# 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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In 2002, a new invasive pest ant in the genus, Nylanderia (formerly Paratrechina), was ABSTRACT 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 cyclophilinlike 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 ( $\approx 300 \pm 50$ individuals/per sample) from three colonies of Caribbean crazy ants were processed separately for RNA and DNA extraction. RNAs of two colonies ( $\approx$ 300 ± 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 fulllength 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  $\mu$ g) 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'-GGTCAACAAAT-CATAAAGATATTGG-3'/HCO2198-R: 5'-TAAAC-TTCAGGGTGACCAAAAAATCA-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'-CCACGGTCTCTTCGTCA-AAT-3'; NpObp-RCA2-28-100 F: 5'-TCTTGTAT-CGCTGAATCTGGC-3'/RCA2-28-263R: 5'-GCAC-GAGCTACTTCCCAGTC-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

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

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	$358: C \rightarrow T$	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; 95% 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-

	1 51 1 101 2 201 2 251 1 301 0 401 2 401 2 551 2 601 1 651 0 701 1	GGTCAACAAF TCAGCACGGA AGGATCACCA TTACTAGACA ATTGGTGGAT TATAGCCTAC SGTTGGACGG ATCAGTGGAC ATTATCCTT SGTGGACGTCA AGCTATTCTT IATTATTAAC IGTGGACGATC TGAAGTTTAA	ТСАТАРАВА: ТААТТБСААС ААТССТСТАА ТGCСТТТАТТ ТТСБАРАТТАТТ ССТСБАРАТА ТАТАСССТСС ТТАССТАТТТ АЛТСАЛТТТТ ТГСАТАРАЛАТ СТТСТТТТАТ САТССАРАТ САТСТТТА	Г АТТGGAATTT ТТСАТААGТ АТААТСАТТТ ТТТАGTGCСТ АТААСАТТТТ АТААСАТААG АGAAATTTTA АТТАGCATCT ТТТСАТТАСА АТТТСААСАА АТТТСААСАА АТТТСААСААСА ССТСССАСТ ССТАСАСАТТА	TATATTTTT ATAATTATC TCAAATTTAC TTATAGTTAT ATTTGATTA ATTTGATGA ATATTTTC TTAATGATGG AATATTTTC TATTGCTGGA GTTTGATCTA TTTAACGGGA CTTTCATTGA TTTTGATTTT	ATTTGCTATT GATTAGAATT AACTCAATAG ACCATTATA GTTCACCAGA TTACCGCCCT TGTAGGGACA ATAATGGACC ATAATGGACC ATAATCCTCAA ACATCACAAA TTTTTATTAC GCAATTACTA TCCTTCGGGA TTGGTCACCC	
Query	7 26	AATTTTATA	TTTTTTATTTGCT	ATTTGAGCAGGG	TAATTGGAACTT	CTATAAGTATAAT	85
Sbjct	: 1	 AATTTTATAT		 ATTTGAGCAGG <b>A</b> A	IIIIIIIIIIII ATAATTGGAACTT		60
Query	86	TATTCGATT	AGAATTAGGATCA	CCAAATCCTCTA	ATTAATAATGATC	AAATTTACAACTC	145
Sbjct	61	TATTCGATT	AGAATTAGGATCA	CCAAATCCTCTAA	ATTAATAATGATC.	AAATTTA <b>T</b> AACTC	120
Query	146	AATAGTTAC	FAGACATGCCTTT	ATTATAATCTTT	TTATAGTTATAC	CATTTATAATTGG	205
Sbjct	121	. AATAGTTAC	FAGACATGC <b>T</b> TTT	ATTATAATCTTT	TTATAGTTATAC	CATTTATAATTGG	180
Query	206	TGGATTTGG	<b>AATTTTTTAGTG</b>	CCTTTAATGTTA	GTTCACCAGATA	TAGCCTACCCTCG	265
Sbjct	: 181	. TGGATTTGGA	AAATTTTTTAGTG	CCTTTAATATTAG	GTTCACCAGATA	TAGC <b>T</b> TA <b>T</b> CCTCG	240
Query	266	AATAAATAA	CATAAGATTTTGA	TTATTACCGCCCI	CAATTTCCTTAC	TTTTATTAAGAAA	325
Sbjct	241	AATAAATAA	CATAAGATTTTGA	TTATTACCACCC	CAATTTCCTTAC	TTTTATTAAGAAA	300
Query	7 326	TTTTATTAA	IGATGGTGTAGGG	ACAGGTTGGACGO	TATACCCTCCAT	TAGCATCTAATAT	385
Sbjct	301	TTTTATTAA	IGATGGTGTAGG <b>A</b>	ACAGGTTG <b>A</b> ACGO	GTATACCC <b>C</b> CCAT	TAGCATCTAATAT	360
Query	7 386	TTTTCATAA	IGGACCATCAGTT	GATTTAGCTATT	TTTCATTACATA	TTGCTGGAATATC	445
Sbjct	: 361	TTTTCATAA	FGGACCATCAGTT	GATTTAGCTATT	TTTCATTACATA	TTGCTGGAATATC	420
Query	7 446	CTCAATTTT	AGGTGCAATCAAT	TTTATTTCAACA	ATTTTAAATATAC	АТСАСАААААТТТ	505
Sbjct	: 421	CTCAATTTI	AGGTGCAATCAAT	TTTATTTCAACA	ATTTTAAATATAC.	ATCACAAAAATTT	480
Query	7 506	TTCAATTGA	TAAAATTCCTCTT	CTTGTTTGATCT	ATTTTTATTACAG	CTATTCTTCTTCT	565
Sbjct	: 481	. TTCAATTGA	PAAAATTCCTCTT	CTTGTTTGATC <b>A</b>	ATTTTTTATTACAG	CTATTCTTCTTCT	540
Query	7 566	TTTATCCCT	ICCAGTTTTAGCG	GGAGCAATTACT	ATATTATTAACTG	АТСGАААТСТТАА	625
Sbjct	541	TTTATCCCT	CCAGTTTTAGCA	GGAGCAATTACT	ATATTATTAACTG.	ATCGAAATCTTAA	600
Query	626	TACTTCTTT	CTTTGATCCTTCG	GGAGGTGGAGAT	CAATTCTTTACC	AACACTTATTTTG	685
Sbjct	601	TACTTCTTT	CTTTGATCC <b>C</b> TC <b>A</b>	.GGAGGTGGAGA <b>C</b>	CAATTCTTTACC.	AACACTTATTTNN	660
Query	7 686	ATTTTTTGG	FCACCCTGAAGTT	TA 709 Ger	nomic DNA		
Sbjct	661	. NNNNNNNNN	fcaccc <b>a</b> gaagti	 'TA 684 <b>cD1</b>	A		
		c		N.	- N. cf. madagascan - N. cf. nambiyops N. cf. N. cf. bu N. cf. bu N. dodo - N. vitie - N. vitie - N. hystrix ividula N. terricola - N. conc. N. fais	ansis vaga purbonica nsis inna onensis	

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.

A							
1	CTCTCCAGCA	GCGAAAACTT	CGACGAGTAC	<b>ATGAA</b> AACCT	TAGGTGAGAC		
51	GACACCCATT	TGAATGTATA	TTTCATACAG	ATTATCGAAA	TCTAACGCGA		
101	TGCCTTTTTT	CAATATTAAT	TCCTCGATCG	GGCAGCTGTT	AACAATGTGG		
151	GAAAATATAT	GTGAAGAGTT	TTTGAAGAGA	ТАААССТААА	GTATGCAAAC		
201	TGGCGATTAT	GAAATTTCAT	CTCCCATTAA	AATTCAAGTG	GTTTTTTTA		
251	GATATGTAAA	ATTTAAAGAG	ATTATCTAAA	CACAATACGA	TATCATGATA		
301	TATTCTTCGT	TCACTATATA	ATCTTATTTA	AATTTTTGTT	TAAGATATAT		
351	TTTTTATGTC	TTAATCTATA	AATCTTTTCA	ATTATAATTC	AGTTACATAT		
401	ACTAATATGA	ATTTGAAGAA	ATAAAGAAGT	TTCGATATTT	TGTTTCGTTT		
451	TAAATTGAAG	AGTTTCTGCA	AAATATTAAG	ATGTTTACGA	GAATAGCAAT		
501	ТААСААААТС	GAAAAACGA	ATC <b>ATCTATG</b>	TAAAAGTGGC	GCCTGGCAAA		
551	TTAGAATTCT	TATAATAGAA	AAAGCTAGTA	AAATTTGAGA	TAATTTTAAA		
601	TAATTCTTAC	TTTTTAGGCG	TAGGTATGGT	GACGCGAAAA	ATGGGTGCCA		
651	CCGTCAGCCC	TGTCGTCGAA	TTGACGGAGA	AAGATGGAGA	GTATACTCTG		
701	AAGACGACTA	GTACCTTCAA	AAGTGCGGAA	ATAAAATTCA	AACTCGGTGA		
751	GGAATTTGAC	GAAGAGACCG	TGGA				
R							
				25 6			
T	CTCTCCAGCAG	GAAAACTTCGA	ACGAGTACATGA	AA 35 Gen	NOMIC DNA(N.	puben.	5)
2.4					/	•	
34	CTCTCCAGCAG	GAAAACTTCGA	ACGATTATATG	AA 68 CD1	A (N.	pubens	5)
C17	000000000000000000000000000000000000000						676
61/	GGCGTAGGTA:	rggrgacgcga	AAATGGGTGC	ACCGTCAGCCG	TGTCGTCGAAT	TGACG	676
7.0							105
16	GGCGTAGGTA'	FGGTGACGCGA	AAAATGGGTGCO	CACCGTCAGCCO	CTGTCGTCGAAT	TGACG	135
C 7 7	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~						700
6//	GAGAAAGATG	JAGAGTATACT	TGAAGACGAC	PAGTACCTTCA	AAGTGCGGAAA	TAAAA	136
100							
136	GAGAAAGA'I'G	JAGAGTATACT(	C'I'GAAGACGAC'	PAGTACCTTCA	AAAGTGCGGAAA	'I'AAAA	195
7 7 7					Com om i a	D313 / 31	
131	TTCAAACTCG	JTGAGGAATTTT	JACGAAGAGACO	GIGGA //4	Genomic	DNA (N.	pupens)
100					- 5173	/ 17	
196	TTCAAACTCG	J'I'GAGGAATTT''I'(	JACGAAGAGACO	JGTGGA 233	CDNA	(N.	pupens)

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 *Dro*sophila 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). C

•						
CCA	1	CTCTCCAGCAGCGAAAACTTCGACGAGTACATGAAAACCTTAGGTGAGACGACACCCATT	60			
RCA	1	CTCTCCAGCAGCGAAAACTTCGACGAGTACATGAAAAACCTTAGGTGAGACGACACCCATT	60			
CCA	61	TGAATGTATATTTCATACAGATTATCGAAATCTAACGCGATGCCTTTTTTCAATATTAAT	120			
RCA	61	TGAATGTATATTTCATACAGATTATCGAAATCTAACGCGATGCCTTTTTTCAATATTAAT	120			
CCA	121	TCCTCGATCGGGCAGCTGTTAACAATGTGGGAAAATATATGTGAAGAGATTTTTGAAGAGA	180			
RCA	121	TCCTCGATCGGGCAGCTGTTAACAATGTGGGAAAATATATGTGAAGAGATTTTTGAAGAGA	180			
CCA	181	TAAACCTAAAGTATGCAAACTGGCGATTATGAAATTTCATCTCCCATTAAAATTCAAGTG	240			
RCA	181	TAAACTTAAAGTATGCAAACTGGCGATTATGAAATTTCATCTCCCATTAAAATTCAAGTG	240			
CCA	241	GTTTTTTTAGATATGTAAAATTTAAAGAGATTATCTAAACACAATACGATATCATGATA	300			
RCA	241	GTTTTTATTAGATATGTAAAATTTAAAGAGATTATCTAAACACAATACGATATCATGATA	300			
CCA	301	TATTCTTCGTTCACTATATAATCTTATTTAAATTTTTGTTTAAGATATATTTTTTATGTC	360			
RCA	301	TATTCTTCGTTCACTATATAATCTTATTTAAATTTTTGTTTAAGATATATTTTTTATGTC	360			
CCA	361	TTAATCTATAAATCTTTTCAATTATAATTCAGTTACATATACTAATATGAATTTGAAGAA	420			
RCA	361	TTAATCTATAAATCTTTTCAATTATAATTCAGTTACATATACTAATATGAATTTGAAGAA	420			
CCA	421	ATAAAGAAGTTTCGATATTTTGTTTCGTTTTAAATTGAAGAGTTTCTGCAAAATATTAAG	480			
RCA	421	ATAAAGAAGTTTCGATATTTTGTTTCGTTTTAAATTAAA	480			
CCA	481	ATGTTTACGAGAATAGCAATTAACAAAATCGAAAAAACGAATCATCTATGTAAAAGTGGC	540			
RCA	481	ATGTTTACGAGAATAGCAATTAACAAAATCGAAAAAACGAATCATCTATGTAAAAGTGGC	540			
CCA	541	GCCTGGCAAATTAGAATTCTTATAATAGAAAAAGCTAGTAAAATTTGAGATAATTTTAAA	600			
RCA	541	GCCTGGCAAATTAGAATTCTTATAATAGAAAAAGCTAGTAAAATTTGAGATAATTTTAAA	600			
CCA	601	TAATTCTTACTTTTTAGGCGTAGGTATGGTGACGCGAAAAATGGGTGCCACCGTCAGCCC	660			
RCA	601	TAATTCTTACTTTTTAGGCGTAGGTATGGTGACGCGAAAAATGGGTGCCACCGTCAGCCC	660			
CCA	661	TGTCGTCGAATTGACGGAGAAAGATGGAGAGTATACTCTGAAGACGACTAGTACCTTCAA	720			
RCA	661	TGTCGTCGAATTGACGGAGAAAGATGGAGAGTATACTCTGAAGACGACTAGTACCTTCAA	720			
CCA	721	AAGTGCGGAAATAAAATTCAAACTCGGTGAGGAATTTGACGAAGAGACCGTGGA 774				
RCA	721	AAGTGCGGAAATAAAATTCAAACTCGGTGAGGAATTTGACGAAGACCGTGGA 774				
Genomic DNA						
Genomic DNA						

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

A 1 51 101 151 201 251 301 351 401 451	TCTTG AGAAA ACACA TATAC ATAAA GAAAA ATTGC TTATG TACAT CA	TATCG ATATAT ACATG ATATA TATATA AAGGG CATAGT TCGAA TTTAG	СТGААТСТGG ТАААТААССА ТАТААСТАТА ТАБТТАСАТG ААТАТАТСАТ GAAATAGATG GAGGAAAATC GTGCATTAAT ATAAATGCAA	CGTGGATCCA CACACATACA TGTACGTGTA TAGATGTAAT AAATCTGTTC TAAATGACGA GGAATTGTAA TAATAGATAA ATGGAGACAT	АСТААССААА САСАТССТАТ ТААСТАТАТС АААТСТАСАТ АСАССТСТС ААААТТАССС СТССССААТА СТАТССААТТ ТСАСТССААТ	GAATAATATA ATATATATAT TATATATATA TAAATATATA TGCTTTCTTA GTGAATCTCA TATTTTACA GTAGCTCGTG	
<b>B</b> 1	TCTTG	<b>TATCG</b>       TATCG	CTGAATCTGGC(            CGGAATCTGGC(	<b>STGGATCCAAG'</b>                     STGGATCCA <b>G</b> G'	<b>F</b> 33 <b>Genor</b>       187 <b>CDNA</b>	nic DNA(N. pubens (N. pubens	) 5)
232 183 292	<b>CAGCT</b>        CAG <b>G</b> T <b>GCTTT</b> 	<b>GTTGT</b>       GTTGT C <b>TTAA</b>	AGAAAATGCGAA             AGAAAATGCGAA TTGCATAGTAAA	AAAAAGGGGAAI 	ATAGATGTAAA             ATAGATGTAAA ATTGTAAGTGC 	rGACGAAAAATTAGCCT	291 242 (N. pubens)
243 411 278	GCTT1 <b>ATAAA</b>             ATAAA	'CTTAA' <b>\TGCAA</b>             \TGCAA	TTGCATAGTAA ATGGAGACATT(             ATGGAGACATT(	AGAAAATCGGA GACTGGGAAGTI              SACTGGGAAGTI	ATT <b>A</b> TAAATGC AGCTCGTGCA           AGGTAGTGCA	<ul> <li>285 CDNA(N. put</li> <li>452 Genomic DN</li> <li>319 CDNA (N</li> </ul>	ens) A(N. pubens) . pubens)
C CCA RCA	1 1 61	TCTTG	TATCGCTGAAT	CTGGCGTGGA:	TCCAAGTAAGC              TCCAAGTAAGC	AAAGAATAATATAAGAA 	AAATAAT 60         AAATAAT 60
RCA CCA RCA	61 121 121	IIIII TAAAT TGTAC IIIIII TGTAC	 AAGCACACACA GTGTATAACTA              GTGTATAACTA	IIIIIIIII TACACACATCO TATGTATATA? IIIIIIIIIIII TATGTATATA?		IIII IIIIIIIIIII FATACACAACATGTATA ATATAGTTACATGTAGA IIIIIIIIIIIIIIIIIIIIIII ATATAGTTACATGTAGA	ACTATA 120 ATGTAAT 180
CCA RCA CCA	181 181 241	AAATC	TACATTAAATA              TACATTAAATA AATGCGAAAAA	ATATAATAAATA              ATATAATAAATA AGGGGAAATA(	ATATAATATAT 	CATAAATCTGTTCACAG                    CATAAATCTGTTCACAG CGAAAAATTAGCCTGCT	GCTGTTG         240   GCTGTTG         240           TTTCTTA         300
RCA CCA RCA	241 301 301	IIIII TAGAA ATTGC IIIIII ATTGC	 AATGCGAAAAA ATAGTAAAGAA              ATAGTAAAGAA	AGGGGAAATA AATCGGAATT AATCGGAATT( AATCGGAATT(			TTCTTA 300 TGTCGAA 360
CCA RCA CCA	361 361 421	GTGCA        GTGCA ATGGA 	TTAATTAATAG               TTAATTAATAG GACATTGACTG 	GGAAGTATCG2              GGAAGTAGCTCG2 	AATTTATTTTT 	ACATACATTTTAGATAA 	AATGCAA 420
RCA	421	ATGGA	GACATTGACTO	GGAAGTAGCT	<b>CGTGCA</b> 452	Genomic DNA	

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%)".

1 TTGGCTCTAT TCCTGCTCGT AGCAGTTTCC TGCGTTTTAG CGGAAGACAG 51 TTACACGACC AAGTTCGACA ATGTCGATTT AGATGCGATC CTAAGGAGCG 101 AACGTCTGCT GAAGAACTAT GTCAACTGTC TGTTAGATAA GGGTTCCTGC 151 ACGCCCGACG GCAAGGAGCT TAAAGAACAC CTTCTGGACG CACTGGAGAC 201 CGAGTGCAGC AAGTGCAGCG AGAAGCAAAG AGAGGGTACC GAGAAGGTTA 251 TCCGGTATCT AGTAAATAAG AAACCAGCAA CATGGGACA В 1 TTGGCTCTATTCCTGCTCGTAGCAGTTTCCTGCGTTTTAGCGGAAGACAGTTACACGACC 60 10 TTGGCTCTATTCCTGCTCGTAGCAGTTTCCTGCGTTTTAGCGGAAGACAGTTACACGACC 69 61 AAGTTCGACAATGTCGATTTAGATGCGATCCTAAGGAGCGAACGTCTGCTGAAGAACTAT 120 70 AAGTTCGACAATGTCGATTTAGATGCGATCCTAAGGAGCGAACGTCTGCTGAAGAACTAT 129 GTCAACTGTCTGTTAGATAAGGGTTCCTGCACGCCCGACGGCAAGGAGCTTAAAGAACAC 121 180 130 GTCAACTGTCTGTTAGATAAGGGTTCCTGCACGCCCGACGGCAAGGAGCTTAAAGAACAC 189 CTTCTGGACGCACTGGAGACCGAGTGCAGCAAGTGCAGCGAGAAGCAAAGAGAGGGTACC 240 181 190 CTTC**C**GGACGCACTGGAGACCGAGTGCAGCAAGTGCAGCGAGAAGCAAAGAGAGGGTACC 249 241 GAGAAGGTTATCCGGTATCTAGTAAATAAGAAACCAGCAACATGGGAC 288 Genomic DNA 250 GAGAAGGTTATCCGGTATCTAGTAAATAAGAAACCAGCAACATGGGAC 297 cNDA(N. pubens)

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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