Morphological Characterization and Molecular Mediated Genetic Variation of Thief Ants (Hymenoptera: Formicidae)

by

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ABSTRACT

The morphological characterization and molecular genetic variation were determined in populations of thief ants, *Solenopsis molesta* (Say). The genetic variations were elucidated using mitochondrial deoxyribonucleic acid (mDNA) sequences of cytochrome oxidase I. DNA from thief ants was extracted with Qiagen's Gentra PUREGENE® DNA Isolation Kit using their solid tissue protocol. Polymerase chain reactions (PCR) were run on the extracted DNA using primers Lep-F1 (forward) and Lep-R1 (reverse). The DNA products were concentrated and purified by Microcon Centrifugal Filter Unit YM-100. Purified DNA samples were sequenced at the University of Arkansas Medical Sciences (UAMS) DNA Sequencing Core Facility. The sequences were edited and aligned using Codon Code Aligner. The contigs wee uploaded to www.Phylogeny.fr and phylogenetic trees were produced (Neighbor joining, Maximum likelihood and Bayesian). The trees displayed variation in genetic makeup of the thief ants from various geographic regions and genetic variation corresponded to the morphologic identification. The thief ants collected from different states were separated into three groups. Ants collected from New York, Indiana and one location in Nebraska formed one group identified as *S. molesta validiuscula*, a second group formed with ants from Louisiana identified as *S. carolinensis* and the third group consisted of ants from South Dakota, Washington, New Jersey Tennessee, Kansas and two other locations in Nebraska identified as *S. molesta molesta.*

Key Words: Thief ants, *Solenopsis* sp., morphology, molecular genetics.

INTRODUCTION

Of the 40 species of common ants in urban environments, 10 species are consiconsidered economic pests. According to a survey of structural pest

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control operators by Field *et al.* (2007), ants are considered the number one urban pest in the United States generating approximately $1.7 billion annually. The most important species include: carpenter ants, *Camponotus* spp. (Mayr), Argentine ants, *Linepithema humile (*Mayr), odorous house ants, *Tapinoma sessile* (Say), pavement ants, *Tetramorium caespitum* L., red imported fire ants, *Solenopsis invicta (*Buren), thief ants, *S. molesta* (Say) plus others (Bennett *et al.* 2005, Klotz *et al.* 2008). Most of these are considered nuisance pests, except the red imported fire ants due to their ability to sting and inject venom into the skin which causes welts or allergic reactions (Rhoades *et al.* 1975).

S. molesta is commonly called a "thief" because they lives near the nests of other ants and "steals" the larvae and food from the other ant colonies to feed its own. Hays (1920) determined the development time from egg to adult for thief ants ranges 52-64 days. Colonies are generally composed of a few hundred to several thousand workers and several queens. The thief ants infest homes, contaminate food products and their presence is unsightly causing distress, especially in elderly residents. Although thief ants do not cause physical harm to occupants of the structures, they are genetically related to red imported fire ants. Therefore, precise separation of thief ants from imported fire ants or possibly from hybrid populations is critical. Numerous molecular studies have been conducted on the red imported fire ant, *S. invicta*, (Krieger & Ross 2003, 2005) and the Argentine ant, *L. humile*, (Rosset *et al.* 2005), but very limited data exist on morphology of *S. molesta* and none on molecular genetic variations.

MATERIALS AND METHODS

Ant Collection

Thief ant workers (*S. molesta*) were collected from various locations in Lancaster County, Nebraska and other states including: Indiana, Kansas, Louisiana, New York, New Jersey, South Dakota, Tennessee and Washington (Fig. 1). Thief ant workers in Nebraska were collected using traps made of cylindrical, plastic culture tubes (17 x 100 mm) (VWR, Chicago, IL) with 16 or 17 entrance holes in their bottom halves (Husen *et al.* 2008). Peanut butter was used as the food source within each trap. Approximately 2-3 grams of peanut butter was placed on a small piece of paper; the paper was rolled and inserted into the collection tube, and collection tubes were placed

around the perimeter of a structure from 5:00 to 7:00 PM and were picked up the following morning from 8:00 to 10:00 AM. Ants were separated to species in the laboratory using a Bausch and Lomb dissecting microscope and specimens were preserved in 95% ethanol and stored at -20^{O}C in VWR freezer (VWR, West Chester, PA) for DNA extraction, COI amplification and sequencing.

Morphological Characterization

Thief ants were identified to genus using numerous keys (Hayes 1920, Creighton 1950, Thompson 1989, Bolton 1994, Pacheco 2007). Identifications were based on 10 specimens from each collection and enumerated with mean lengths (mm) of antennae and antennal club formed with apical and preapical segments (Fig. 2), and two-segmented petiole connecting gaster and thorax (Fig. 3). Measurements were denoted in mm at 250x magnification, using a micrometer in the ocular lens of a Wild dissection microscope. Other morphological characters supplementing identification were body color, head shape, density of hairs on head and body, and prominence of eyes. Morphology was further illustrated by the following measurements: i) Total

Fig. 1. Geographic collection points for thief ants.

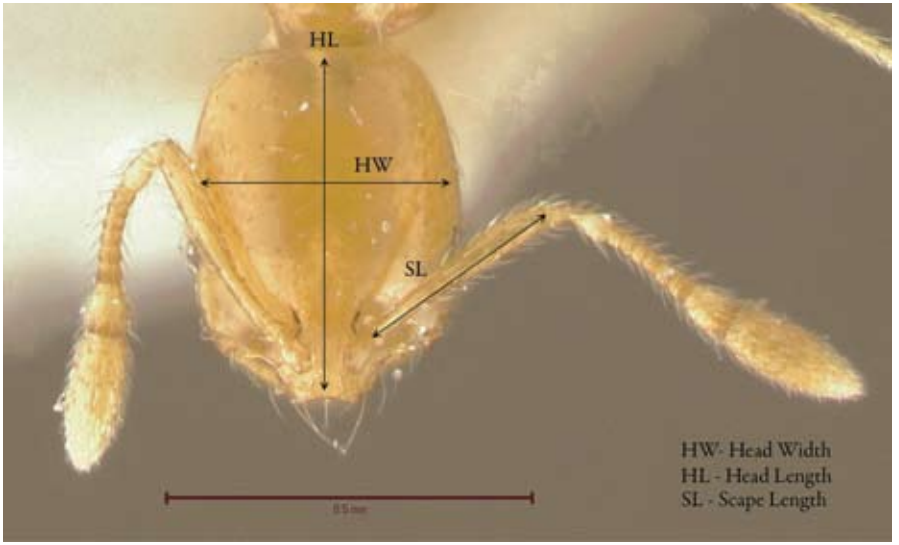

Fig. 2. Morphological characters and various measurements of *Solenopsis* spp. head in frontal view. (Image: R. Narain).

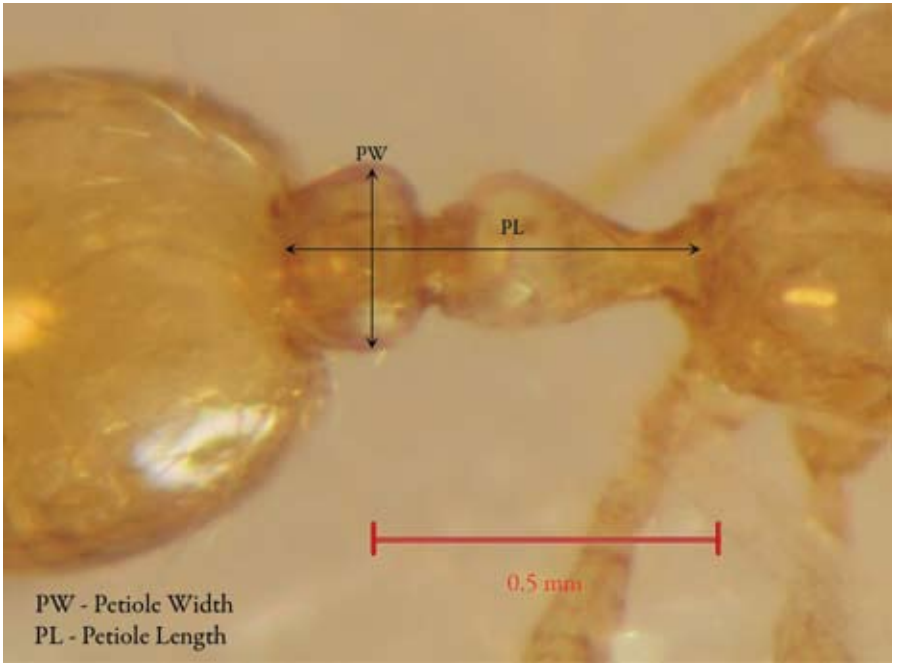

Fig. 3. Morphological characters and various measurements of *Solenopsis* spp. petiole dorsal view. (Image: R. Narain).

length (TL) from head to tip of gaster; ii) Head length (HL) posterior border (top of head) to anterior margin of clypeus (just before mandibles) (Fig. 2); iii) Head width (HW) maximum width excluding eyes (Fig. 2); iv) Scape length (SL) excluding basal condyle (Fig. 2); v) Petiole length (PL) maximum length of nodes measured in dorsal view, starting at posterior edge of thorax and ending at anterior edge of gaster (Fig. 3); and vi) Petiole width (PW) maximum width of node measured in dorsal view (Fig. 3). The indices used in further illustrations were calculated as follows: a) Cephalic index (CI), HW/HL x 100; b) Scape index (SI), SL/HL x 100; and c) Petiolar index (PI), PL/PW x 100. The measurements were recorded as number of units on the micrometer in the left ocular lens then converted to mm using a calibration scale. One unit viewed through the ocular lens at 250x magnification was equivalent to 0.4 mm on the calibration scale, so a recording of 4.68 units is equivalent to 1.87 mm.

DNA Extraction and Isolation

Ants were stored at -20^{O}C in 95% ethanol were removed and the ethanol was allowed to evaporate. Ten thief ants were used per extraction with a minimum of three extractions per location. DNA from thief ants was extracted using a PUREGENE® DNA Isolation Kit (Invitrogen 2010) using a modified tissue protocol from their manual. Standard primers (Smith *et al.* 2007) *(Forward Primer >LepF1 ATTCAACCAATCATAAAGATATTGG: Reverse primer >LepR1 TAAACTTCTGGATGTCCAAAAAATCA*) (Invitrogen, Carlsbad, CA) were used to amplify and sequence the mitochondrial cytochrome oxidase-I (COI) from thief ants (*S. molesta*). The extracted DNA, once rehydrated was stored at 4.0^{O}C until PCR amplification was completed. The concentration of the extracted DNA was determined with the aid of a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and an equivalent of 80-100 ng/µl was used as template for the PCR reaction.

Polymerase Chain Reaction (PCR) Amplification Protocol

The PCR reactions were performed in a total volume of 25.0 µl: 23.0 µl Master Mix (MM) and 2.0 µl DNA template, and run on Applied Biosystem Veriti 96 Well Thermocycler (Applied Biosystem, Foster City, CA) The MM was prepared by combining the equivalent volume of each reagent into a 1.5 ml micro centrifuge tube. The equivalent volume was determined by the

number of PCR reactions to be run at that time. All solutions were allowed to thaw to room temperature and mixed, by uptake and release solution in pipette tip for at least 50 times, before extracting aliquot for MM. The MM was comprised a final concentration of 3.0 mM magnesium chloride, 400.0 µM dNTP mix, 0.2 µM forward primer, 0.2 µM reverse primer and 0.05 Units/µl JumpStart™ REDTaq® (Sigma, St. Louis, MO).

The MM was made homogenous by an uptake and release solution in pipette tip 100+ times before aliquot volume needed for PCR reaction was transferred into 0.2 ml thin walled DNA free micro-centrifuge tube and DNA template was added. Before amplification, DNA template and MM were also mixed by pipettor 20 times and pulse centrifugation. After each PCR reaction, tubes were prepared (MM + template). During sample preparation, DNA template, PCR master mix and prepared samples were kept on ice to reduce template DNA breakdown until all samples were ready and thermocycler program check completed.

PCR Program and Sequencing

The PCR program used was optimized from Smith *et al.* (2007). The program consisted of an initial denaturation step at 94.0°C for 1min, then 5 cycles of denaturation for 40 seconds (sec.) at 94.0°C, an annealing step at 45.0°C for 40 sec., with an elongation step which lasted for 1min at 72.0°C. This was followed by the main PCR cycles which consisted of 35 cycles of denaturing at 94.0°C for 40 sec.; annealing at 51.0°C for 40 sec., and extension for 1min at 72.0°C. The PCR program was completed with a final extension/

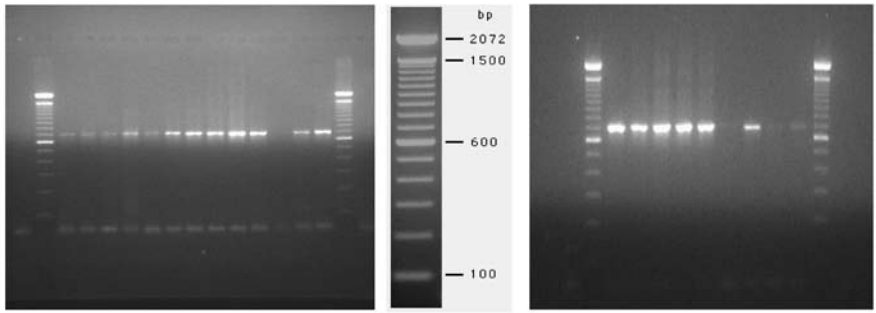

Fig. 4. Images (a and c) of 1% Agarose gel showing PCR amplified COI from thief ants and (b) Invitrogen 100bp DNA Ladder used to estimate number of base pairs (Invitrogen 2010)

elongation soak at 72.0°C for 5 min. The samples stayed in the thermocycler at 4.0°C (max 12 hr) until they were removed for the next step.

After completion of the amplification process, 5.0 µl PCR product was loaded into 1.0% agarose gel in 0.5x TBE, stained with 0.1% ethidium bromide, electrophoresed in 0.5X TBE buffer solution at 100 volts for approximately 1 hr. The gel was viewed and photographed (Fig. 4) on a Bio-Rad Gel Doc System (Bio-Rad, Hercules, CA). Successfully amplified products were concentrated and purified using Microcon Centrifugal Filter Unit YM-100 (Fisher Scientific, Pittsburgh, PA). Another 1.0% agarose gel was run to determine the approximate concentration of the clean PCR product by comparing the distance travelled by the cleaned PCR product and intensity of the bands to that of a Low Mass DNA Ladder (Invitrogen Carlsbad, CA). Purified samples were diluted to 20 ng/ml and sent to the University of Arkansas Medical Sciences (UAMS) DNA Sequencing Core Facility for sequencing. Sequences received from UAMS were edited and aligned using Codon Code Aligner (Dedham, MA). The contigs were uploaded to Phylogeny.fr (Dereeper *et al.* 2008) where phylogenetic trees were produced. The trees obtained (Figs 6, 7 and 8) from the sequences submitted indicated the relationship and variation in thief ant genetic make up for the location sampled.

RESULTS AND DISCUSSION

Morphological and Genetic Variation

Thief ant specimens were identified with morphological characters (Table 1) as *Solenopsis molesta validiuscula, S. carolinensis* and *S. molesta molesta*. *Solenopsis molesta validiuscula* was identified from one collection site from each of three states: New York, Indiana and Nebraska. *Solenopsis molesta molesta* was identified from two collection sites in Nebraska, two collection sites in Tennessee, and one collection site from each of the following states: Kansas, South Dakota, Washington and New Jersey. *Solenopsis carolinensis* was collected from two locations in Louisiana (Table 2). The thief ants species identified from these nine states are not an exhaustive list, but were based on specimens collected. There could be additional thief ant species or subspecies found in those states.

The latest revision of the thief ants by Pacheco (2007) listed a total of 83 species. From a previous 149 available taxa, the author recognizes 72 valid

Table 1: Morphometric measurements of thief ants.

	Location	Total Length	Head Length	Head Width	Scape Length	Petiolar Length	Petiolar Width	Cephalic Index[a]	Scape Index[b]	Petiolar Index[c]
1	LA71051	1.677	0.466	0.321	0.279	0.298	0.124	68.979	59.928	243.170
2	LA70714	1.690	0.458	0.342	0.248	0.251	0.122	74.672	54.148	205.229
3	SD57701	1.791	0.474	0.327	0.289	0.271	0.123	68.987	60.886	220.292
4	TN37721	1.808	0.518	0.403	0.276	0.268	0.134	77.838	53.282	200.599
5	TN37996	1.853	0.511	0.384	0.282	0.264	0.131	75.294	55.278	202.917
6	NE68521	1.820	0.512	0.402	0.264	0.268	0.132	78.516	51.563	203.030
7	NJ08901	1.822	0.524	0.336	0.270	0.256	0.126	74.122	51.527	202.532
8	KS66503	1.828	0.518	0.420	0.285	0.278	0.132	81.208	55.042	210.399
9	WA99224	1.866	0.524	0.405	0.338	0.299	0.132	77.328	64.504	225.982
10	NE68505	1.872	0.496	0.400	0.290	0.288	0.131	80.645	58.468	219.817
11	NY11741	1.949	0.576	0.436	0.291	0.293	0.157	75.676	50.579	186.441
12	NE68583	1.988	0.516	0.380	0.268	0.250	0.128	73.751	51.986	195.751
13	IN47907	2.028	0.512	0.426	0.278	0.268	0.135	83.203	54.297	198.813

[a]Cephalic index = Head width/Head length x 100
[b]Scape index = Scape length/ Head length x 100
[c]Petiolar index = Petiole length/Petiole width x 100

species and identified an additional 11 new species. One possible reason as to why there were 149 taxa listed previously was the use of synonymy, for example, listed below are several of the synonyms of *S. carolinensis: S. texana carolinensis* (Forel 1901); *S. molesta* var. *castanea* (Wheeler 1908); *S. texana r. truncorum* (Creighton 1950). Similar situations of synonymy are found in other thief ant populations. Another reason for taxa overestimation could be because of the wide range of color and size of thief ants within a species. It is still possible that the number of species Pacheco (2007) identified in his dissertation may be changed, either by addition of new species or review and re-identification of the species. One way to counter this problem is the use of morphological and molecular genetic identification simultaneously.

Solenopsis molesta Species Complex

Workers of most species in this complex are around 1.0-2.5 mm long and yellow to light brown ants. The head is somewhat elongated, two-node petiole

Table 2: Identification of thief ant specimens collected from different states.

Site Location and Specimen Identification			
	State	Zip code	Specimen Identification
1	Louisiana	71051	*Solenopsis carolinensis*
2	Louisiana	70714	*Solenopsis carolinensis*
3	South Dakota	57701	*Solenopsis molesta molesta*
4	Tennessee	37721	*Solenopsis molesta molesta*
5	Tennessee	37996	*Solenopsis molesta molesta*
6	Nebraska	68521	*Solenopsis molesta molesta*
7	New Jersey	08901	*Solenopsis molesta molesta*
8	Kansas	66503	*Solenopsis molesta molesta*
9	Washington	99224	*Solenopsis molesta molesta*
10	Nebraska	68505	*Solenopsis molesta molesta*
11	New York	11741	*Solenopsis molesta validiuscula*
12	Nebraska	68583	*Solenopsis molesta validiuscula*
13	Indiana	47907	*Solenopsis molesta validiuscula*

connecting the gaster to the thorax and 10 segmented antennae with apical two segments forming a large club. Thief ants have small stingers and generally have small eyes (Say 1836, Hayes 1920, Creighton 1950, Thompson 1989, Pacheco 2007). Some species in *S. molesta* complex are difficult to conclusively identify with morphological features without the species' queen and male. This limitation could be resolved with the use of DNA barcoding.

Solenopsis carolinensis Forel

Workers are small, yellow with the hairs on the posterior tibia are usually semi-erect, scape length 0.23-0.26 mm, head length 0.30-0.35 mm, a relatively narrower petiole (petiolar 0.12-0.13 mm, and the post petiolar width 0.25-0.26 mm. The lateral clypeal teeth are well developed, and the extra-lateral processes are developed at least into an angle. Eyes are nearly circular, normal size between 0.03-0.04 mm, mostly brown to black (Say 1836, Hayes 1920, Creighton 1950, Thompson 1982, 1989) (Fig. 5). The S. *carolinensis* species identified based on morphometric data in this study had been previously identified as the same species by Hopper-Bui (2010).

Solenopsis molesta molesta Say and *Solenopsis molesta validiuscula* Emery

The workers are small, yellow or light brown species, with two well-developed clypeal teeth and underdeveloped extra-lateral teeth (only small bumps). The smaller segments of the funiculus are about 0.12 mm long. This species of ants can be separated from *S. carolinensis* by the longer length of the smallest segments of the funiculus.

It is difficult to separate subspecies of *S. molesta validiuscula* from *S. molesta molesta*. The workers of *S. molesta molesta* are often smaller (1.7-1.8 mm long) than *S. molesta validiuscula* (1.9- 2.0 mm long), although the sizes of the workers overlap. The cephalic punctures of *S. molesta validiuscula* are nearly always moderately coarse, much larger than the hairs which arise from them, the punctures are often finer in *S. molesta molesta*, often difficult to see, and not much larger in diameter than the hairs which arise from them. The pedicel of *S. molesta validiuscula* is about 2/3 the length of the scape. The two species are nearly identical, but based on these characters; it appears that they are both valid subspecies and do not appear to be a synonym of *S. molesta* (Say 1836, Hayes 1920, Creighton 1950, Thompson 1989, Bolton 1994). Morphometric data generated with this research agreed with previously published work by the authors mentioned above.

Fig. 5. Thief ants collected from Louisiana (LA 70714), Identified as *Solenopsis carolinensis* (Image: R. Narain).

Solenopsis molesta validiuscula is relatively larger than S. *molesta molesta*, which is relatively larger than *S. carolinensis*. Color ranges from yellow in *S. carolinensis* to yellow or light brown in *S. molesta molesta* and to a darker brown in *S. molesta validiuscula*.

Molecular Based Species Genetic Variation

Phylogenetic analysis using programs on www.phylogeny.fr separates the COI sequences collected into three groups, which correspond to the morphological identification using the measurements of key ant features and calculations of cephalic, antennae scape and petiole ratios. *Tetramorium caespitum* and *Myrmica* spp. were used as out-groups for the phylogenetic trees.

The phylogenetic tree produced by Maximum likelihood analysis (Fig. 6) illustrates that the sequences analyzed fall into identifiable groups. One group consists of *S. carolinensis* from the two locations in Louisiana. The second group consists of *S. molesta validiuscula* from New York, Indiana and Nebraska. The final group was *S. molesta molesta* from two collection sites in Nebraska, two collection sites in Tennessee, and one collection site from Kansas, South Dakota, Washington and New Jersey. Bayesian analysis (Fig. 7) and Neighbor-joining (Fig. 8) trees showed similar groupings with

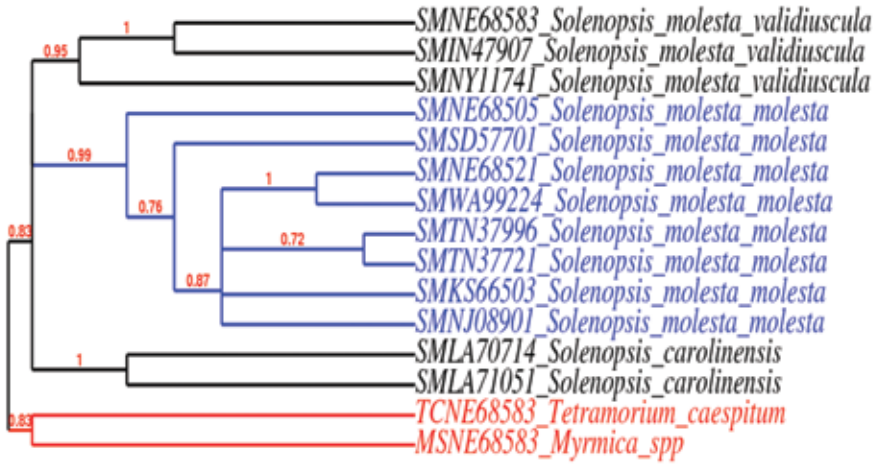

Fig. 6. Maximum Likelihood phylogenetic tree branch supporting values of thief ants collected from nine states (13 locations) across its distribution range, rooted with *Tetramorium caespitum* and *Myrmica* spp.

the only differences being the supporting values displayed on the branches of each tree.

Since the morphological data generated in this research agrees with published data, these phylogenetic trees confirm the morphological identifications and are descriptive of the genetic variation of these thief ant species. The sequences generated could be used for identification of these species in future research.

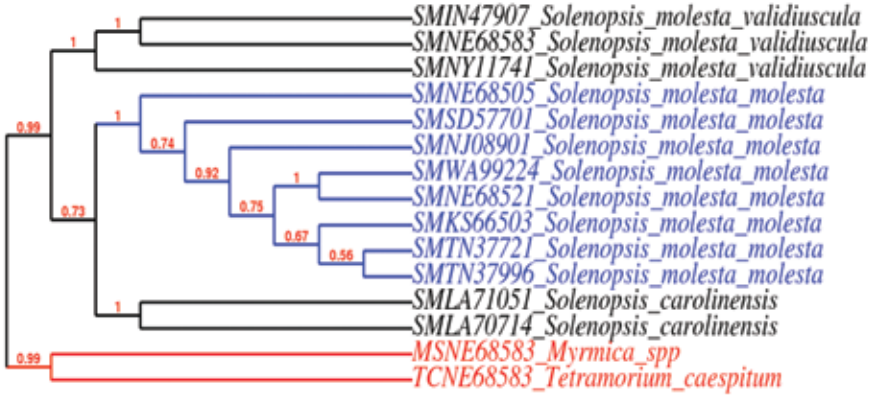

Fig. 7: Bayesian phylogenetic tree with branch supporting values of thief ants collected from nine states (13 locations). Analysis done on phylogeny.fr program MrBayes, rooted with *Tetramorium caespitum* and *Myrmica* spp.

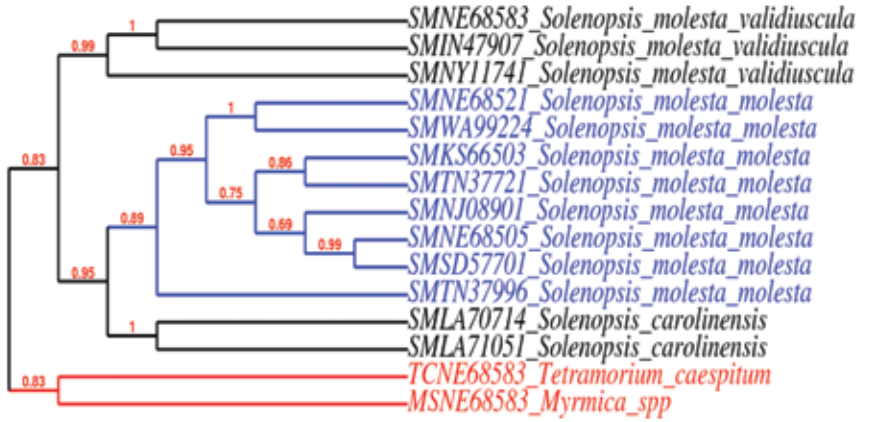

Fig. 8: Neighbor joining profile with branch supporting values of thief ants collected from nine states (13 locations) across their geographic distribution range.

SUMMARY AND CONCLUSIONS

This research shows that the use of COI sequences to identify thief ant species/subspecies that are difficult to key out via the dichotomous keys is a feasible process. Specimens that are very minute, such as thief ants, or disintegrated due to age could be identified once COI sequences from previously identified specimen have been sequenced and the sequences deposited in gene banks, or via comparison of related species using phylogenetic trees. These COI sequences would be used to identify unknown or undetermined species. Thief ants were found in all states sampled, distribution of species in the complex could vary within each State.

The geographic distribution of thief ants varies; some species are likely to be localized, such as *S. carolinensis*, which was collected only from Louisiana. Other species were more universally distributed. *Solenopsis molesta molesta* was identified within seven states and *S. molesta validiuscula* was identified from specimens collected in three states. The morphologic identification corresponded to genetic variation found within the samples analyzed. The thief ants collected from Louisiana were morphologically identified as *S. carolinensis* and genetic variability supported this distinction.

COI sequences generated and the protocol used in this research could be reproduced on thief ant specimens collected in other locations. This could aid in identification of the species, reducing the hassle and aggravation associated with morphologic identification of such tiny ants.

Significance of Research

Based on literature reviewed, it was determined that this is the first attempt to correlate morphologic and genetic variation of thief ant species, and also the first submission of COI sequences from thief ants to GenBank. A search for nucleotide sequences from *Solenopsis molesta* in GenBank on May 11, 2010 (http://www.ncbi.nlm.nih.gov/sites/entrez) returned seven entries. None of these entries were COI sequences of thief ants.

This study also sought to update the geographic distribution map of the *S. molesta* species complex. Previous geographic distribution data suggested that *S. molesta validiuscula* were more common in the Western states. This research determined this to not be the case. *Solenopsis molesta validiuscula*

was found in Nebraska, Indiana and New York suggesting that this species was always present in these states or its distribution range has increased.

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