Molecular Comparisons Suggest Caribbean Crazy Ant From Florida and Rasberry Crazy Ant From Texas (Hymenoptera: Formicidae: Nylanderia) Are the Same Species

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ABSTRACT In 2002, a new invasive pest ant in the genus, Nylanderia (formerly Paratrechina), was found in Houston, TX. This invasive ant has been causing significant economic and ecological damage in infested areas. Because of the morphological and behavioral similarities to Nylanderia pubens Forel (Caribbean crazy ant) found in Florida, this ant was named Nylanderia sp. nr. pubens (Rasberry crazy ant). So far, morphometric and phylogenetic analyses have not determined if the two ants are the same or separate species. To determine the relationships between the two populations, a molecular approach was undertaken. Five novel genes with various functions from N. pubens and N. sp. nr. pubens were cloned, sequenced, and identified, including a chemosensory protein (NpCsp), the cyclophilin-like protein (NpClp), the fatty acid binding protein (NpFabp), the ferritin 2-like protein (NpFlp), and an odorant binding protein (NpObp). The cDNA sequences of NpCsp, NpFabp, NpFlp, and NpObp, shared 100% identity between N. sp. nr. pubens and N. pubens. The cDNA of NpClp shared 99% identity, with the only difference at the nucleotide position 358. Comparisons of four partial genomic DNA sequences from Caribbean and Rasberry crazy ants indicated 100% identity for a 710-bp partial genomic DNA sequence of cytochrome oxidase subunit I gene, 99% identity for a 774-bp, and a 452-bp and 710-bp partial genomic DNA sequence containing only coding regions, and 100% identity for a 289 bp partial genomic DNA sequence of NpCsp containing only coding region. This study showed that N. sp. nr. pubens in Texas is the same, or at most an intraspecific variant or ecotype of the species in Florida.

KEY WORDS Nylanderia pubens, cDNA, genomic DNA, ecotype, Paratrechina pubens

Nylanderia pubens (Forel), originally described as Paratrechina pubens Forel, is an exotic species native to the Caribbean Islands and South America (Trager 1984, LaPolla et al. 2010). In the United States, N. pubens has been reported in southern Florida for at least 60 yr (Trager 1994). In 2002, a pest ant in the genus Nylanderia was found in Houston, TX (Meyers 2008). It was morphologically similar to N. pubens and N. fulva (Mayr) (Meyers 2008). However, because of taxonomic uncertainty, this ant has neither been identified as N. pubens, nor N. fulva, and instead was designated as Nylanderia sp. nr. pubens (Meyers 2008). In the popular media, N. sp. nr. pubens was called the Rasberry crazy ant, after Tom Rasberry, the discoverer of this ant in Texas, and more recently the hairy crazy ant. Both of these names are unofficial common names. Similarly, N. pubens from Florida has been unofficially called the Caribbean crazy ant and the brown crazy ant (Warner and Scheffrahn 2004, Wetterer and Keularts 2008, MacGown and Layton 2010, Calibeo and Oi 2011).

Although the economic and ecological impact of N. sp. nr. pubens is not fully known yet, some of their biological and behavioral characteristics indicate that N. sp. nr. pubens can potentially be a significant pest. In addition to the tremendous numbers, they are polygyne, unicolonial, and omnivorous. It was found that N. sp. nr. pubens displaced both native and introduced ants, indicating that they can cause deleterious ecological effects (Meyers 2008). They also may cause wildlife to move out of infested areas. The economic impact of N. sp. nr. pubens also can be substantial. Failures of electrical equipment have been attributed to large numbers of these ants by shorting circuits and clogging switching mechanisms. In some cases, the ants have caused thousands of dollars in damage and repair costs (http://urbanentomology.tamu.edu/ants/exotic_tx.cfm). Unfortunately, typical control tactics for urban pest ants do not work well for N. sp. nr. pubens because of their tremendously large population densities. N. sp. nr. pubens spreads at ≈ 30 m per

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month in urban areas (Meyers 2008) and 207.4 m/yr in rural areas (D. McDonald unpublished, personal communication). Without measures to contain the movement of N. sp. nr. \textit{pubens}, it will likely continue to extend its geographic range.

There is an urgent need to determine if N. sp. nr. \textit{pubens} is the same species as \textit{N. pubens} infesting Florida. In this study, five novel genes with various functions and four genomic DNA fragments, as well as a mitochondrial gene from N. sp. nr. \textit{pubens} and \textit{N. pubens} were cloned, sequenced, identified and compared, to contribute toward the species determination of N. sp. nr. \textit{pubens}. Some genes showed diverse sequences in other insects such as odorant-binding proteins and chemosensory proteins (Pelosi et al. 2005).

**Materials and Methods**

**Ant Collection.** Caribbean crazy ants, \textit{N. pubens}, were collected in Alachua and Duval Counties, FL, and Rasberry crazy ants, N. sp. nr. \textit{pubens}, were collected in Harris and Brazoria Counties, TX. Workers of Caribbean crazy ants and Rasberry crazy ant were preserved in RNAlater solution (Ambion, Austin, TX) for RNA analysis, or in 100% ethanol (Sigma-Aldrich, St. Louis, MO) for DNA analysis. Samples (\(\approx 300 \pm 50\) individuals/per sample) from three colonies of Caribbean crazy ants were processed separately for RNA and DNA extraction. RNAs of two colonies (\(\approx 300 \pm 50\) individuals/per sample) from Rasberry crazy ant were separately extracted for gene cloning. The Caribbean crazy ants and Rasberry crazy ant samples preserved in ethanol from the locations described above were used for DNA extractions.

Because there are no genomic data for \textit{N. pubens} available in GenBank, it was important to clone genes for this study. First, mRNA from Caribbean crazy ants and Rasberry crazy ants was extracted and purified. Then, cDNA libraries were synthesized and genes were cloned from these cDNA libraries. Subsequently, gene-specific polymerase chain reaction (PCR) primers were designed using genomic DNA as templates, to check the partial genomic DNA information for the genes. The sequences of cloned cDNA and DNA fragments were analyzed and deposited in GenBank of the National Center for Biotechnology Information (NCBI).

**RNA Extraction.** Total RNAs were extracted using TRizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Poly (A) + RNA was isolated by applying Oligotex-dT suspension (QIAGEN, Valencia, CA). RNA samples were quantified using NanoPhotometer (IMPLEN, Westlake Village, CA). Puriﬁed genomic DNA samples from Caribbean crazy ants and Rasberry crazy ants were used as templates for PCR ampliﬁcation.

**PCR for Cloning.** Primers designed from the genes (NpCol, NpFabp, NpObp and NpFabp) were used to generate PCR products using genomic DNA as template. NpCol—LC01490-F: 5’-GGTCACCAACTCATAAGAGATATTGG-3’/HCO2198-R: 5’-TAAAACCCTCAGGCGTACAAAAATCA-3’. NpFab—RCA2-9-10 F: 5’-TTGCTCTATTCCTGCTGCGT-3’/RCA2-9-25R: 5’-GCCAGCATGTGCCTGCTCTTCTT-3’. NpFabp—RCA2-34-34 F: 5’-CTCTCCACCGAGCGAAAACTTT-3’/RCA2-34-232R: 5’-CCACCGTTCTCTCTGCTGACT-3’. NpObp—RCA2-28-100 F: 5’-TCCTGCTTACGGCAGATGCTG-3’/RCA2-28-263R: 5’-GCCAGCCTACTCTCAGCTC-3’. PCR conditions were 95°C for 4 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 3 min, finishing with an extension step at 72°C for 10 min. PCR products were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA). The partial sequences of genomic DNA were analyzed using NCBI nucleotide blast program.

**Sequence Data Processing.** A multiple sequence alignment of chemosensory protein, cyclophilin-like protein, fatty acid binding protein, ferritin 2-like protein, odorant binding protein, and orthologues or paralogs from other insects were compared with the MEGA
5.05 program (http://www.megasoftware.net). MEGA5 default distance was used to construct the phylogenetic trees. Five phylogenetic trees were constructed using the Neighbor-joining method with MEGA 5.05 program (Tamura et al. 2011). The Neighbor-joining is based on the minimum-evolution criterion, and also a bottom-up clustering method for the creation of phenetic trees (Saitou and Nei 1987).

**Results**

**cDNA Sequence From Caribbean Crazy Ants and Rasberry Crazy Ants.** To examine and compare the genetic identity of Caribbean crazy ants and Rasberry crazy ants, five genes of Caribbean crazy ants and RCA were cloned and sequenced from their cDNA libraries and then deposited in GenBank at the NCBI. NCBI BLAST program was used to run the Standard Nucleotide BLAST, to align two sequences and to analyze these cDNA sequences. In cDNA coding regions, NpCsp, NpFabp, NpFlp and NpObj shared 100% identity between the Caribbean crazy ants and Rasberry crazy ants (Table 1, Fig. 7S). NpClp shared 99% identity between Caribbean crazy ants and Rasberry crazy ants. The only difference was the nucleotide at the position of 358: C for Caribbean crazy ants and T for Rasberry crazy ants.

**Identification of Chemosensory Protein Gene.** The chemosensory protein cDNA is 378 bp in length and is calculated to encode a protein of 126 amino acids with a molecular mass of 14.41 kDa. Comparison with chemosensory protein nucleotide sequences from other ant species showed 52% identity to Camponotus floridanus Buckley with 87% coverage; 77% identity to Acromyrmex echinatior Forel with 96% coverage; 78% identity to Harpegnathos saltator (T. C. Jerdon) with 88% coverage. However, there was no significant identity to Linepithema humile Mayr and Solenopsis invicta Buren. Comparison with chemosensory protein nucleotide sequences from other insects showed 74% identity to Bombus ignitus Smith with 73% coverage; 74% identity to Anopheles gambiae Giles with 52% coverage; and 71% identity to several species of Drosophila with 53% coverage (Supp. Table S1, Supp. Figure S1). All of the accession numbers of nucleotides and proteins of five genes in different species were reported in the Supplementary Table S1-S5.

The NPCSP protein sequence had 75% identity to C. floridanus with 96% coverage; 76% identity to A. echinatior with 98% coverage; 73% identity to H. saltator with 98% coverage; 74% identity to S. invicta with 95% coverage; 56% identity to D. ananassae Fallen and D. virilis with 98% coverage; 57% identity to Stomoxys calcitrans L. with 96% coverage; 58% identity to Tribolium castaneum Herbst with 96% coverage; 51% identity to Apis mellifera L. with 99% coverage; 54% identity to Culex quinquefasciatus Say with 98% coverage; 53% identity to An. gambiae with 98% coverage; and 50% identity to Aedes aegypti L. with 96% coverage (Supp. Table S1).

**Identification of Cyclophilin-like Protein Gene.** The cyclophilin-like protein cDNA is 492 bp in length and is calculated to encode a protein of 164 amino acids with a molecular mass of 18.96 kDa. Comparison with cyclophilin-like protein nucleotide sequences from other ants showed 91% identity to C. floridanus with 98% coverage; 87% identity to S. invicta with 98% coverage; 89% identity to A. echinatior with 100% coverage; and 87% identity to H. saltator with 100% coverage. NpClp also showed 81% identity to A. mellifera with 98% coverage; 75% identity to Gryllus pennsylvaniae Buemeister and C. firmus Scudder with 90% coverage; 73% identity to most species from Drosophila genus with 94% coverage; 73% identity to Ae. aegypti with 81% coverage and 60–78% identity to many other insects with 25–69% coverage (Supp. Table S2, Supp. Figure S2).

NPCCL protein was showed 97–98% identity to three different ants, including S. invicta with 99% coverage; 97% identity to C. floridanus with 99% coverage; 98% identity to A. echinatior with 98% coverage; 92% identity to A. mellifera with 99% coverage; 89% identity to Nasonia citripennis Ashmead with 99% coverage; 95% identity to H. saltator with 99% coverage; 80–82% identity to most species from the genus Drosophila, with 93–99% coverage; 77% identity to Ae. aegypti with 99% coverage; 79% identity to An. gambiae with 99% coverage; and 76% identity to Culex tarsalis L. with 99% coverage (Supp. Table S2).

**Identification of Fatty Acid Binding Protein Gene.** The fatty acid binding protein cDNA is 402 bp in length and is calculated to encode a protein of 133 amino acids with a molecular mass of 14.97 kDa. Comparison with fatty acid binding protein nucleotide sequences from other ants showed 84% identity to C. floridanus with 96% coverage; 81% identity to A. echinatior with 95% coverage. NpFab also showed 77% identity to A. mellifera with 96% coverage; 69% identity to N. vitripennis with 95% coverage; 68% identity to Periplaneta americana L. with 91% coverage; and 67% identity to Drosophila mojavensis Patterson with 67% coverage (Supp. Table S3, Supp. Figure S3).

NPFABP protein was showed 87% identity to C. floridanus with 99% coverage; 85% identity to A. echi-
natior with 99% coverage; 80% identity to H. saltator with 99% coverage; 80% identity to A. mellifera with 97% coverage; 73% identity to N. vitripennis with 97% coverage; 63% identity to Drosophila melanogaster Meigen with 93% coverage; 63% identity to Cx. quiquefasciatus with 92% coverage; 62% identity to An. gambiae with 92% coverage; and 64% identity to Ae. aegypti with 93% coverage (Supp. Table S3).

Identification of Ferritin 2-like Protein Gene. The ferritin 2-like protein cDNA is 668 bp in length and is calculated to encode a protein of 221 amino acids with a molecular mass of 25.01 kDa. Comparison with fer-
Fig. 2. Genomic DNA sequence analysis of NpCoI of Caribbean crazy ants and Rasperry crazy ants. A. Genomic DNA sequence of NpCoI. Bold sequences stand for coding sequence. Genomic DNA sequence alignments of NpCoI of Caribbean crazy ant (CCA) and Rasperry crazy ant (RCA) showed 100% identity. B. BLAST results showed that “Identities/H11005 654/684 (96%)” for genomic DNA and cDNA sequences of NpCoI. Query was genomic DNA of N. pubens, whereas Sbjct was cDNA of N. pubens. There were 18 positions of the sequence which are different between genomic DNA and cDNA. Nucleotides were changed from T to C (4), G to A (5), T to A (3), C to T (5), and G to T (1). C. Phylogenetic trees were constructed using the Neighbor-joining tree-making method for nucleic acid sequences of insect orthologs using the MEGA 5.05 program.
ritin 2-like protein nucleotide sequences from other ants showed 84% identity to C. floridanus with 100% coverage; 77% identity to A. echinatior with 97% coverage. However, there is no significant identity to S. invicta.

Comparison with ferritin 2-like protein nucleotide sequences from other insects found 65% identity to A. mellifera with 86% coverage; 67% identity to N. vitripennis with 55% coverage; and 72% identity to Asobara tabida Nees (Supp. Table S4, Supp. Figure S4).

NPFLP protein was showed 80% identity to C. floridanus with 99% coverage; 81% identity to A. echinatior with 99% coverage; 72% identity to H. saltator with 99% coverage. However, there is no significant identity to S. invicta. Comparison with ferritin 2-like protein nucleotide sequences from other insects found 65% identity to A. mellifera with 86% coverage; 67% identity to N. vitripennis with 55% coverage; and 72% identity to Asobara tabida Nees (Supp. Table S4, Supp. Figure S4).

Identification of Odorant Binding Protein Gene.
The complete odorant binding protein cDNA of C. floridanus (NP0bp) was amplified and sequenced and has been deposited in GeneBank (Table 1). The odorant binding protein cDNA is 441 bp in length and is calculated to encode a protein of 147 amino acids with a molecular mass of 16.485 kDa. According to the NCBI BLAST databases and sequence analysis, there is no similarity between NP0bp and Obp nucleotide sequences of the ants C. floridanus, S. invicta, S. geminate, S. megergates, S. macdonoghi, and S. saevissima. NP0bp nucleotide sequences compared with those of other insects showed 80% identity to D. melanogaster with 12% coverage (Supp. Table S5, Supp. Figure S5).

Although there is no significant identity to C. floridanus in the nucleotide sequences, NOP0BP protein showed 81% identity to C. floridanus with 99% coverage; 30% identity to S. invicta with 96% coverage; 28% identity to S. richteri with 96% coverage; 25–29% identity to most species from Solenopsis genus with 90–96% coverage (Supp. Table S5).
Molecular Phylogenetic Analysis. The molecular phylogeny corroborates the distinct lineage between \textit{N. pubens} and \textit{C. floridanus} (Fig. 1A–E, Supp. Table S1–S5). The phylogenetic trees of five genes \textit{NpCsp}, \textit{NpClp}, \textit{NpFabp}, \textit{NpFlp}, and \textit{NpObp} for nucleic acid sequences from other insect orthologs or paralogs supported that \textit{N. pubens} have the closest relationship to the ant \textit{C. floridanus} (Fig. 1A–E, Supp. Table S1–S5). Additionally, the mitochondrial gene \textit{NpCOI} showed a close relationship within the genus of \textit{Nylanderia} (Fig. 2D, Supp. Table S6).

DNA Sequence From Caribbean Crazy Ants and Rasberry Crazy Ants. To further compare the genetic identity of the Caribbean crazy ant and Rasberry crazy ant samples collected from Florida and Texas, four pairs of primers were designed for examining the genomic DNA sequence levels. PCR products of partial genomic DNAs were cloned and sequenced. Some of the DNA sequences contained noncoding regions of the gene. The genomic DNA from Caribbean crazy ants and Rasberry crazy ant were 99% to 100% identical to each other (Figs. 2–5). For example, 710 bp
Fig. 4. Genomic DNA sequence analysis of NpObp of Caribbean crazy ant and Rrasberry crazy ant. A. Genomic DNA sequence of NpObp. Bold sequences stand for coding sequences and the rest are introns in the genomic sequence. B. BLAST analysis of genomic DNA (bold sequence) and cDNA sequences of NpObp. Nucleotides are changed at positions 11 (T to G), 31 (A to G), 235 (C to G), 327 (G to A), 331 (G to A), 445 (C to G), and 447 (C to A). There are three cDNA fragments which showed “Identities/H11005 31/33 (94%)"; “Identities/H11005 100/103 (97%)" and “Identities/H11005 40/42 (95%)" respectively. C. Genomic DNA sequence alignments of NpObp of Caribbean crazy ant (CCA) and Rrasberry crazy ant (RCA). There are two-positions where nucleotides were changed from T to C, which showed “Identities/H11005 450/452 (99%)".
partial genomic DNA sequence of cytochrome oxidase subunit I (CoI) gene showed 100% identity between Caribbean crazy ants and Rasberry crazy ants. However, the genomic DNA sequence of \(NpCoI\) compared with cDNA of \(NpCoI\) showed 18 positions of nucleotide change, which might indicate RNA editing during RNA molecular processing (Fig. 2B). In another example, a 773 bp partial genomic DNA sequence of \(NpFabp\) containing a noncoding region, and coding regions of 35 bp plus 157 bp (Fig. 3B), showed 99% identity between Caribbean crazy ants and Rasberry crazy ants (Fig. 3C). A third, the 452 bp partial genomic DNA sequence of \(NpObp\) that included cDNA coding regions of 35, 102, and 42 bp (Fig. 4B), also revealed 99% identity between Caribbean crazy ants and Rasberry crazy ants.

Discussion

Morphological evidence alone was not enough to identify Rasberry crazy ant to species (Meyers 2008). Chemical analysis using gas chromatography–mass spectrometry (GC–MS) showed that Caribbean crazy ants and Rasberry crazy ants had an almost perfect match in their chemical profiles (Chen and Zhao, unpublished data). Although it is unusual for two species to have identical chemical profiles, they cannot be used as the direct evidence that Caribbean crazy ants and Rasberry crazy ants are the same species. One concern is that not all compounds can be detected at a particular GC-MS condition. A more reliable method to examine the identification of Caribbean crazy ants and Rasberry crazy ants is to analyze its gene sequences. For this purpose, five novel genes (\(NpCsp\), \(NPClp\), \(NpFabp\), \(NpFlp\) and \(NpObp\)) from Caribbean crazy ants and Rasberry crazy ants were cloned for the molecular analysis of Caribbean crazy ants and Rasberry crazy ants, as well as for comparison with orthologues or paralogs of several other insects.

Chemosensory protein is a class of small, soluble proteins secreted into the sensillar lymph of chemosensory organs (Angeli et al. 1999). According to the genomic database and sequence analysis, the \(NpCsp\) nucleotide sequence was found to have 74–82% identity to other ants including \(C. floridanus\), \(A. echinatior\), and \(H. saltator\). The genomic analysis also revealed that the \(NpCsp\) gene shared higher identity to some ants such as \(C. floridanus\), \(H. saltator\), and \(A. echinatior\), than the other ants such as \(L. humile\) and \(S. invicta\), and the bumblebee species \(B. ignitus\) and \(B. terrestris\) (Smith et al. 2011, Wurm et al. 2011). Although there was no significant identity with chemosensory protein genes between \(N. pubens\) and \(L. humile\) or \(S. invicta\), the NPCBP protein was found to possess a 74% identity to \(S. invicta\).
The cyclophilin-like protein (CLP) gene, is a member of the highly conserved, ubiquitous family of peptidylprolyl isomerases, which plays an important role in protein folding and immunosuppression by cyclosporin A (Carson et al. 2009). NpClp nucleotide sequence in Rasberry crazy ant showed a 91% identity to C. floridanus. Interestingly, the NCPLP protein sequence showed 98% identity to S. invicta, although the identity of the nucleotide sequences was relatively low ≈87%.

The fatty-acid-binding proteins (FABPs) are a superfamily of carrier proteins for fatty acids and other lipophilic substances (Chmurzynska 2006). These proteins are thought to facilitate the transfer of fatty acids between extracellular and intracellular membranes, for example, to transport lipophilic molecules from outer cell membrane to certain intracellular receptors (Tan et al. 2002, Weisiger 2002). The NPFPABP protein sequences showed an 87% identity to C. floridanus.

Ferritin, a ubiquitous intracellular protein consisting of 24 subunits, serves to store iron in a nontoxic form, and to transport it to required areas (Theil 1987). At the nucleotide sequence level, the NpFlp showed an 84% identity to C. floridanus. However, in the protein sequences, NPFLP showed an 80% identity to C. floridanus (Bonasio et al. 2011).

Odorant binding proteins (OBP) are abundant small soluble proteins secreted in the nasal mucus of many animal species and in the sensillar lymph of chemosensory sensilla of insects. The aqueous solubility of hydrophobic odorants is greatly enhanced via odorant binding proteins which exist in the extracellular fluid surrounding the odorant receptors (Vogt et al. 1991, Yang et al. 2011). There was no significant nucleotide similarity between NpOlp and Obp from Solenopsis, including S. invicta (Gotzek et al. 2011, Wurm et al. 2011). However, the NOPOB was found to have an 81% identity to C. floridanus (Bonasio et al. 2011).

Four genes with very different functions shared 100% identity between the Caribbean crazy ant and Rasberry crazy ant (Table 1). It is very unlikely that two different species would have four genes with exactly the same sequences. Cyclophilin-like protein (NpClp) shared 99% identity between Caribbean crazy ants and Rasberry crazy ants. The only difference was the nucleotide at position 358 (C to T), which did not change the amino acid sequence of NCPLP. In addition, three partial genomic DNA sequences showed high similarity between Caribbean crazy ants and Rasberry crazy ants (Figs. 2–4).

Mitochondrial genes can be used for the analysis of the genetic and phenotypic diversity and the relationship between species in terms of plesiomorphy and convergent evolution (Schlick-Steiner et al. 2006, Bataille et al. 2009). Our genomic DNA sequence of NpCol1 revealed 100% identities between Caribbean crazy ants and Rasberry crazy ant. These data showed that Caribbean crazy ants from Florida and the Texas Rasberry crazy ants are the same species, or, at most, they are intraspecific variants of the same species. Because genotypic comparisons of N. pubens and closely related species from their native ranges were not made, actual species identification cannot be established at this time. Nevertheless, confirmation that Caribbean crazy ants and Rasberry crazy ants are virtually identical will aid the development of control methods and regulatory policies for this invasive ant in the United States.

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