7/25/14	Nea Makri, Greece
R. urbis	DQ866972.1	7/26/06	Igoumenitsa, Greece
	JQ231196.1	12/3/11	Castellaneta, Italy
R. speratus	KM245821.1	7/25/14	Kamigamo, Kyoto, Japan
	KM245819.1	7/25/14	Motoyama, Kochi, Japan
R. chinensis	AB050705.1	11/1/2000	Beijing, China
	JX142148.1	6/6/12	Changping, Beijing, China
	FJ423454.1	10/28/08	Beijing, China
R. kanmonensis	KM245812.1	7/25/14	Ejio Park, Yamaguchi, Japan
	KM245811.1	7/25/14	Ejio Park, Yamaguchi, Japan
R. hesperus	DQ018960.1	4/26/05	CA, USA
	AY623447.1	5/12/04	CA, USA
	KM245769.1	7/25/14	Placerville, CA, USA
	KM245770.1	7/25/14	Novato, CA, USA
R. tibialis	AY808094.1	11/1/04	AZ, USA
	AF525355.1	6/28/02	AZ, USA
	HM208248.1	5/6/10	CA, USA
R. lucifugus	JQ231194.1	12/3/11	Pisticci, Marina, Italy
	JQ231192.1	12/3/11	Marina di Lesina, Italy
	EF591507.1	5/3/07	Corsica, Bastia, France
R. banyulensis	KM245779.1	7/25/14	Teruel, Spain
	KM245778.1	7/25/14	Cassis, France
R. grassei	MN107083.1	7/24/19	France

	MT188739.1	3/12/20	Foret Coubre, Point Espagnols, France

CHAPTER 4

ABUNDANCE AND DISPERSION OF RETICULITERMES COII HAPLOTYPES IN GEORGIA, USA

[1]Allison Johnson and B.T. Forschler. To be submitted to PLoS One.

Abstract

Reticulitermes are economically and ecologically important insects in the southeastern USA yet understanding dispersal and species-specific abundance of subterranean termites in their endemic range is confounded by their plastic reproductive biology and resulting phenotypic plasticity making identification without morphologically diagnostic castes difficult. We employed COII mtDNA from references identified using an integrative taxonomic approach to examine the abundance and dispersion of COII haplotypes from samples collected in a statewide survey of termites from wood on ground in Georgia, USA. Here we show that COII is a valid and useful molecular marker to identify samples to species, resulting in a monophyletic maximum likelihood phylogeny. More common haplotypes had a clustered distribution within the state, which supports assumptions about *Reticulitermes* dispersal based on their limited ability to sustain fight. *Reticulitermes nelsonae* and *R. flavipes* haplotypes were the most variable between the described species within the state, suggesting the presence of potentially undescribed species. Results from these basic genetic comparisons suggest the potential for species specific primers to be utilized in a novel form of *Reticulitermes* identification through a multiplex PCR-based assay.

Introduction

Reticulitermes subterranean termites are profound structural pests in the built environment on almost every continent [1], yet little is known about their species-specific abundance in their endemic range. The biodiversity of subterranean termites USA has likely been underestimated [2, 3] highlighting the necessity for further ecological research on *Reticulitermes* distributions. Difficulties associated with understanding colony size and dispersion of *Reticulitermes* have been attributed to their subterranean lifestyle and obstacles identifying terminal castes to species [4, 5] including notable morphological plasticity [6,7, 8, 9] and recent species descriptions limiting the function of dichotomous keys created before 2012 [10, 11].

Castes traditionally used to identify *Reticulitermes* using morphology are either found seasonally or constitute 1-2% of the colony [6, 7, 12]. Mitochondrial DNA has been successfully used to alleviate some of these confounding attributes of *Reticulitermes* identification through gene sequencing to infer species designations of workers, which are customarily accessible and abundant in a colony [13, 14, 15, 16, 17].

The objective of this study was to examine the abundance and relationship of *Reticulitermes* using cytochrome oxidase II [COII] haplotypes identified from Georgia, USA. COII is a globally accepted mitochondrial barcoding gene [18] that has been successfully used in *Reticulitermes* identification [19, 2, 20]. We employed COII sequences from alates collected and identified by the Household and Structural Entomology Research Program using an integrative taxonomic approach, combining flight phenology and morphology, to serve as a reference sequence in a maximum likelihood phylogeny [6]. Identifying samples in a phylogeny by association with an ITA-verified reference in this genus increases confidence that species identifications are appropriate [6, 5].

COII sequences were compared to infer intraspecies and interspecies relationships. and illuminate dispersion patterns of haplotypes using Clarke and Evans aggregation scores. We conducted an NCBI BLASTn analysis with sequence data to investigate if haplotypes recovered in this study were previously recorded.

Materials and Methods

Sample Collection

Samples were collected by one person between January 2015 to March 2017 from wood on ground near state highways and roads within a county (n=159). The wood of live or dead standing hardwood or softwood trees, limbs or branches on the ground, stumps, or leaf litter at the base of trees was examined for termites. Termites collected for each sample were stored in 100% ethanol and a sample included any castes available including workers, nymphs, soliders and alates. Samples were collected at 5-17 sites from each county with a range of 1-7 samples within 100-m radius of a chosen GPS coordinate at each site.

Sample Preparation

DNA was extracted from the entire body of a single worker per sample using adapted instructions from the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Adapted protocol includes the addition of a Proteinase K (17 µL of 20 mg/mL) digestion at 65ºC (120 min). DNA was amplified for COI/COII using a 10ul volume PCR protocol; 7.2 µL nuclease-free H2O, 1 µL 5PRIME HotMaster Taq Buffer (Quantabio, MA, USA), .2 µL each of forward and reverse primers (Integrated DNA Technologies Inc. Coraville, IA, USA), .2 µL dNTP Solution Mix (New England Biolabs, MA, USA), and .2 µL 5PRIME HotMaster Taq Polymerase (Quantabio, MA, USA) per reaction. COII PCR conditions were set in an Eppendorf Mastercycler X50s (Hamburg, Germany) at 95°C for 2 mins, 95°C for 15s, 53°C for 15s, and

68°C for 45s (35 cycles) followed by a final extension at 68°C for 5:00 mins. COI followed 95°C for 3 mins, 95°C for 15s, 50°C for 15s, and 68 °C for 45s (35 cycles) with a final extension at 68°C for 5:00 mins. Published primers for the entire length of the 685 bp COII mtDNA gene (modified A-t Leu and B-t Lys) [18, 21]and partial COI (C1-J-2195 and TL2-N-3014) [22] were used for the PCR protocols. Impurities were removed using an EXO I and CIP enzymatic digestion (New England Biolabs, Ipswich, MA, USA) before purified PCR product was sent to Eurofins Genomics (Eurofins, Louisville, KY, USA) for 96-Well Microplate Sanger sequencing. Returned sequences were curated in Geneious Prime v2020.2 [23] and aligned using MUSCLE v3.8.31 [24].

Consensus Haplotypes

Unique haplotypes were recovered in R 4.1.2 (The R Foundation for Statistical Computing, Vienna, Austria) using PEGAS [25] and APE [26] from 4,371 mitochondrial cytochrome oxidase subunit II (*mt*COII) sequences curated in Geneious Prime v2022 [2323] and aligned using MUSCLE [2424]. Consensus haplotypes were inferred to species using a maximum likelihood tree with a GTR + G + I nucleotide substitution model (Figure 1) including 55 ITA-verified references (Table S2). Haplotypes were then organized by species and given an accession name based on the species and a unique numerical identifier, e.g. ‘F1, N1, V1’ (Table 1).

Comparative Analyses

Nucleotide composition, diversity, parsimony informative and polymorphic sites, conservative mutation sites, average pairwise differences, haplotype diversity, and estimated sequence divergence, were calculated for haplotypes recovered within each species using DnaSP v.6.12.03 [27] and MEGA v.11 [28, 29]. Base pair changes in the three largest haplotypes for each species

were examined in a graphic multiple sequence alignment using *ggmsa* [30] in R (The R Foundation for Statistical Computing, Vienna, Austria) (Figure 2).

Colony Estimation

We tested the assumption that samples with the same COII haplotype within the same site or at sites more than 1 km apart could be differentiated into separate colonies using a partial segment (767 BP) of the COI mtDNA gene. We examined multiple samples that had the same haplotype from 3-15 sites per COII haplotype more than 1km apart (Table S1). At least one site per the 10 haplotypes examined contained multiple samples with the same haplotype at that site (Table S1). A total of 91 samples from 10 COII haplotypes were sequenced for partial COI. Reads were curated in Geneious Prime v2022 [20] aligned using MUSCLE [2124] and haplotypes were recovered using PEGAS [25].

Haplotype Dispersion

Spatial clustering of haplotypes represented by more than 10 colonies for each species were calculated using Clarke and Evans R aggregation scores and distributions visualized with kernel density estimation maps (Figures S2a-d). Haplotypes were compared to COII *mt*DNA accessions deposited in GenBank using NCBI Nucleotide BLASTn to identify sequences that had 100% query matches with previously reported accessions submitted for the full (685bp) and partial length of the COII mtDNA gene (Table S3).

Results

Consensus Haplotype Analysis

PEGAS analyses recovered 1,134 unique COII haplotypes [22]. The GTR + G + I nucleotide substitution model maximum likelihood tree was monophyletic with a log likelihood of -14899.47 (Figure 4.1). Parsimony probability from 2,000 ultrafast bootstraps ranged from 42-100%. The proportion of species identified in accordance with references included 46% or 522 *R. flavipes*, 29.5% or 332 *R. nelsonae*, 12.9% or 147 *R. virginicus*, 10.9% or 124 *R. malletei*, and 0.62% or 7 *R. hageni*. There were three internodal clades containing sequences identified as *R. flavipes* and *R. nelsonae* (Figure 1). The ultrafast boostrap support within those species claded ranged from 63-100% for *R. flavipes* and 64-100% for *R. nelsonae*.

Comparative Analyses

The average nucleotide frequency for sequences was A= 0.39, T= 0.23, C= 0.24, and G= 0.14 (Table 3). All species were characterized by low nucleotide diversity ranging from 0.001-0.02 (Table 3). *Reticulitermes. nelsonae* had the highest nucleotide diversity (0.02), followed by *R. flavipes* (0.1), *R. virginicus* (0.008), *R. malletei* (0.005), and lastly *R. hageni* (0.002). The proportion of polymorphic sites was greater in *R. flavipes* (34%) and *R. nelsonae* (30%), that combined represented 75% of the survey samples (Table 4). There were 37 conservative mutations identified in the 135 unique *R. flavipes* haplotypes, followed by 33 in R. nelsonae, 13 in *R. virginicus*, and 9 in *R. malletei.* The *R. hageni* haplotypes provided no conservative mutations (Table 4). Haplotype diversity, which estimates the probability two randomly sampled haplotypes would be different followed the same order as nucleotide diversity within species; *R. nelsonae* had the highest haplotype diversity between species (0.986) and R. hageni the lowest (0.48).

Values for intraspecific distance ranged from 0.15-4.82% for Rf, 0.15-5.26% Rn, 0.15-1.90% Rv, 0.15-1.90%% Rm, 0.15-0.32% Rh (Table 4). Average interspecific distances between species were over 2% for all comparisons (Table 5).

The *ggmsa* highlighted 15 synonymous mutations in the third codon position that were conserved for *R. flavipes*, 1 for *R. nelsonae*, 8 *R. virginicus*, 4 *R. malletei*, and 9 *R. hageni.* These regions were selected in haplotypes recovered for each species to observe if the silent mutation was conserved across the species. Three out of the 15 fixed character states originally detected for *R. flavipes* were conserved among all haplotype, followed by 1 *R. nelsonae*, 5 *R. virginicus*, 3 *R. malletei* and 8 *R. hageni* (Figure 2). Gene fragment length between conserved mutations for species that had more than one fixed character state were recorded and arranged by unestimated product sizes (Table 6).

Colony Estimation

A list of sites and recovered COI haplotypes can be found in supplemental materials (Table S1). The assumption of defining colonies based on haplotypes by distance was justified because all 12 sites where we sequenced 2 or more samples from the same site provided the same COI haplotype across all 4 species. Over half (59%) of the disparate sites with the same COII haplotypes had different COI haplotypes. A total of 3,487 colonies were identified from 4,371 samples assuming the same COII haplotype at a site was the same colony while the same haplotype at collection sites over 1km away represented different colonies; 1,817 were Rf, 816 Rn, 393 Rv, 437 Rm, and 24 Rh (Table 3).

Haplotype Dispersion

We originally investigated the dispersion of 116 haplotypes with at least 5 colonies. Examining haplotypes represented by less than10 colonies increased the probability of a Clarke

and Evans aggregation estimate indicative of a dispersed distribution. Clarke and Evans aggregation indices for 52 haplotypes represented by at least 10 colonies are listed in Figures S2. All those haplotypes provided R-scores less than 1, an indication of a clustered dispersion (Figure S2a-d).

BLASTn Analysis

Results displayed 139 queries that were identical haplotypes from this survey: 54 identical to the full length (685bp) of the COII gene, and 85 partial (544-677 BP) submissions (Table S3). Six accessions identical to the full length of the COII for *R. flavipes* were from collection sites outside of GA (Table S3). F4 matched 4 accessions designated as *R. santonensis* from locations in France and Italy, 2 *R. flavipes* accessions from IN and Italy, and an *R. arenincola* accession from Sand Dunes, Indiana. Haplotype F55 is identical to 4 accessions, two *R. santonensis* from Italy and Germany, and two *R. flavipes* from LA and Uruguay (Table S3).

Reticulitermes hageni, *R. virginicus*, and *R. nelsonae* haplotypes from this survey were identical to full sequences that were collected from NC, AR, IN and FL (Table S3) as well as Georgia. *R. malletei* haplotypes recovered from this survey were identical to full length COII accessions collected in Georgia.

Discussion

The GenBank BLASTn analysis revealed there is little to no evidence of association between haplotype and geography for *R. flavipes* haplotypes. *R. flavipes* is an invasive termite [1]; haplotypes found in this survey were identical to specimens collected from the Central US, South America and Europe. Yet in our survey of Georgia which is within their endemic range, haplotypes were clustered (Figures S2a-d), which supports the assumption *Reticulitermes*

dispersal would be aggregated considering they are 'relatively weak fliers' [31] and distance traveled is dependent on abiotic factors like wind and air currents [31]. The BLASTn analysis additionally supports the synonomy of *R. arenincola* and *R. flavipes* because our Georiga haplotypes F4 and F36 were identical to GenBank assessions attributed to *R. arenincola*. Those same haplotypes were attributed to accesssions collected in Indiana, Georgia, Italy, and France identified to *R. santonensis* or *R. flavipes*. There is further examination required as to what constitutes a species in this genus, but the data agrees with the synonmy of R. santonensis [32] and supports the call to synonmize *R. arenincola* as *R. flavipes* [9].

Both *R. nelsonae* and *R. flavipes* displayed high intraspecific polymorphism, indicated by more polymorphic and conservative mutations than the other 3 *Reticulitermes* species examined (Table 4). Similar proportions of intraspecific polymorphism using COII has been recorded in programs researching *Reticulitermes flavipes* [19], supporting literature that suggests COII may have higher variability in *Reticulitermes* than other insects [20].

The biodiversity of *Reticulitermes* is only beginning to be examined in the southeastern USA [3, 13, 19, 33]. Type locales for the 2 most recently described species were in Georgia, USA [10, 11]. Sequences identified as R. *nelsonae* and *R. flavipes* from this collection present evidence of additional potential undescribed species in Georgia. Average intra- and interspecific sequence distances were over 2%, suggesting these groups are composite and contain multiple lineages [34, 35], which were shown in the maximum likelihood phylogeny internodal clades with strong bootstrap support (Figure 1). Clarke and Evans aggregation scores were calculated for the haplotypes within internodal clades for each species revealing all but one internodal clade, which was the largest in *R. flavipes* comprised of 375 haplotypes, were geographically clustered within the state (Figure 3).

Jenkins et al. (2000) [2] described two samples with unique hydrocarbon phenotypes (BH25 and HH11) that separated into their own clade using mtDNA genetic markers suggesting that these two collections may "represent at least one new taxon in *Reticulitermes*". These samples were both identified as *R. nelsonae* using COII mtDNA in Lim and Forschler (2012) [13]. Sequences from BH25 and HH11 separate into the two larger internodal clades with 133 and 188 haplotypes within *R. nelsonae* and are supported by 97 and 100% bootstrap values (Figure 1).

Reticulitermes have an XY inheritance system implying an examination of mtDNA genes will not indicate paternal inheritance patterns, but because of the sex-linked nature of mitosis, inheritance might show maternal influences from hybridization and provide a clue to gene flow between these sympatric species [36, 37]. *Reticulitermes virginicus* males have been recorded to 'readily' mate with *R. flavipes* females [38] and interspecific hybridization under laboratory conditions has been recorded in five *Reticulitermes* species [38, 39, 40, 41]. Higher average pairwise differences in *R. flavipes,* R. *nelsonae,* and *R. virginicus* compared to the other two species collected in this survey could be related to hybridization in the field because of their flight time overlap, *R. flavipes* alates flying November to May, *R. nelsonae* February to May, *R. virgincus* April to June, [13] and recorded compatibility [38].

Results from the visual multiple sequence alignment (Figure 1) highlight the potential for an additional method of identification for *Reticulitermes* generated from ITA-verified species identifications. Four out of the five described *Reticulitermes* species have conserved silent mutations that, when targeted, produce unique partial gene sequences (Table 6). We presume species specific primers could be created to encompass those conserved mutations and utilized in

a multiplex PCR-based electromorph assay to identify samples as an alternative to sequencing the COII gene.

Results from this survey reinforce that COII is a useful marker for species identification validated in an integrative taxonomic approach. The inheritance patterns influenced by *Reticulitermes* reproductive biology is still greatly unknown and supports further examination of what constitutes a species as illustrated by the haplotype data in respect to *R. flavipes* and *R. nelsonae.* The potential for a species species PCR-based assay generated from an integrative taxonomic approach should inspire future research with these insects.

References

1. Evans, T. A.; Forschler, B. T.; Grace, J. K. Biology of Invasive Termites: A Worldwide Review. *Annual Review of Entomology* 2013, *58* (1), 455–474. DOI:10.1146/annurev-ento-120811-153554
2. Jenkins, T.M.; Haverty, M.I.; Basten, C.J.; Nelson, L.J.; Page, M.; Forschler, B.T. Correlation of mitochondrial haplotypes with cuticular hydrocarbon phenotypes of sympatric Reticulitermes species (Isoptera: Rhinotermitidae) from the southeastern United States. *J. Chem. Ecol.* 2000, *26*, 1525-1542. DOI:10.1023/A:1005548111591
3. Sillam-Dussès, D, and Forschler, B. T. A Dominant and Undescribed Species of Reticulitermes in Sapelo Island (Georgia, USA). *Sociobiology* 2010. *56*. 137-147.
4. Thorne, Barbara & Traniello, James & Adams, E. & Bulmer, Mark. Reproductive dynamics and colony structure of subterranean termites of the genus Reticulitermes (Isoptera Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. *Ethol Ecol Evol* 1999, *11*, 149-169. DOI:10.1080/08927014.1999.9522833.
5. Haverty, M.I. and L.J. Nelson. Cuticular hydrocarbons of Reticulitermes (Isoptera: Rhinotermitidae) from northern California indicate undescribed species. *Comp. Biochem. Physiol.* 1997, *118B*, 869-880.
6. Nelson, L., L. Cool, C. Solek, and M. Haverty. Cuticular Hydrocarbons and Soldier Defense Secretions of Reticulitermes in Southern California: A Critical Analysis of the Taxonomy of the Genus in North America. *J. Chem. Ecol*. 2008, *34*, 1452–1475. DOI 10.1007/s10886-008-9548-6

7. Nutting, W.L. Insecta: Isotopera. In: Dindal D.L., editor. *Soil Biology Guide*, 1st ed.; John Wiley & Sons, New York, NY, USA, 1990; pp. 997-1032.

8. Hostettler, N., D. Hall, and R. Scheffrahn. Intracolony Morphometric Variation and Labral Shape in Florida Reticulitermes (Isoptera: Rhinotermitidae) Soldiers: Significance for Identification. *Fla. Entomol*. 1995, *78*: 119-129. DOI:10.2307/3495675

9. Ye, W., Lee, C., Scheffrahn, R. H., Aleong, J. M., Su, N., Bennett, G. W., & Scharf, M. E. Phylogenetic relationships of nearctic Reticulitermes species (Isoptera: Rhinotermitidae) with particular reference to Reticulitermes arenincola Goellner. *Mol. Phylogenet. Evol.* 2004, *30*(3), 815-822. DOI: 10.1016/S1055-7903(03)00230-6

10. Clément, J.-L.; Howard, R.W.; Blum, M.S.; Lloyd, H. L'isolement spécifique des termites du genre Reticulitermes (Isoptera) du sudest des Etats-Unis. Mise en èvidence grâce à la chimie et au comportement d'une espèce jumelle de R. virginicus = R. malletei sp. nov. et d'une semi-species de R. flavipes. *C R Acad Sci* 1986, *302*, 67–70.

11. Lim S.Y.; Forschler, B.T. A new species of *Reticulitermes* Holmgren, 1913 (Isoptera: Rhinotermitidae) from the Southeastern United States. *Insecta Mundi* 2012, O252:1-4.

12. Haverty, M. The proportion of soldiers in termite colonies: A list and a bibliography (Isoptera). *Sociobiology* 1977, *2*, 199-216.

13. Lim S.Y., and B.T. Forschler. Reticulitermes nelsonae, a New Species of Subterranean Termite (Rhinotermitidae) from the Southeastern United States. *Insects* 2012, *3*, 62-90.

14. Forschler, Brian & Jenkins, Tracie. Evaluation of subterranean termite biology using genetic, chemotaxonomic, and morphometric markers and ecological data: A testimonial for multi-disciplinary efforts. *Trends Entomol.* 1999, *2*, 71-80.

15. Jenkins, T.M., Haverty, M.I., Basten, C.J., Nelson, L.J., Page, M., and Forschler, B.T. Correlation of mitochondrial haplotypes with cuticular hydrocarbon phenotypes of sympatric Reticulitermes species (Isoptera: Rhinotermitidae) from the southeastern United States. *J. Chem. Ecol.* 2000, *26*, 1525-1542.

16. Cameron SL, Lo N, Bourguignon T, Svenson GJ, Evans TA. A mitochondrial genome phylogeny of termites (Blattodea: Termitoidae): robust support for interfamilial relationships and molecular synapomorphies define major clades. Mol Phylogenet Evol. 2012 Oct;65(1):163-73. DOI:10.1016/j.ympev.2012.05.034. Epub 2012 Jun 7. PMID: 22683563.

17. Bourguignon, T.; Lo, N.; Šobotník, J.; Sillam-Dussès, D.; Roisin, Y.; Evans, T. A. Oceanic Dispersal, Vicariance and Human Introduction Shaped the Modern Distribution of the Termites *Reticulitermes* , *Heterotermes* and *Coptotermes*. *Proceedings of the Royal Society B: Biological Sciences* **2016**, *283* (1827), 20160179.

18. Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. Annals of the Entomological Society of America 87: 651–701.

19. Garrick, R.; Collins, B.; Yi, R.; Dyer, R.; Hyseni, C. Identification of Eastern United States Reticulitermes Termite Species via PCR-RFLP, Assessed Using Training and Test Data. *Insects* **2015**, *6* (2), 524–537. DOI: 10.3390/insects6020524

20. Luchetti, A.; Marini, M.; Mantovani, B. Mitochondrial Evolutionary Rate and Speciation in Termites: Data on European Reticulitermes Taxa (Isoptera, Rhinotermitidae). *Insectes Sociaux* **2005**, *52* (3), 218–221. DOI: 10.1007/s00040-005-0806-5

21. Liu, H., and A.T. Beckenbach 1992. Evolution of the Mitochondrial Cytochrome Oxidase II Gene among 10 Orders of Insects. Molecular Phylogenetics and Evolution 41: 41-52.

22. Frohlich DR, Torres-Jerez I I, Bedford ID, Markham PG, Brown JK. A phylogeographical analysis of the bemisia tabaci species complex based on mitochondrial DNA markers. Mol Ecol. 1999 Oct;8(10):1683-91. DOI:10.1046/j.1365-294x.1999.00754.x. PMID: 10583831.

23. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)*, *28*(12), 1647–1649. DOI:10.1093/bioinformatics/bts199

24. Edgar, R.C. MUSCLE: multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research*, Volume 32, Issue 5, 1 March 2004, Pages 1792–1797, DOI:10.1093/nar/gkh340

25. Paradis E (2010). "pegas: an R package for population genetics with an integrated–modular approach." *Bioinformatics*, **26**, 419-420.

26. Paradis E, Schliep K (2019). "ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R." *Bioinformatics*, **35**, 526-528.

27. Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Datasets. Mol. Biol. Evol. 34: 3299-3302. DOI: 10.1093/molbev/msx248

28. Tamura K, Stecher G, and Kumar S 2021 MEGA11: Molecular Evolutionary Genetics Analysis version 11. Molecular Biology and Evolution 38:3022-3027. DOI: 10.1093/molbev/msab120

29. Nei M. and Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

30. Zhou L.; Feng,T.; Xu, S.; Gao, F.; Lam, TT.; Wang, Q.; Wu, T.; Huang, H.; Zhan, L.; Li, L.; Guan, Y.; Dai, Z.; Yu, G. ggmsa: a visual exploration tool for multiple sequence alignment and associated data. Briefings in Bioinformatics. DOI:10.1093/bib/bbac222

31. Thorne, B.L. Biology of Subterranean Termites of the Genus Reticulitermes. In NPMA Research Report on Subterranean Termites. NPMA, Dunn Loring VA, 1999. pp 5-8.

32. Austin, J. W., A. L. Szalanski, R. H. Scheffrahn, M. T. Messenger, S. Dronnet, and A.-G. Bagneres. 2005b. Genetic Evidence for the Synonymy of Two Reticulitermes Species: Reticulitermes flavipes and Reticulitermes santonensis. Annals of the Entomological Society of America 98: 395-401.

33. Hyseni, C.; Garrick, R. C. The Role of Glacial-Interglacial Climate Change in Shaping the Genetic Structure of Eastern Subterranean Termites in the Southern Appalachian Mountains, USA. *Ecology and Evolution* **2019**, *9* (8), 4621–4636. DOI: 10.1002/ece3.5065

34. Hubert, N.; Hanner, R. DNA Barcoding, Species Delineation and Taxonomy: A Historical Perspective. *DNA Barcodes* **2015**, *3* (1). 10.1515/dna-2015-0006

35. Kornet, D.J.; McAllister W. The Composite Species Concept: A Rigorous Basis for Cladistic Practice in Current Themes in Theoretical Biology. Springer. 2005. pp 95-127.

36. Jenkins T.M., Dean R.E., Verkerk R., Forschler B.T. Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate European subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy, and introduction dynamics. *Mol. Phylogenet. Evol.* 2001; 20:286–293. DOI:10.1006/mpev.2001.0966.

37. White, D. J., Wolff, J. N., Pierson, M., & Gemmell, N. J. 2008. Revealing the hidden complexities of mtDNA inheritance. *Molecular ecology*, *17*(23), 4925–4942. DOI:10.1111/j.1365-294X.2008.03982.x

38. Howard, R.W., E.J. Mallette, M.I. Haverty, and R.V. Smythe. 1982. Laboratory evaluation of within-species, between-species, and parthenogenetic reproduction in Reticulitermes flavipes (Kollar) and R. virginicus (Banks). Psyche. 88: 75-87.

39. Clément, J.L. 1979. Hybridization experimentale entre *Reticulitermes santonensis* Feytaud et *Reticulitermes lucifugus* Rossi. Ann. Sci. Nat., Zool. 1: 251-260.

40. Lefebvre, T., Châline, N., Limousin, D. et al. From speciation to introgressive hybridization: the phylogeographic structure of an island subspecies of termite, Reticulitermes lucifugus corsicus. BMC Evol Biol 8, 38 (2008). DOI:10.1186/1471-2148-8-38

41. Wu, Jia, Huan Xu, Ali Hassan, and Qiuying Huang. 2020. "Interspecific Hybridization between the Two Sympatric Termite Reticulitermes Species under Laboratory Conditions" Insects 11, no. 1: 14. DOI:10.3390/insects11010014

Table 4.1. List of haplotypes for each species of *Reticulitermes* collected in this survey listed in order of colonies collected, from the most abundant species (Rf) to the least (Rh).

Rf	Colonies	Rn	Colonies	Rv	Colonies	Rm	Colonies	Rh	Colonies
F1	193	N1	39	V1	62	M1	104	H1	17
F2	185	N2	33	V2	19	M2	90	H2	2
F3	160	N3	32	V3	18	M3	50	H3-H7	1
F4	69	N4, N5	26	V4	17	M4	16		
F5	51	N6	20	V5	16	M5	8		
F6	40	N7	19	V6, V7	13	M6, M7	6		
F7	38	N8	18	V8, V9	11	M8, M9	5		
F8	31	N9, N10	15	V10	7	M10-M12	4		
F9	30	N11	14	V11-V13	6	M13-M18	3		
F10	21	N12	13	V14-V16	5	M19-M30	2		
F11	20	N13	12	V17-V23	4	M29-M124	1		
F12	17	N14	11	V24-V28	3				
F13	16	N15, N16	9	V29-V42	2				
F14-F17	15	N17-N20	8	V43-V147	1				
F18-F20	14	N21-N24	7						
F21	13	N25, N26	6						
F22, F23	11	N27-N34	5						
F24	10	N35-N46	4						
F25-F28	9	N47-N58	3						
F29-F33	8	N59-N96	2						
F34-F40	7	N97-N332	1						
F41-F45	6								
F46-F56	5								
F57-F67	4								
F68-F85	3								
F86-F131	2								
F132-F522	1								

Table 4.2. Nucleotide frequencies for consensus sequences from each species of *Reticulitermes* collected in the survey, and average nucleotide frequencies for all sequences.

Species	Nucleotide Frequency			
	A	T	C	G
Rf	39.3	23.74	23.22	13.74
Rn	39.4	23.10	23.56	13.95
Rv	39.3	23.31	23.49	13.89
Rm	39.4	23.28	23.51	13.82
Rh	39.2	22.87	23.91	13.97
Average	39.3	23.3	23.5	13.9

Table 4.3. A comparative table of nucleotide diversity within species, including unique haplotypes recovered, total number of colonies collected, nucleotide diversity (π), parsimony informative sites, polymorphic sites, conservative mutations sites, average pairwise differences between haplotypes (k), Tajima's test of neutrality statistic (D), and haplotype diversity (*Hd*) for five species of *Reticulitermes* collected in this survey.

Species	Unique Haplotypes (h)	Total # of Colonies	Nucleotide Diversity (π)	Parsimony Informative Sites	Polymorphic Sites	Conservative Mutation Sites	Haplotype Diversity
Rf	522	1817	0.01412	176	231	37	0.958
Rn	334	816	0.02304	166	204	33	0.986
Rv	147	393	0.00757	46	91	13	0.953
Rm	124	437	0.00493	47	94	9	0.869
Rh	7	24	0.00159	3	13	0	0.478

Table 4.4. Range of intraspecific sequence divergence estimates calculated in MEGA v11 [29].

	R. flavipes	*R. hageni*	*R. virginicus*	*R. nelsonae*	*R. malletei*
R. flavipes	0.15-4.82%				
R. hageni		0.15-0.32%			
R. virginicus			0.15-1.90%		
R. nelsonae				0.15-5.26%	
R. malletei					0.15-1.90%

Table 4.5. Average interspecific sequence divergence estimates calculated in MEGA v11 [29].

	R. flavipes	*R. hageni*	*R. virginicus*	*R. nelsonae*	*R. malletei*
R. flavipes					
R. hageni	5.66%				
R. virginicus	5.79%	4.23%			
R. nelsonae	4.62%	3.92%	3.28%		
R. malletei	5.02%	4.29%	3.84%	2.42%	

Table 4.6. Targeted COII regions from *Reticulitermes* that encompass silent conserved mutations in the third codon position for R. flavipes, R. virginicus, R. malletei and *R. hageni* that could be used to create species specific primers, and the estimated amplicon size that each pair of primers would produce in a multiplex PCR assay.

Species	COII Region	Estimated Product Size
Rf	166-420	204
Rv	207-513	306
Rm	153-573	420
Rh	129-249	120

Figure Captions

Figure 4.1. Collapsed Maximum-Likelihood GTR+G+I tree topology, with a log likelihood of -14899.47, used to identify the survey samples to species including 1,132 full length (685bp) COII haplotypes recovered in from PEGAS, highlighting the 55 full length ITA-Verified references by species clade (Table S2). A Coptotermes formosanus COII sequence (AY683221) was used as the extant group. Branch numbers represent percentage of posterior probability from 1,000 UF Bootstrap iterations.

Figure 4.2. Graphic multiple sequence alignment created using *ggmsa* [30] in R (The R Foundation, Vienna Austria) of three most common haplotypes of *R. flavipes*, *R. hageni, R. virginicus, R. malletei,* and *R. nelsonae* collected from WoG in wildland sites from in Georgia, USA. Conserved mutations in the third base pair position are identified for each species using a black rectangle.

Figure 4.3 Kernel density estimation maps of internodal clades identified in a GTR+G+I maximum likelihood tree (Figure 1) including species identification, number of haplotypes in each clade, and Clarke and Evans R clustering score. R <1 indicates a clustered distribution, while R scores >1 are attributed to dispersed distributions.

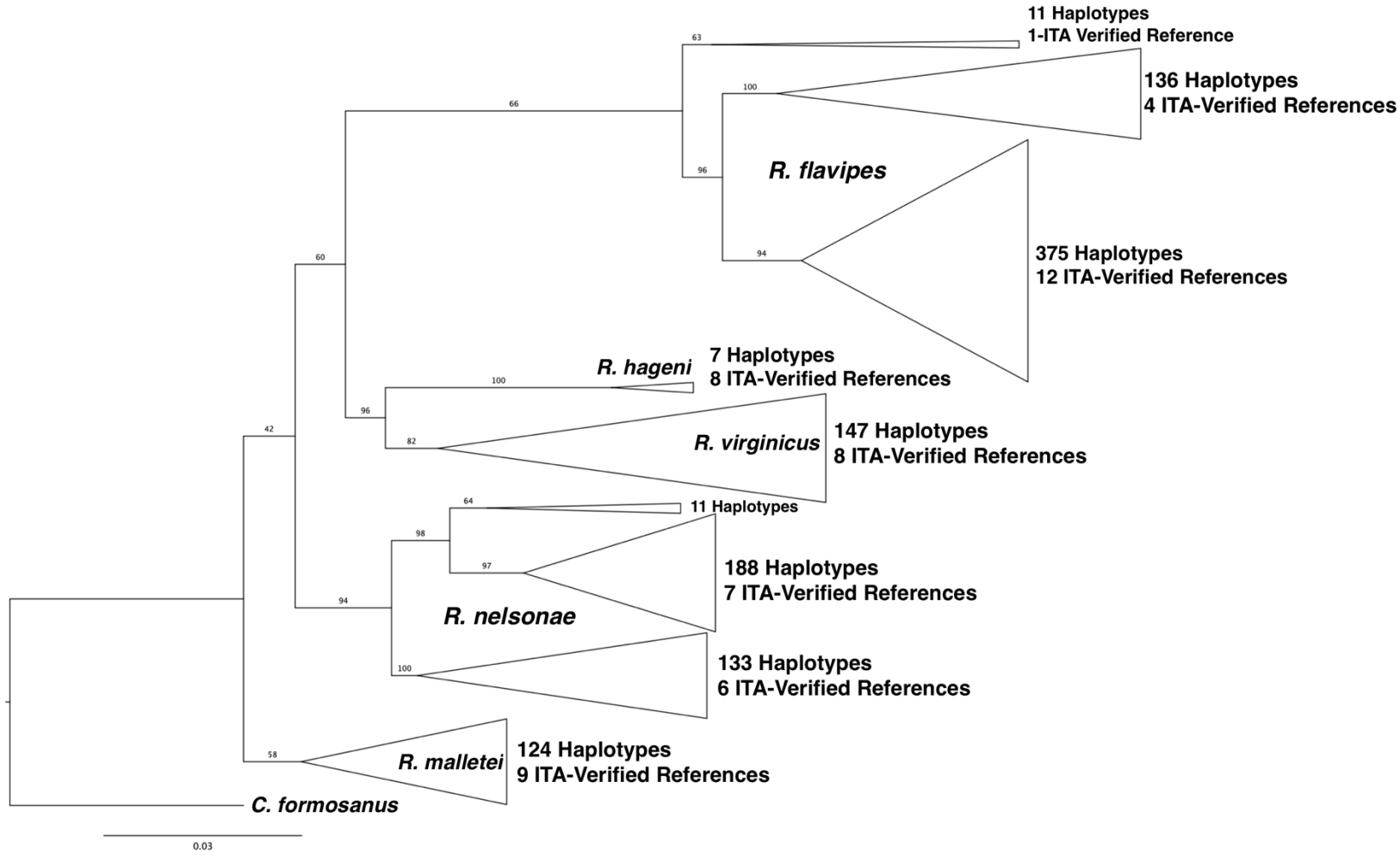
11 Haplotypes
1-ITA Verified Reference
136 Haplotypes
4 ITA-Verified References
R. flavipes
375 Haplotypes
12 ITA-Verified References
R. hageni
7 Haplotypes
8 ITA-Verified References
R. virginicus
147 Haplotypes
8 ITA-Verified References
11 Haplotypes
188 Haplotypes
7 ITA-Verified References
R. nelsonae
133 Haplotypes
6 ITA-Verified References
R. malletei
124 Haplotypes
9 ITA-Verified References
C. formosanus
0.03

Figure 4.1

Figure 4.2

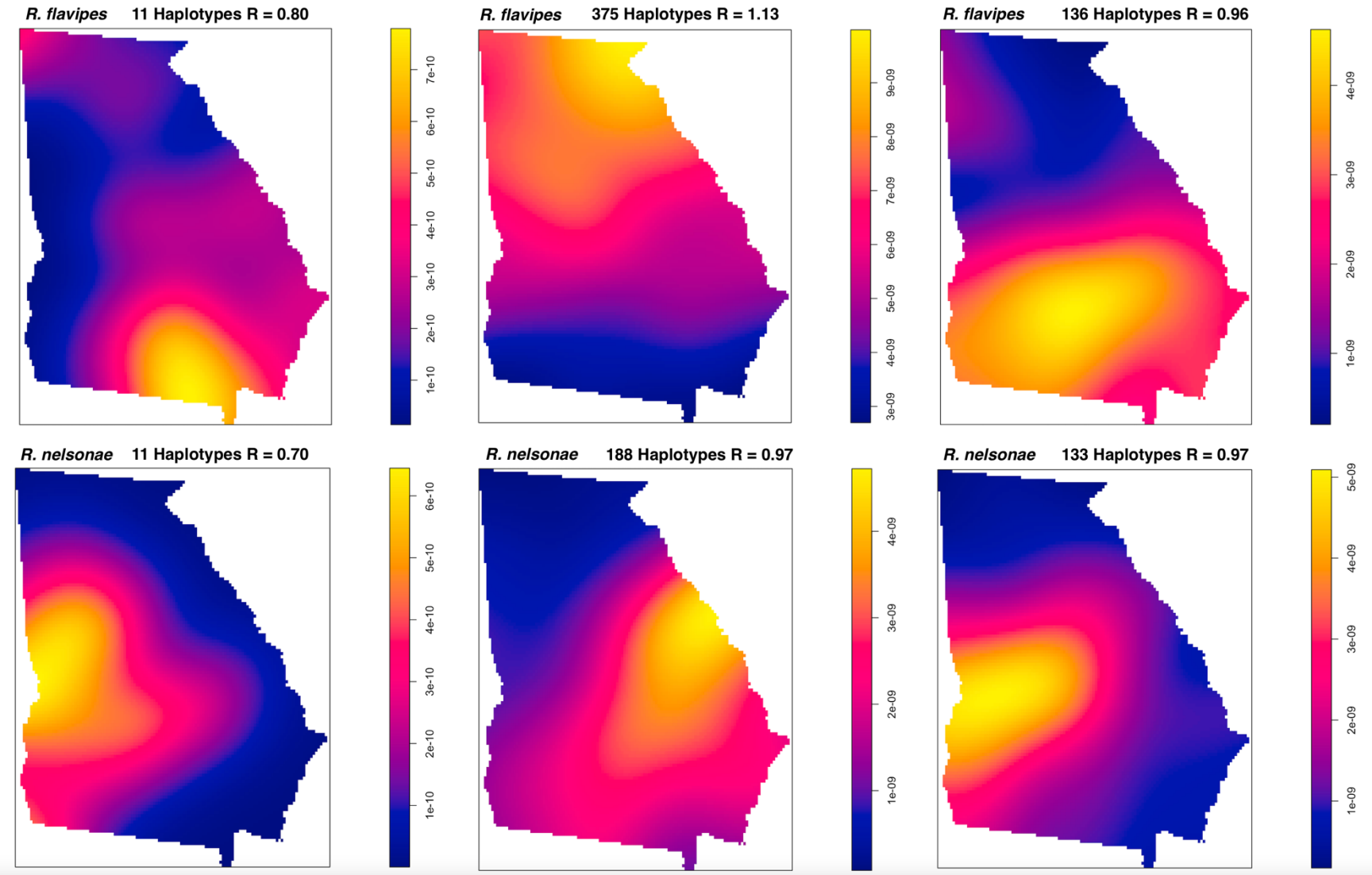
R. flavipes 11 Haplotypes R = 0.80
1e-10
2e-10
3e-10
4e-10
5e-10
6e-10
7e-10
R. flavipes 375 Haplotypes R = 1.13
3e-09
4e-09
5e-09
6e-09
7e-09
8e-09
9e-09
R. flavipes 136 Haplotypes R = 0.96
1e-09
2e-09
3e-09
4e-09
R. nelsonae 11 Haplotypes R = 0.70
1e-10
2e-10
3e-10
4e-10
5e-10
6e-10
R. nelsonae 188 Haplotypes R = 0.97
1e-09
2e-09
3e-09
4e-09
R. nelsonae 133 Haplotypes R = 0.97
1e-09
2e-09
3e-09
4e-09
5e-09

Figure 4.3

APPENDIX C: SUPPLEMENTARY MATERIALS

Table S4.1. List of COI haplotypes recovered using PEGAS [20] from 91 samples representing 4 species in a colony estimation analysis. Samples are arranged by COI haplotype, corresponding COII haplotype, county, and site and sample number.

COI Haplotype	COII Haplotype	County	Site and Sample
C1	F1	Crawford	2-4
C1	F1	Crawford	6-1
C1	F1	Fayette	6-1
C1	F1	Gwinnett	4-4
C1	F1	Gwinnett	4-3
C1	F1	Oglethorpe	2-3
C1	F1	Randolph	2-1
C1	F1	Union	5-1
C2	F1	Oglethorpe	3-3
C3	F1	Rabun	2-4
C4	F1	Fayette	8-3
C5	F1	Butts	2-1
C6	F1	Coweta	3-1
C7	F2	Mitchell	9-1
C7	F2	Mitchell	9-2
C8	F2	Bartow	5-2
C8	F2	Bleckley	8-1
C8	F2	Cobb	2-3
C9	F2	Spalding	2-2
C10	F2	Union	5-3
C11	F2	Bartow	2-3
C11	F2	Meriwether	3-1
C11	F2	Meriwether	8-2
C11	F2	Union	7-2
C11	F2	Union	8-1
C12	F2	Meriwether	8-6
C13	F2	Walker	1-1
C14	F2	Walker	8-2
C15	F2	Meriwether	4-4
C16	F4	Walker	8-1
C17	F4	Candler	12-1
C18	F4	Candler	4-2
C18	F4	Decatur	2-3
C19	F4	Colquitt	4-2
C20	F4	Colquitt	6-1
C20	F4	Colquitt	6-2
C21	F4	Bartow	9-3
C22	F10	Bibb	5-1

C23	F10	Atkinson	8-2
C23	F10	Atkinson	8-3
C24	F10	Camden	10-2
C25	F10	Gilmer	10-1
C26	F10	Jefferson	9-4
C27	F33	Bryan	1-2
C28	F33	Clinch	10-1
C29	F33	Colquitt	10-1
C30	F33	Jeff Davis	6-1
C30	F33	Jeff Davis	6-3
C31	M1	Oglethorpe	6-1
C32	M1	Bartow	4-1
C32	M1	Bartow	4-2
C32	M1	Bartow	4-3
C33	M1	Pickens	2-1
C33	M1	Pickens	5-3
C34	M1	Pickens	7-2
C35	M1	Whitfield	5-1
C36	M1	Butts	5-1
C37	M1	Clarke	7-2
C38	M3	Clarke	5-2
C39	M3	Oconee	8-2
C39	M3	Oconee	8-1
C40	M3	Banks	11-1
C41	M3	Barrow	9-1
C41	M3	Harris	6-2
C42	N5	Mitchell	7-1
C43	N5	Monroe	2-1
C44	N5	Paulding	1-4
C45	N5	Johnson	1-2
C45	N5	Pulaski	5-2
C45	N5	Pulaski	5-3
C46	N5	Pulaski	6-1
C47	N5	Stewart	3-2
C47	N5	Stewart	6-1
C48	N5	Turner	8-4
C49	N5	Calhoun	8-2
C49	N5	DeKalb	4-1
C50	N5	Chattahoochee	2-2
C50	N5	Chattahoochee	2-3
C51	N5	Coweta	7-2
C52	N5	Lee	8-1
C53	N5	Meriwether	7-1
C54	V1	Brantley	3-2
C54	V1	Wilkinson	4-2
C55	V1	Brantley	7-1
C55	V1	Brantley	7-2

C56	V2	Berrien	9-1
C57	V2	Sumter	3-1
C58	V5	Grady	2-1
C58	V5	Grady	5-2
C58	V5	Grady	5-3
C59	V5	Tift	5-2

Table S4.2. ITA-Verified References included in a GTR+G+I ML tree used to infer species designation of samples collected in this survey.

COI			
Species	**Accession No.**	**Date Collected**	**Location**
C. formosanus	AY027472	2001	USA
R. flavipes	JN207470	2/6/07	Sapelo Island, GA, USA
	JN207471	2007	Sapelo Island, GA, USA
	JN207472	11/13/07	Sapelo Island, GA, USA
	JN207473	11/23/07	Sapelo Island, GA, USA
	JN207474	10/23/07	Athens, GA, USA
	JN207475	4/13/09	Athens, GA, USA
	JN207476	4/25/09	Athens, GA, USA
	JN207477	4/21/09	Athens, GA, USA
	F182	3/12/14	Athens, GA, USA
	F192	3/20/19	Athens, GA, USA
R. hageni	JN207483	2007	Athens, GA, USA
	H13	8/20/13	Clarke, Co. USA
R. virginicus	JN207478	11/13/07	Sapelo Island, GA, USA
	JN207479	1/30/08	Sapelo Island, GA, USA
	JN207480	2007	Athens, GA, USA
R. nelsonae	JN207486	2/6/07	Sapelo Island, GA, USA
	JN207487	2007	Sapelo Island, GA, USA
	JN207488	1/30/08	Sapelo Island, GA, USA
	JN207489	2/6/07	Sapelo Island, GA, USA
	JN207490	2/6/07	Sapelo Island, GA, USA
	JN207491	2/6/07	Sapelo Island, GA, USA
	N5	2/6/07	McIntosh Co, GA, USA
	N9	3/31/11	Branford, FL, USA
	N10	4/18/13	McIntosh, GA, USA
R. malletei	JN207484	2007	Athens, GA, USA
	JN207485	10/23/07	Athens, GA, USA
	M47	5/14/12	Athens, GA, USA
COII			
Species	**Accession #**	**Date Collected**	**Location**
C. formosanus	AY683218	6/12/03	Savannah, GA, USA
R. flavipes	JF796216	2009	Athens, GA, USA
	JF796217	8/25/09	Thomasville, GA, USA
	JF796218	7/6/09	USA
	JF796219	3/31/10	Branford, FL, USA
	JF796220	11/5/10	Branford, FL, USA
	F2	4/19/96	Pike Co., GA, USA
	F24	3/28/02	Clarke Co., GA, USA
	F42	4/19/12	Clarke Co., GA, USA

	F50	2/13/13	Clarke Co., GA, USA
	F51	2/20/13	Clarke Co., GA, USA
	F52	2/28/13	Athens, GA, USA
	F58	4/13/13	Athens, GA, USA
	F174	3/10/13	Athens, GA, USA
	F182	3/12/14	Athens, GA, USA
	F192	3/20/19	Athens, GA, USA
	F193	4/27/19	Athens, GA, USA
	FJB	3/4/21	Athens, GA, USA
R. hageni	AF107486	8/97	Barnesville, GA, USA
	JF796224	2007	Athens, GA, USA
	JF796225	2007	Athens, GA, USA
	EU689026	2008	USA
	H11	8/5/07	Athens, GA, USA
	H13	8/20/13	Clarke Co., GA, USA
	H18	8/15/16	Athens, GA, USA
	H19	8/15/16	Athens, GA, USA
R. virginicus	JF796234	5/26/05	Madison, FL, USA
	JF796223	2009	Thomasville, GA, USA
	JF796222	7/5/09	Sapelo Island, GA, USA
	JF796221	5/30/08	Athens, GA, USA
	V41	5/10/16	Athens, GA, USA
	V44	5/13/17	Athens, GA, USA
	V46	5/14/17	Athens, GA, USA
	V47	5/14/17	Athens, GA, USA
R. nelsonae	JF796235	N/A	Sapelo Island, GA, USA
	JF796229	2009	Croatan NF, NC, USA
	JF796233	12/20/06	Apopka, FL, USA
	JF796232	6/18/09	GA, USA
	JF796231	3/31/10	Branford, FL, USA
	JF796230	2009	Thomasville, GA, USA
	EU689013	2008	GA, USA
	N5	2/6/07	McIntosh Co, GA, USA
	N9	3/31/11	Branford, FL, USA
	N10	4/18/13	McIntosh, GA, USA
	N11	4/8/13	McIntosh Co, GA, USA
	N12	5/29/10	Athens, GA, USA
R. malletei	JF796226	10/23/07	Athens, GA, USA
	JF796228	2009	NC, USA
	GU550074	N/A	USA
	JF796227	5/21/10	Athens, GA, USA
	M11	4/26/00	Winston, MS, USA
	M47	5/14/12	Athens, GA, USA
	M49	6/24/12	Athens, GA, USA
	M62	5/26/14	Athens, GA, USA

	M44	4/22/12	Athens, GA, USA

Table S4.3. Full and partial GenBank accessions matching haplotypes recovered from this survey during a BLASTn analysis including the survey numerical identifier, total number of colonies collected for those haplotypes, matching accession(s) from GenBank, the GenBank species designation, whether it was a full or partial COII submission, and the reported collection locations of accessions. [α]denotes accessions that provided a location but specified they are lab reared colonies.

Survey Haplotype	# of Colonies	Matching Accession(s)	GenBank Species Designation	Partial/Full Match	Location(s)
F1	193	EU689010 AY168203	*R. flavipes*	Full	Sapelo Island, Ga Indiana
F3	185	JQ280681	*R. flavipes*	Partial	Destin, FL
F4	69	MT188740 MK922263 EF206315 AF291742 AY168209 AY168210 FJ806884	*R. santonensis* *R. arenincola* *R. flavipes* *R. santonensis*	Full Partial	Foret de la Coubre, France Foret de la Coubre, France Elevage, Dijon, France Biscardrosse, Italy Dune Acres, Indiana Lafayette, Indiana Foret de la Coubre, France
F7	38	JF796220 OL875053	*R. flavipes*	Full	Branford, FL Florida
F8	30	JQ280620	*R. flavipes*	Partial	Pearl River, LA
F9	30	JQ280712	*R. flavipes*	Partial	Charleston, SC
F10	21	EU689011	*R. flavipes*	Full	Sapelo Island, GA
F11	20	JQ280663	*R. flavipes*	Partial	Ostulee State Park, FL
F12	18	JQ280675	*R. flavipes*	Partial	Sopchoppy, FL
F21	11	JQ280682	*R. flavipes*	Partial	Newport, FL
F29	8	EF206314	*R. flavipes*	Full	Louisiana
F33	7	EU689012	*R. flavipes*	Full	Sapelo Island, GA
F34	7	AY808077	*R. flavipes*	Full	Fort Clinch State Park, FL
F36	7	AY453589	*R. arenincola*	Partial	Indiana
F37	7	JF796217	*R. flavipes*	Full	Thomasville, GA

F38	7	JQ280622	*R. flavipes*	Partial	Mississippi
F46	5	JQ280671	*R. flavipes*	Partial	Blackriver Kurl, FL
F51	5	JQ280662	*R. flavipes*	Partial	Ostulee, FL
F55	5	MT211874 AF291743 EF206314 AY808080 AY027474	*R. santonensis* *R. santonensis* *R. flavipes* *R. flavipes* *R. santonensis*	Full Partial	Berlin, Germany[α] Marsiglia, Italy[α] Louisiana Montevideo, Parque Miramar, Uruguay Bordeaux, France
F57	4	EU689007	*R. flavipes*	Full	Sapelo Island, GA
F69	3	JF796216	*R. flavipes*	Full	Athens, GA
F78	3	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F81	3	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F83	3	JQ280674	*R. flavipes*	Partial	Apalachicola, FL
F90	2	KR870360	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F97	2	KR870355	*R. flavipes*	Partial	Johns Mountain Wildlife Management Area, GA
F115	2	KR870360	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F116	2	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F214	1	JQ280711	*R. flavipes*	Partial	Charleston, SC
F242	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F248	1	KR870360	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F253	1	KR870364	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F264	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F266	1	KR870355	*R. flavipes*	Partial	Johns Mountain Wildlife Management Area, GA
F270	1	JQ280623	*R. flavipes*	Partial	Hancock County, MS

F276	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F286	1	JQ280712	*R. flavipes*	Partial	Charleston, SC
F308	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F309	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F310	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F320	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F327	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F341	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F401	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F402	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F405	1	AY808085	*R.flavipes*	Full	Plantation, FL
F408	1	EU689004	*R. flavipes*	Full	Sapelo Island, GA
F429	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F441	1	JQ280623	*R. flavipes*	Partial	Hancock County, MS
F449	1	KR870363	*R. flavipes*	Partial	Great Smoky Mountain National Park, TN
F474	1	EU689006	*R. flavipes*	Full	Sapelo Island, GA
F478	1	JQ280620	*R. flavipes*	Partial	Pearl River, LA
F485	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F491	1	KR870353	*R. flavipes*	Partial	Chattahoochee National Forest, GA
F519	1	JQ280712	*R. flavipes*	Partial	Charleston, SC
H1	17	EU689026 DQ493729	*R. hageni*	Full	Sapelo Island, GA North Carolina

H2	2	AY168208 AF107486	*R. hageni*	Full	Conway, AR Barnesville, GA
H3	1	JF796224	*R. hageni*	Full	Athens, GA
M1	104	JF796226	*R. malletei*	Full	State Botanical Garden – Athens, GA
M2	89	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M3	49	GU550074	*R. malletei*	Full	Sapelo Island, GA
M6	6	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M7	6	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M9	5	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M16	3	AY53642	*R. virginicus*	Full	Jasper-Pulaski Wildlife Area, IN
M17	3	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M22	2	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M24	2	MK295605	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M25	2	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M28	2	MK295605	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M30	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M32	1	MK295605	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M37	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M39	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M47	1	MK295605	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA

M52	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M53	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M55	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M61	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M73	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M80	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M85	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M95	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M98	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M104	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M109	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M110	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
M111	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M114	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M115	1	KR870407	*R. malletei*	Partial	Great Smoky Mountain National Park, TN
M124	1	KR870404	*R. malletei*	Partial	Johns Mountain Wildlife Management Area, GA
N2	33	JF796229 EU689016	*R. nelsonae*	Full	Croatan National Forest, NC Sapelo Island, GA
N3	32	KM245772	*R. nelsonae*	Partial	Fontainebleau Park Forest, LA
N5	26	KM245777	*R. sp*	Partial	Athens, GA

N9	15	JF796232	*R. nelsonae*	Full	Georgia
N18	8	EU689013	*R. sp*	Full	Sapelo Island, GA
N19	8	KU925238 EU689014	*R. nelsonae* *R. sp*	Full	Cumberland Island, GA Sapelo Island, GA
N77	2	EU689018	*R. sp*	Full	Sapelo Island, GA
N169	1	EU689020	*R. sp*	Full	Sapelo Island, GA
N317	1	KR870403	*R. nelsonae*	Partial	Talladega National Forest, AL
V2	19	EU689027	*R. virginicus*	Full	Sapelo Island, GA
V6	13	EF206318	*R. virginicus*	Full	Oconee County, GA
V7	13	KR870401	*R. virginicus*	Partial	Cherokee National Forest, TN
V8	11	EU689024	*R. virginicus*	Full	Sapelo Island, GA
V9	9	AF107487	*R. virginicus*	Full	Sapelo Island, GA
V11	6	EF206319	*R. virginicus*	Full	Wakulla County, FL
V17	4	EU689025	*R. virginicus*	Full	Sapelo Island, GA
V19	4	KR870399	*R. virginicus*	Partial	Johns Mountain Wildlife Management Area, GA
V21	4	KM245775	*R. virginicus*	Partial	Wakulla Spring State Park, FL
V37	2	MK295607	*R. virginicus*	Partial	Johns Mountain Wildlife Management Area, GA
V50	1	MK295607	*R. virginicus*	Partial	Johns Mountain Wildlife Management Area, GA
V51	1	KR870399	*R. virginicus*	Partial	Johns Mountain Wildlife Management Area, GA
V97	1	KR870401	*R. virginicus*	Partial	Cherokee National Forest, TN
V98	1	JF796221	*R. virginicus*	Full	Athens, GA
V110	1	JF796223	*R. virginicus*	Full	Thomasville, GA
V141	1	AF525348	*R. sp*	Partial	Columbia, MO
V143	1	KR870400	*R. virginicus*	Partial	Chattahoochee National Forest, GA

Supplementary Figure Captions

Figure S4.1. Collapsed Maximum-Likelihood GTR+G+I tree topology used to identify 91 samples to species using partial COI mtDNA sequence and 28 ITA-Verified COI references by species clade (Table S4.2). A *Coptotermes formosanus* COI sequence (AY027472) was used as outgroup. Branch numbers represent percentage of posterior probability from 1,000 UF Bootstrap iterations.

Figure S4.2a. Kernel density estimation maps of commonly collected *Reticulitermes* haplotypes (F1-F15) collected in Georgia, USA including haplotype name and Clarke and Evans R clustering score. R <1 indicates a clustered distribution, while R scores >1 are attributed to dispersed distributions.

Figure S4.2b. Kernel density estimation maps of commonly collected *Reticulitermes* haplotypes (F16-N6) collected in Georgia, USA including haplotype name and Clarke and Evans R clustering score. R <1 indicates a clustered distribution, while R scores >1 are attributed to dispersed distributions.

Figure S4.2c. Kernel density estimation maps of commonly collected *Reticulitermes* haplotypes (N7-V7) collected in Georgia, USA including haplotype name and Clarke and Evans R clustering score. R <1 indicates a clustered distribution, while R scores >1 are attributed to dispersed distributions.

Figure S4.2d. Kernel density estimation maps of commonly collected *Reticulitermes* haplotypes (V8-H1) collected in Georgia, USA including haplotype name and Clarke and Evans R clustering score. R <1 indicates a clustered distribution, while R scores >1 are attributed to dispersed distributions.

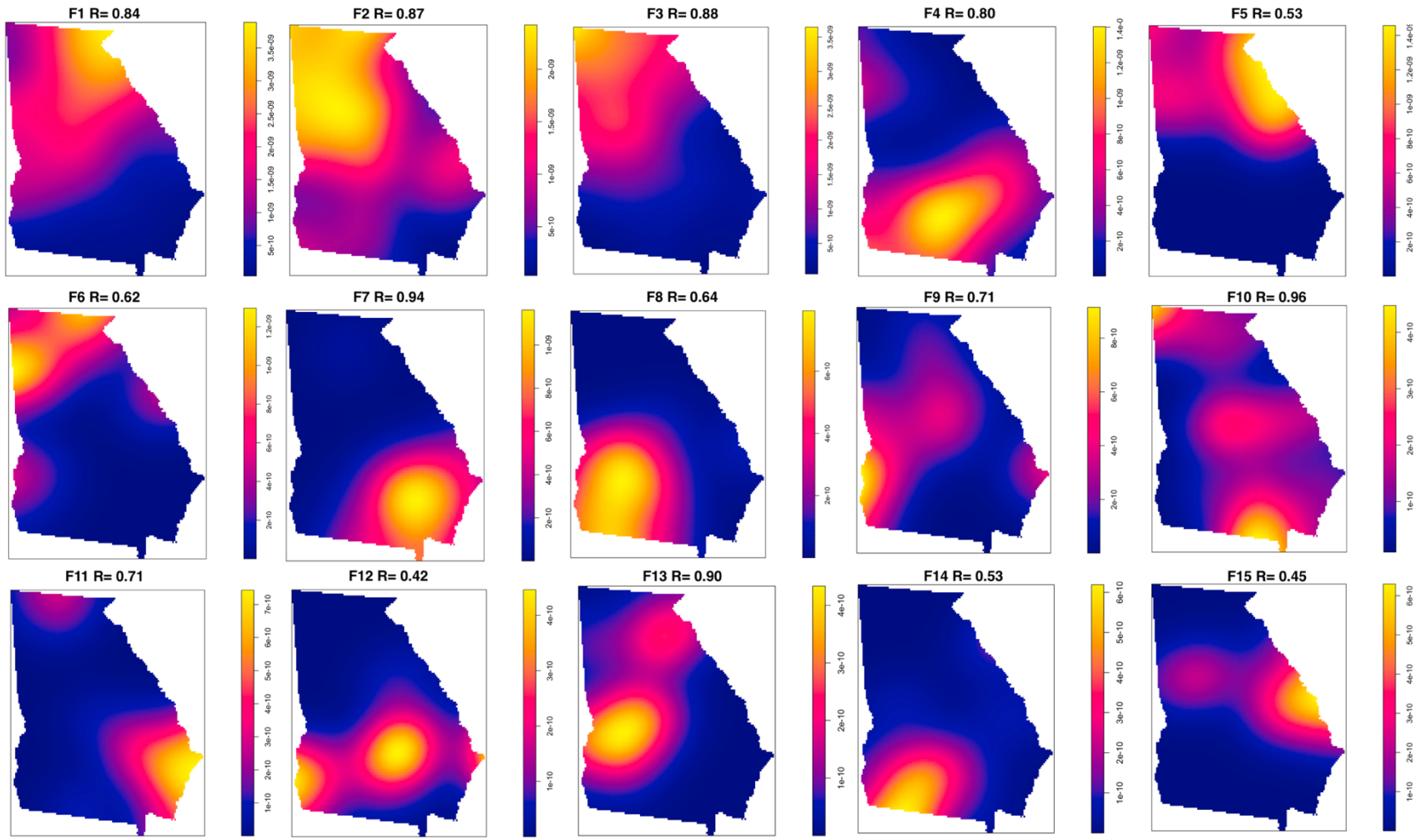
F1 R= 0.84
F2 R= 0.87
F3 R= 0.88
F4 R= 0.80
F5 R= 0.53
F6 R= 0.62
F7 R= 0.94
F8 R= 0.64
F9 R= 0.71
F10 R= 0.96
F11 R= 0.71
F12 R= 0.42
F13 R= 0.90
F14 R= 0.53
F15 R= 0.45

Figure S4.2a

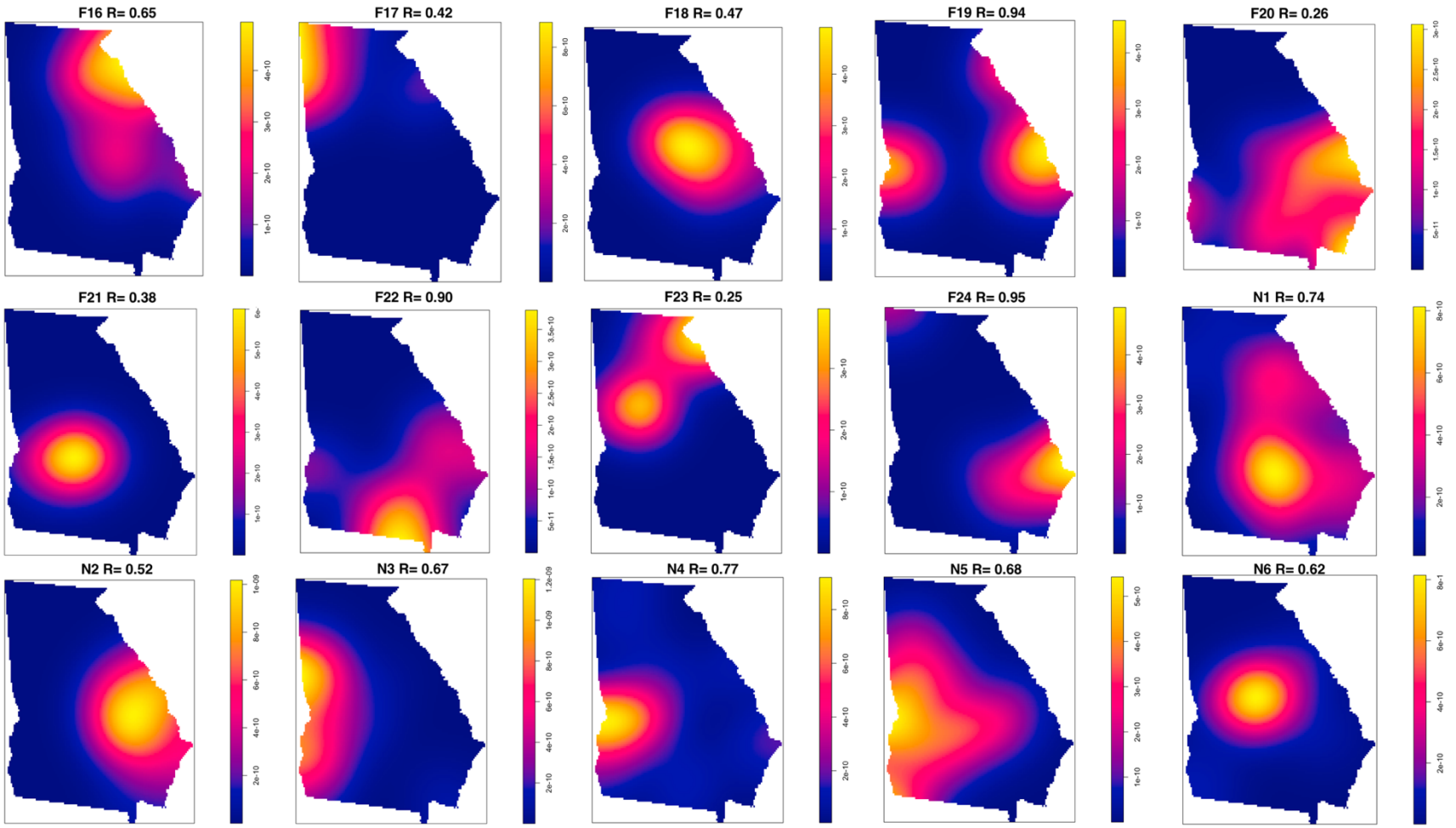
F16 R= 0.65
F17 R= 0.42
F18 R= 0.47
F19 R= 0.94
F20 R= 0.26
F21 R= 0.38
F22 R= 0.90
F23 R= 0.25
F24 R= 0.95
N1 R= 0.74
N2 R= 0.52
N3 R= 0.67
N4 R= 0.77
N5 R= 0.68
N6 R= 0.62

Figure S4.2b

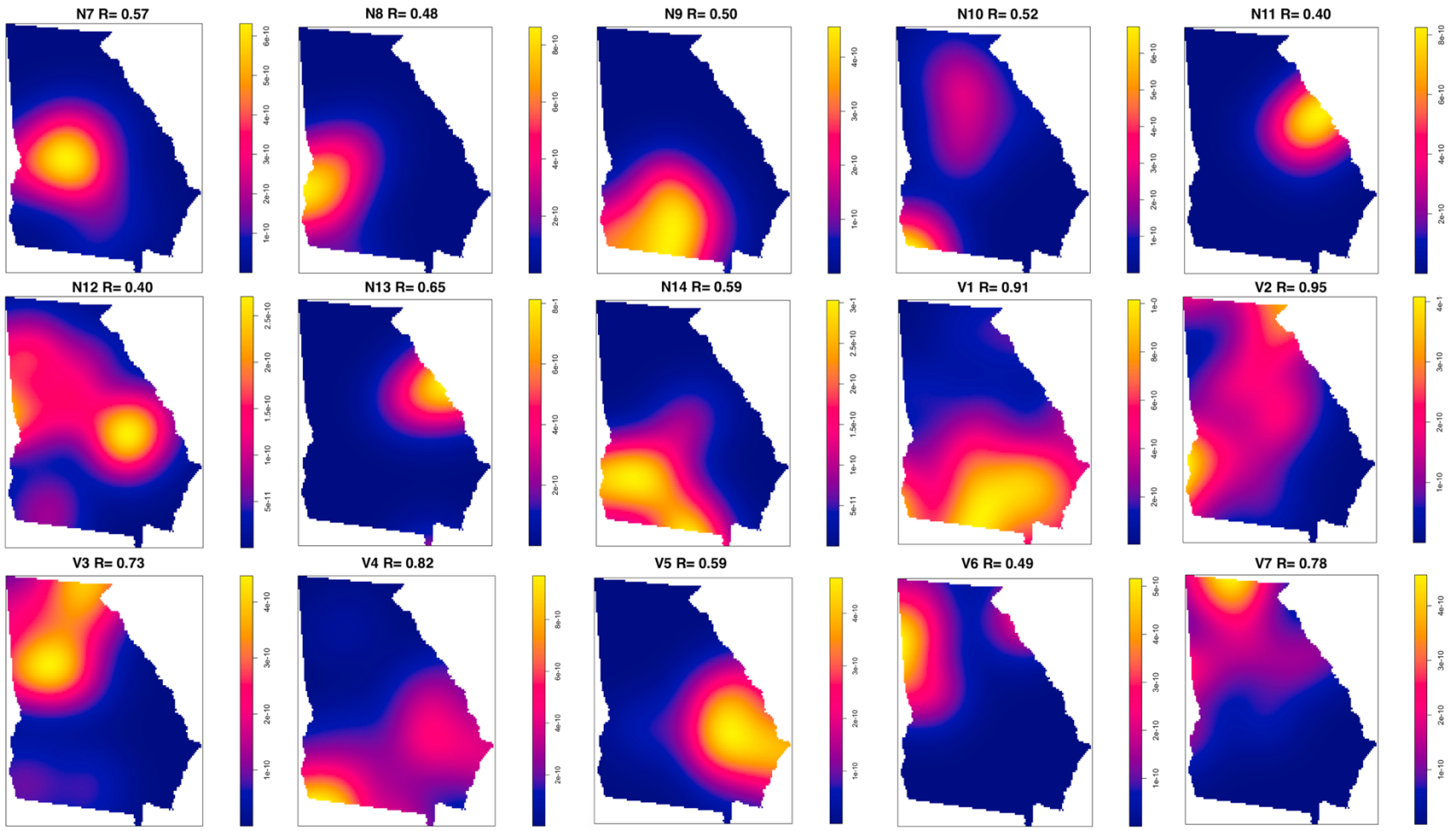
N7 R= 0.57
N8 R= 0.48
N9 R= 0.50
N10 R= 0.52
N11 R= 0.40
N12 R= 0.40
N13 R= 0.65
N14 R= 0.59
V1 R= 0.91
V2 R= 0.95
V3 R= 0.73
V4 R= 0.82
V5 R= 0.59
V6 R= 0.49
V7 R= 0.78

Figure S4.2c

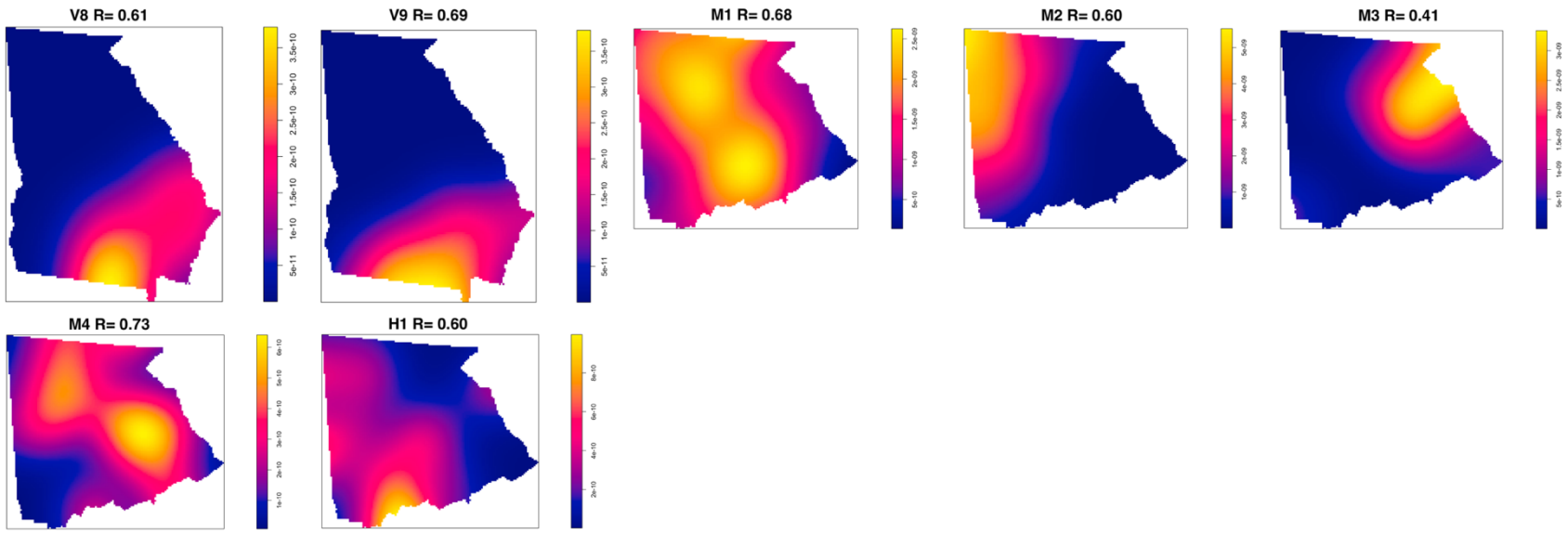

Figure S4.2d

CHAPTER 5

CONCLUSIONS

The objective of this dissertation was to identify subterranean termites, using an integrative taxonomic approach, collected from across the state of Georgia, USA with samples from all 159 counties to illustrate the distribution of *Reticulitermes* in their endemic range. The results highlight that the biodiversity and distribution of *Reticulitermes* in the United States is underrepresented in the literature. This work includes descriptions of qualitative morphometric characters that can be used in future surveys to alleviate the cost of sequencing molecular markers that are the most useful method of species identification. Lastly, this research illuminated several aspects of mtDNA diversity and distribution within this genus of subterranean termite.

Qualitative characters of soldier mandible pairs can be used successfully to identify *R. flavipes*, *R. virginicus*, and *R. nelsonae*, but deferring to an observer with experience identifying *Reticulitermes* will provide the results that aligns with a mtDNA barcode. The implementation of the online 'SMP' trainings and survey emphasized the variability inherent to participant responses in citizen science projects, revealing there are some who 'can' and some who 'cannot' categorize specimens using qualitative characters and demonstrates the importance of assessing participant reliability before analyzing citizen science data.

An examination of GenBank accessions in chapter three further supports the critical need for a revision of the genus. Chapter three provides data that supports an appeal for the *Reticulitermes* research community to employ a COI or COII barcoding marker for species using an integrative taxonomic approach in deference to accepting species identifications through sequence similarity in NCBI BLAST results. In a simple proof of concept exercise, I found 15 mtDNA accessions with species identifications that did not align with their respective clade in a phylogeny grounded in reference sequence determined to an IZCN-verified species using dichotomous keys. Chapter three illuminated the distribution of Rn in Georgia and demonstrated its range is underestimated in the southeast and continental US while that of Rh has been exaggerated in the literature.

A basic genetic analysis of mtDNA in chapter four revealed there is further examination required as to what constitutes a species in this genus. The data support synonymizing *R. arenincola, R. sanontensis,* and *R. flavipes* and add that the matching haplotype (F4) collected in Georgia is abundant, as it was the 4th most frequently collected Rf haplotype from this survey. *R. nelsonae* must also be further examined to determine if the species is genetically diverse or a potential cryptic species complex. Results from this genetic analysis should motivate taxonomists to redefine species boundaries in *Reticulitermes* before describing additional species using molecular data.

In conclusion, I recommend the adoption of an integrative taxonomic approach to identify samples in future surveys of *Reticulitermes* for an anticipated revision of the genus.