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 partial genomic DNA sequence of *NpFabp* and *NpObp* containing noncoding 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, *Partrechina 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 1984). 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. *pubens,* it will likely continue to extend its geographic range.

There is an urgent need to determine if *N. sp.* nr. *pubens* is the same species as *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. *pubens* and *N. pubens* were cloned, sequenced, identified *and compared,* to contribute toward the species determination of *N.* sp. nr. *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, *N. pubens,* were collected in Alachua and Duval Counties, FL, and Rasberry crazy ants, *N.* sp. nr. *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 (≈300 ± 50 individuals/per sample) from three colonies of Caribbean crazy ants were processed separately for RNA and DNA extraction. RNAs of two colonies (≈300 ± 50 individuals/per sample) of 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 *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). The RNA samples were used for cDNA synthesis and GeneRacer cloning.

GeneRacer Cloning. The GeneRacer Kit was used to amplify full-length genes of 5′ and 3′ cDNA ends by slightly modifying the manufacturer's instructions (Invitrogen, Carlsbad, CA). PCR products of full-length genes were inserted and ligated into the cloning vectors using the TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA). Ligations of PCR products and cloning vectors were then transformed into One Shot TOP10 Competent Cells (Invitrogen, Carlsbad, CA) and grown overnight on Luria-Bertani plates containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside). Clones were isolated and grown overnight in LB-ampicillin broth at 37°C and 235 RPMs in an Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ).

Gene Sequencing of GeneRacer Library. Clones of the GeneRacer library were purified with QIAprep Miniprep (QIAGEN, Valencia, CA). The plasmid DNAs (0.5 μg) were then digested by using *Eco*RI enzyme (2.5 U) for 1.5 h and were run on a 1% agarose gel to confirm the DNA insert. Selected clones were then sent to the DNA Sequencing Core at the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida (Gainesville, FL) to be sequenced and analyzed using the NCBI BLASTN program to identify sequence homologies. The sequences were submitted into NCBI GenBank and the Accession Numbers are GU980916-GU980928, HQ636472-HQ636478 and JF815100-JF815104. After obtaining >15 full length cDNA sequences for *N. pubens,* five nucleus-encoded genes chemosensory protein (*NpCsp*), fatty acid binding protein (*NpFabp*), ferritin 2-like protein (*NpFlp*) and odorant binding protein (*NpObp*) and one mitochondrial encoded gene (*NpCoI*) were selected for this study.

DNA Extraction. Genomic DNA was extracted using DNeasy Blood & Tissue Kit, by slightly modifying the manufacturer's instructions (QIAGEN Science, Germantown, MD). DNA samples were quantified using a 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 *NpCbp*) were used to generate PCR products using genomic DNA as template. *NpCol*—LCO1490-F: 5′-GGTCAACAAATCATAAAGATATTGG-3′/HCO2198-R: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′. *NpCsp*—RCA2–9-10 F: 5′-TTGGCTCTATTCCTGCTCGT-3′/RCA2-9–297R: 5′-GTCCCATGTTGCTGGTTTCT-3′; *NpFabp*—RCA2–34-34 F: 5′-CTCTCCAGCAGCGAAAACTT-3′/RCA2-34–232R: 5′-CCACGGTCTCTTCGTCAAAT-3′; *NpObp*—RCA2–28-100 F: 5′-TCTTGTATCGCTGAATCTGGC-3′/RCA2–28-263R: 5′-GCACGAGCTACTTCCCAGTC-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 performed with the MEGA

Table 1. Comparison of cDNA sequence between Caribbean crazy ant and Rasberry crazy ant by using NCBI nucleotide blast program

Gene name	Accession no. (CCA)	Accession no. (RCA)	Coding region (bp)	Protein amino acids	Predicted aa Molecular mass (KDa)	Nucleotide difference at the specific point	Identities
NpCsp	JF815104	GU980916	378	126	14.41	0	100%
NpCLP	grp3458287	GU980926	492	164	18.96	358: C → T	99%
NpFabp	JF815103	GU980922	402	133	14.97	0	100%
NpFlp	JF815100	GU980917	663	221	25.01	0	100%
NpObp	JF815101	HQ636478	441	147	16.485	0	100%

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 *NpObp* 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 82% 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 ignites* 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 Supplemental 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 quiquefasciatus* 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 pennsylvanicus* 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).

NPCLP 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 vitripennis* 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-*

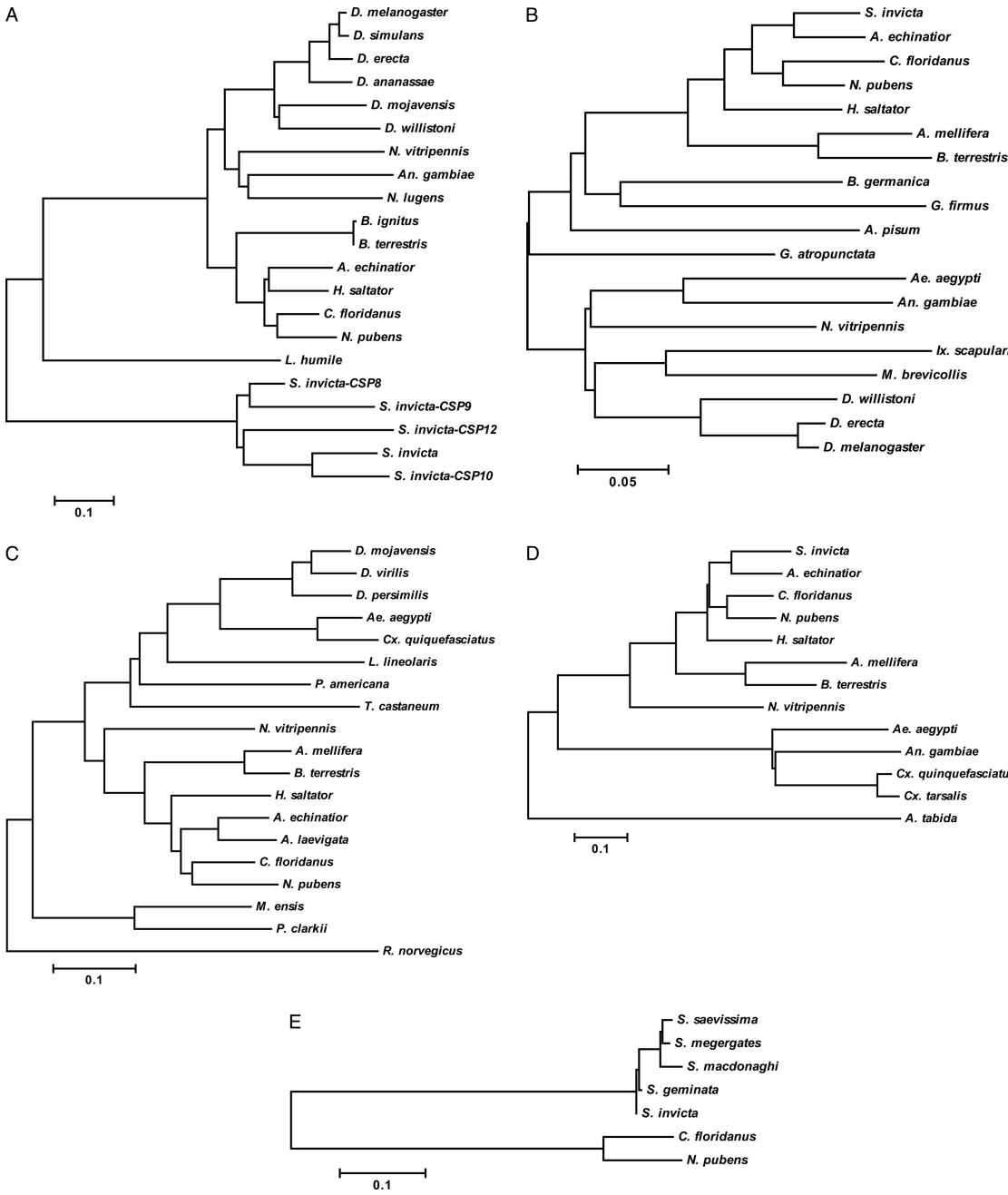

Fig. 1. Phylogenetic trees were constructed for nucleic acid sequences of insect orthologues or paralogs using the Neighbor-joining tree-making method in MEGA 5.05 program (A, B, C, D, and E stand for *NpCsp*, *NpClp*, *NpFabp*, *NpFlp*, and *NpObp*, respectively). The scale bar indicates the number of changes inferred as having occurred along each branch. Accession numbers of nucleic acid sequences of insect orthologs or paralogs used in this analysis are listed in supplemental Table 1A–E.

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 Rasberry 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 Rasberry crazy ant (RCA) showed 100% identity. B. BLAST results showed that "Identities = 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.

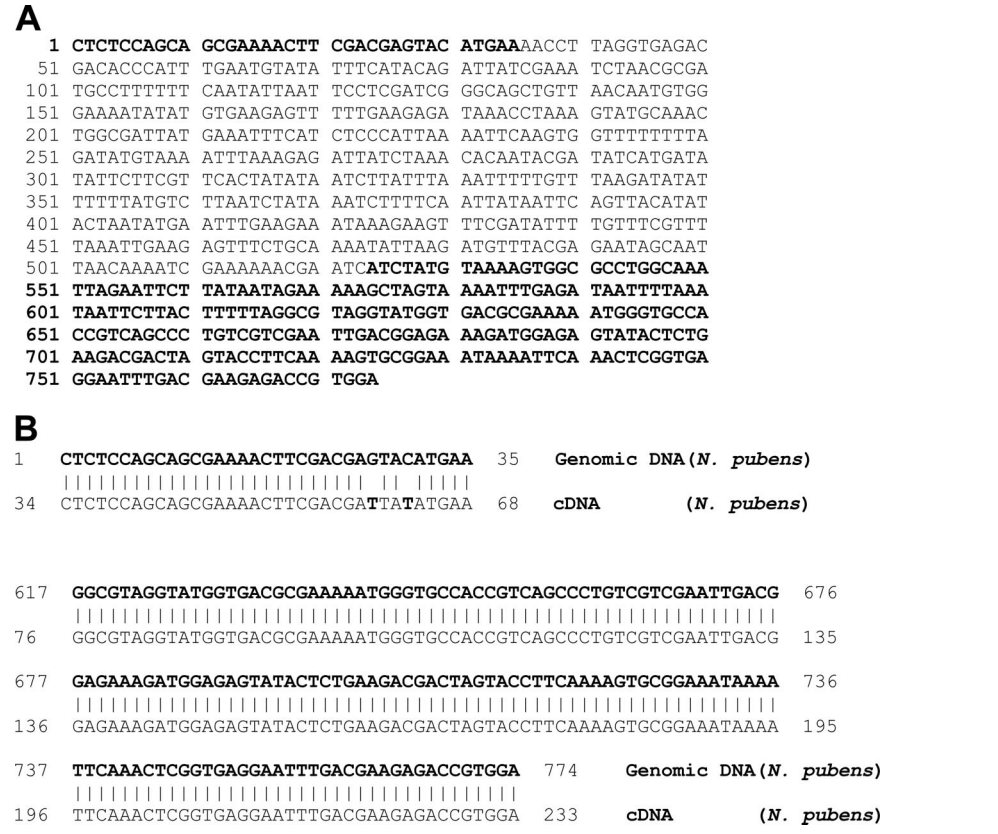

Fig. 3. Genomic DNA sequence analysis of *NpFabp* of Caribbean crazy ant and Rasberry crazy ant. A. Genomic DNA sequence of *NpFabp*. Bold sequences stand for coding sequence. The rest part of the sequence stands for an intron in the genomic sequence. B. The alignments of genomic DNA and cDNA sequences of *NpFabp*. Nucleotides are changed from G to T and C to T at positions 27 and 30, respectively, showing "Identities = 33/35 (94%)" and "Identities = 158/158 (100%)". C. Genomic DNA sequence alignments of *NpFabp* of Caribbean crazy ant and Rasberry crazy ant. There are three positions of the sequence that are different between Caribbean crazy ant (CCA) and Rasberry crazy ant (RCA): 185 C to T; 246 T to A, and 457 G to A, which showed "Identities = 771/774 (99%)".

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; 78% identity to *H. saltator* with 93% 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 (EFN61070.1); 75% identity to *A. echinatior* with 97% coverage; 72% identity to *H. saltator* with 99% coverage; 68% identity to *S. invicta* with 99% coverage; 59% identity to *N. vitripennis* with 99% coverage; 56% identity to *A. tabida* with 99% coverage; 53% identity to *A. mellifera* with 98% coverage; 40–45% identity with most species from *Drosophila* genus with 93–99% coverage; 34% identity to *Cx. quiquefasciatus* with 91% coverage; 36% identity to *An. gambiae* with 97% coverage; and 37% identity to *Ae. aegypti* with 80% coverage (Supp. Table S4).

Identification of Odorant Binding Protein Gene. The complete odorant binding protein cDNA of *N. pubens* (*NpObp*) 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 *NpObp* and *Obp* nucleotide sequences of the ants *C. floridanus, S. invicta, S. geminate, S. megergates, S. macdonoghi, and S. saevissima. NpObp* 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, NPOBP 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).

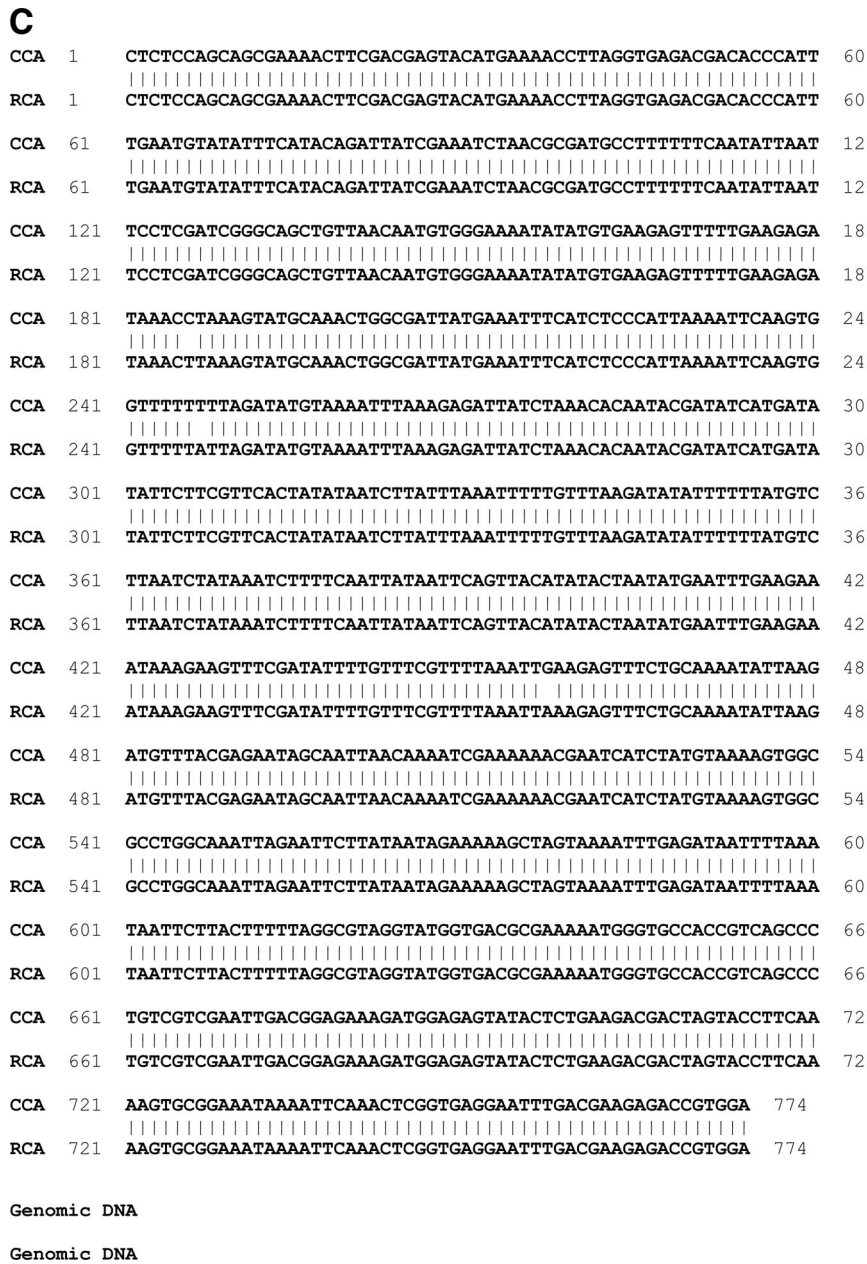

Fig. 3. (Continued).

Molecular Phylogenetic Analysis. The molecular phylogeny corroborates the distinct lineage between *N. pubens* and *C. floridanus* (Fig. 1A–E, Supp. Table S1-S5). The phylogenetic trees of five genes *NpCsp NpClp*, *NpFabp*, *NpFlp*, and *NpObp* for nucleic acid sequences from other insect orthologs or paralogs supported that *N. pubens* have the closest relationship to the ant *C. floridanus* (Fig. 1A–E, Supp. Table S1-S5). Additionally, the mitochondrial gene *NpCOI* showed a close relationship within the genus of *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

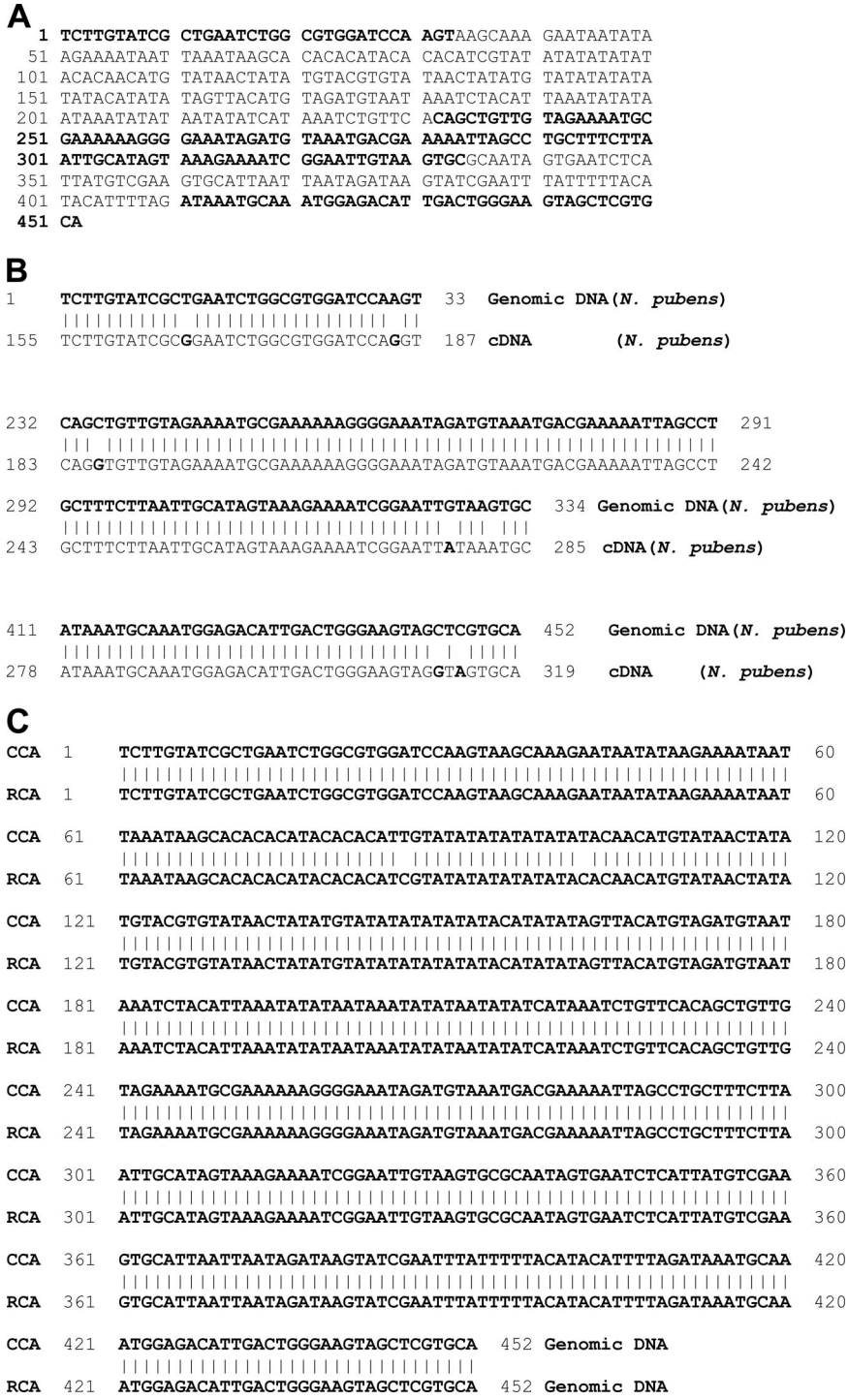

Fig. 4. Genomic DNA sequence analysis of *NpObp* of Caribbean crazy ant and Rasberry crazy ant. A. Genomic DNA sequence of *NpObp*. Bold sequences stand for coding sequences and the rest are introns in the genomic sequence. B. BLAST analysis of genomic DNA (bold sequence) and cDNA sequences of *NpObp*. 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 = 31/33 (94%)"; "Identities = 100/103 (97%)" and "Identities = 40/42 (95%)" respectively. C. Genomic DNA sequence alignments of *NpObp* of Caribbean crazy ant (CCA) and Rasberry crazy ant (RCA). There are two-positions where nucleotides were changed from T to C, which showed "Identities = 450/452 (99%)".

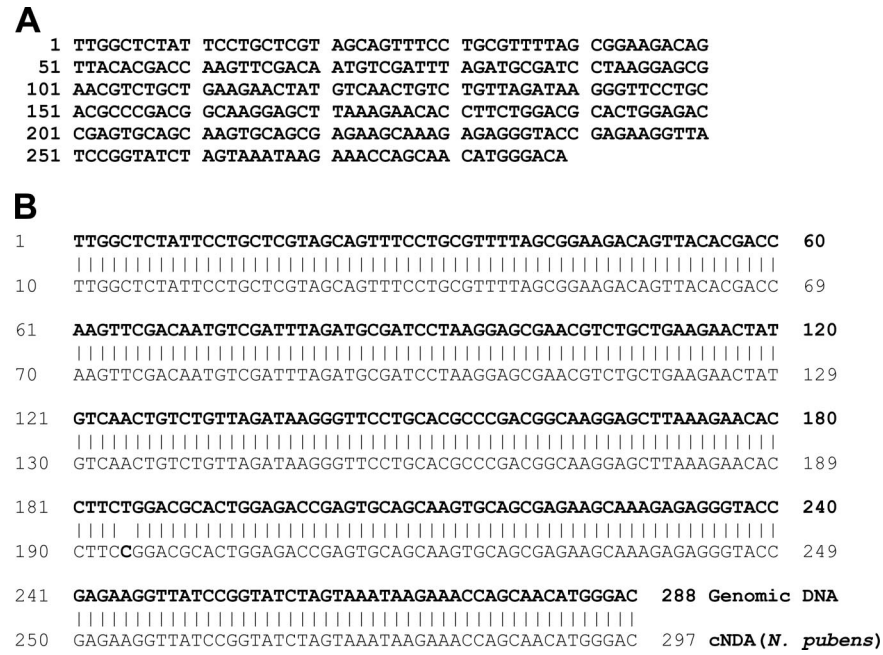

Fig. 5. Genomic DNA sequence analysis of *NpCbp* of Caribbean crazy ant and Rasberry crazy ant. A. Genomic DNA sequence of *NpCbp*. Bold sequence stands for coding sequence. No intron occurred in this genomic sequence. Genomic DNA sequence alignments of *NpCbp* of Caribbean crazy ant and Rasberry crazy ant, which showed "Identities = 289/289 (100%). B. The alignments of genomic DNA (bold letters) and cDNA sequences of *NpCbp*. Nucleotide was changed from T to C at position 85, showing "Identities = 287/288 (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 (Fig. 4C). Finally, a 288 bp partial genomic DNA sequence of *NpCsp* containing a coding region for only one nucleotide change at the position 185 (Fig. 5B), also showed 100% 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. salator*, 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 NPCLP 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 extra- and intracellular membranes, for example, to transport lipophilic molecules from outer cell membrane to certain intracellular receptors (Tan et al. 2002, Weisiger 2002). The NPFABP 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 *NpObp* and *Obp* from *Solenopsis*, including *S. invicta* (Gotzek et al. 2011, Wurm et al. 2011). However, the NPOBP 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 NPCLP. In addition, three partial genomic DNA sequences also 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 *NpCoI* 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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