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CLADISTIC ANALYSIS OF PALEO-ISLAND POPULATIONS OF THE FLORIDA HARVESTER ANT (HYMENOPTERA: FORMICIDAE) BASED UPON DIVERGENCE OF MITOCHONDRIAL DNA SEQUENCES

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ABSTRACT

To examine the relationships of geographically isolated paleo-island populations of *Pogonomyrmex badius* (Latreille 1802) in Florida we generated a phylogeographic hypothesis based on mitochondrial DNA (mtDNA) sequences. We found at least three distinct mtDNA lineages and a positive correlation between genetic and geographic distances. The relationships between nowadays isolated *P. badius* populations might resemble a long lasting separation due to either restricted gene flow caused by inbreeding, paleo-climatic events or the impact of novel invasive species. The current depletion of the only representative of the ant genus *Pogonomyrmex* in the south-eastern USA makes a more fine-scaled mapping of the remaining, small *P. badius* populations necessary to identify evolutionary distinct units for conservation purposes.

Key Words: *Pogonomyrmex badius*, Cytochrome c Oxidase I, Cytochrome b, genetic distance, geographic distance, restricted gene flow, phylogeny.

RESUMEN

Para examinar las relaciones de poblaciones de *Pogonomyrmex badius* (Latreille 1802) de paleo-islas geograficamente aisladas en Florida nosotros generamos una hipótesis filogeografica basada sobre las secuencias de ADN mitocondrial (mtDNA). Nosotros encontramos por lo menos tres linajes distintos de mtDNA y una correlación positive entre las distancias genéticas y geográficas. Las relaciones entre las poblaciones de *P. badius* aisladas de hoy dia puede representar una separación de largo plazo debido al flujo de genes restringidos causado por la reproducción entre individuos de la misma familia, los eventos paleo-climáticos o el impacto de nuevas especies invasoras. La reducción actual de la unica hormiga representativa del género *Pogonomyrmex* en el sureste de los Estados Unidos hace necesario que se traze un mapa de una escala mas precisa para las poblaciones pequeñas de *P. badius* restantes para identificar las distintas unidades evolucionarias para propósitos de conservación.

The paleogeographic history of Florida and its islands during Pleistocene with regular flooding of major parts of the Florida peninsula is well documented (Faught & Carter 1998; Froede 2002; Cunningham et al. 2003; Portell et al. 2003). The general pattern is that animals expanded their range from their refuges with the end of the last glaciation period. This historical isolation of different populations of the same species could have created distinct strains (Ribera & Vogler 2004) with their own history and genetic composition. The genetic distinctiveness of those "paleo-island" populations might be further increased if the population structure is highly viscose.

Demographic responses to climate change and resulting range changes usually result in genetic manifestation, making them genetically traceable with adaptively neutral genetic markers (Hewitt 2000; Lessa et al. 2003). Therefore, the genetic analysis of current Florida paleo-island populations might provide us with insights into historical events. In between numerous exotic ant species inhabiting the Florida peninsula, *Pogonomyrmex badius* (Latreille 1802) is considered "the closest approach to an endemic genus" (Deyrup & Trager 1986; Deyrup et al. 1988). *Pogonomyrmex badius* was isolated in the xeric uplands of central Florida during Pleistocene (Deyrup & Trager 1986). These isolated populations came again into contact after climatic changes at the end of the Pleistocene. Recently, colonization of Florida by aggressive, invasive species like *Solenopsis invicta* (Whitcomb et al. 1972; Deyrup et al. 2000; Cherry 2001) and abundant agricultural land use resulted again in a restriction of *P. badius* to more or less isolated island populations in remnant sand hill and scrub habitats. Therefore, the current populations of *P. badius* are the remains of formerly larger populations with a very restricted gene flow between them.

Retained ancestral polymorphisms can yield distinct phylogenetic relationships (Bulgin et al. 2003). Therefore, assuming restricted gene flow between isolated "island" populations of *P. badius* even since Pleistocene, a DNA based cladogram should resemble the historical events of the withdrawal of *P. badius* into ice-age refuges before and after introduction of invasive species. Genetic distances should increase with geographic distances if the phylogenetic pattern between island populations is based on ancient colonization events rather than splitting of a wide population zone and subsequently random, but incoherent mutation events. To examine the relationships of geographically isolated populations of *Pogonomyrmex badius* in Florida, we analyzed population samples phylogenetically using mitochondrial DNA sequences.

Materials and Methods

Collection of Specimens

Specimens of *Pogonomyrmex* (*sensu stricto*) *badius* (Table 1) were collected from six populations throughout Florida (USA) and preserved in 70-100% Ethanol for later DNA analyses. Similarly, *Pogonomyrmex* (*Ephebomyrmex*) *imberbiculus* and four additional *Pogonomyrmex* (*sensu stricto*) species (Table 1) were collected as outgroup-specimens for later phylogenetic analyses. Subgenus-classification followed Bolton (1995), whereas determination of species followed the keys of Taber (1998) and Cole (1968), and was confirmed by independent researchers where possible (Table 1). Nestmates from the analyzed workers are deposited as pinned voucher specimens in the collections of Harvard University (Cambridge, MA, USA) and collections of P.S. Ward at University of California in Davis (USA) and preserved in 100% Ethanol (stored at -70°C) at Museum Koenig (Bonn, Germany).

DNA-Isolation

DNA was extracted from workers with their gasters removed by phenol/chloroform extraction (Gadau et al. 1996), with the DNeasy Kit (Quiagen; following manufacturers tissue-protocol A for insects), or the Puregene Kit (Biozym/Gentra Systems, following the protocol of Gadau et al. 2003). The latter two methods worked well for specimens conserved in 70% Ethanol, which was problematic for the phenol/chloroform extraction method. Genomic and mitochondrial DNA was not separated by this method. DNA was dissolved in low TE-buffer and the success of DNA-isolation was tested on agarose gels. Good samples were diluted 1:10 with HPLC-water to a final concentration of approximately 5-10 µM.

PCR

We amplified fragments of the Cytochrome c Oxidase I (COI, 1054 bp) and Cytochrome b (CytB, 439 bp) mitochondrial genes (Table 2). PCR reactions were performed on a Biometra thermocycler (heating rate 5°C/s) with the following primer pairs (degenerate positions of primer-sequences are placed within brackets; numbers in

Table 1. Specimens of *Pogonomyrmex* used for MTDNA sequence analyses (cytochrome c oxidase I; cytochrome oxidase b); Acc.-No. = accession numbers (numbers indicate collectors of specimen/determinators of species names: 1 = Annett Endler, 2 = Alexander Mikheyev; 3 = Christoph-P. Strehl (CPS); 4 = Juergen Gadau (JG); 5 = Juergen Liebig; 6 = Phil S. Ward; 7 = Robert A. Johnson (RAJ); 8 = Susanne Hoyer; 9 = Stefan P. Cover; 10 = Z. Punsak); Location = place of collection, connected to GPS-data; COI/CytB = genebank accession numbers for Cytochrome C Oxidase I sequences/Cytochrome B sequences corresponding to samples.

Species	Acc.-No.	Location	Sequence name [COI/CytB]
P. badius	CPS125 [10/7]	Titusville, FL (N28°32' W80°50')	TIV [AY510637/-]
P. badius	CPS199 [3,5/3,5]	Lake Placid, FL (N27°11' 37.7" W81°20' 42.1")	ABS [AY510636/AY538614]
P. badius	CPS200 [1,3/1,3]	Withlacoochee, FL (N 28°48' 42.4" W 82°29' 6.3")	WIT [AY510633/AY538616]
P. badius	CPS201 [1,3/1,3]	Ocala Natl. Park, FL (N29°16' 26.5" W81°49' 5.4")	OCA [AY510635/AY538619]
P. badius	CPS203 [3/3]	Fort Pierce, FL (N27°28' 26.5" W80°17' 32.0")	FTP [AY510638/AY538621]
P. badius	CPS204 [1,3/1,3]	Lake Placid, FL(N27°13' 11.8" W81°22' 48.5")	LKP [AY510634/AY538615]
P. badius	CPS230 [2/2]	Tallahassee, FL (N30°27' W83°20')	TA1 [AY510631/AY538618]
P. badius	CPS234 [2/2]	Tallahassee, FL (N30°27' W83°20')	TA2 [AY510632/AY538617]
P. barbatus	CPS56-30 [3,4,8/3,7]	Phoenix, AZ (N33°32' 40.7" W111°38' 3.6")	BRB [AY510639/AY538620]
P. californicus	RAJ2269 [7/6]	La Chocera, Mexico (N30°30.96' W116°2.46')	CAL [AY510649/AY538625]
P. huachucanus	CPS123 [4,3/6,9]	Portal, AZ (N31°55' 56.1" W109°12' 26.2")	HUA [AY510657/AY538623]
P. occidentalis	JG27 [3,4/3,5]	Seligman, AZ (N35°19' 37.5" W112°52' 35.7")	OCC [AY510667/AY538622]
P. (*Ephebomyrmex*) *imberbiculus*	CPS268 [3/3]	Portal, AZ (N31°55' 49.7" W109°7' 59.4")	IMB [AY510614/AY538624]

TABLE 2. PAIRWISE DISTANCE BETWEEN EACH SEQUENCE (SEQU.), CALCULATED USING PAUP 4.0B10 (SWOFFORD 1998); BELOW DIAGONAL: TOTAL CHARACTER DIFFERENCES; ABOVE DIAGONAL: *P*-DISTANCE MATRIX; SEQUENCE NAMES: COMPARE TABLE 1.

Sequ.	IMB	TA1	TA2	WIT	LKP	OCA	ABS	FTP	BRB	CAL	HUA	OCC	TIV
IMB	—	0.18201	0.18137	0.18880	0.18953	0.18620	0.18942	0.18823	0.18510	0.19351	0.19411	0.20116	0.15907
TA1	252	—	0.00000	0.02617	0.04885	0.02602	0.05106	0.04973	0.10452	0.09134	0.14300	0.11041	0.03139
TA2	253	0	—	0.02601	0.04792	0.02585	0.04989	0.04893	0.10242	0.09122	0.14113	0.10949	0.03136
WIT	268	37	37	—	0.04096	0.00480	0.04457	0.04048	0.10688	0.09232	0.14897	0.11422	0.01766
LKP	267	69	68	60	—	0.04108	0.00140	0.00348	0.10624	0.09520	0.14732	0.11279	0.00314
OCA	258	36	36	7	59	—	0.04396	0.04120	0.10786	0.09608	0.15098	0.11011	0.01555
ABS	260	70	69	64	2	63	—	0.00285	0.10552	0.09749	0.14736	0.11312	0.00311
FTP	260	69	68	58	5	58	4	—	0.10587	0.09886	0.14342	0.11299	0.00314
BRB	258	145	143	155	153	154	150	150	—	0.11654	0.14747	0.12224	0.09953
CAL	254	121	121	126	130	129	130	135	158	—	0.16228	0.11672	0.09293
HUA	275	201	200	219	215	217	210	205	213	221	—	0.16021	0.13272
OCC	273	150	149	161	159	154	157	158	171	156	225	—	0.10316
TIV	50	10	10	7	1	6	1	1	34	32	46	36	—

brackets following the 3' end of each primer refer to the next nucleotide positions relative to the sequence of the *Apis mellifera* mitochondrial genome published by Crozier & Crozier 1993, GeneBank-accession number NC_001566.1): LCO (sense) 5'-GGTCAACAAATCATAAAGATATTGG-3' [1835] and HCO (anti-sense) 5'-TAAACTTC-AGGGTGACCAAAAAATCA-3' [2492] (Folmer et al. 1994), Jerry (sense) 5'-CAACATTTATTTTGA-TTTTTT-3' [2502] (modified bee-primer Ca-J-2183 of Simon et al. 1994) and Ben3R (anti-sense) 5'-GC(AT)AC(AT)AC(AG)TAATA(GT)GTATCATG-3' [2888] (Brady et al. 2000) for CoxI; CB1 (sense) 5'-TATGTACTACCATGAGGACAAATATC-3' [11426] and CB2 (anti-sense) 5'-ATTACACCTC-CTAATTTATTAGGAAT-3' [11858] (bee-primers CP-J-10933 and CB-N-11367 of Simon et al. 1994) for CytB. The reaction volume was 25 µl, containing 2 µl of 1:10 diluted DNA-extraction, 2.5 µl of 10× PCR Buffer (750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, 0.52 µM of each primer, and 1.0U Taq DNA polymerase (MBI Fermentas, Lithuania). Cycling parameters were 3 min at 95°C for initial denaturation, followed by 33 cycles of denaturing 30 sec at 95°C, annealing 60 sec at 45°C, and elongation 30 sec at 72°C; two final steps of elongation 90 sec at 72°C and cooling down to 4°C were added. Amplicons were purified by ammonium acetate-precipitation (Sambrook et al. 1989) or with the Quiaquick purification kit (Quiagen). Sequencing reactions were performed by SeqLab (Göttingen, Germany).

Sequence Analysis

Obtained sequences were analyzed on Personal Computers. Proof reading was accomplished by comparing the forward and reverse amplicons and aligning them in a text-program with subsequent use of ClustalX (Thompson et al. 1997). Statistical analysis was performed with the programs PAUP 4.0b10 (Swofford 1998) and Mega 2.1 (Kumar et al. 2001). All sequences are deposited in GenBank (Table 1). For comparing population pairings we analyzed both types of sequences (COI, CytB) together or separately. Gene-trees were constructed in PAUP 4.0b10 using the Neighbor-Joining method (uncorr. *p*-distance; Kimura 2-parameter; HKY85; 100,000 bootstrap replicates), or Maximum Parsimony method (branch and bound search, 1000 bootstrap replicates).

We tested for a correlation of genetic distances with geographic distances between the analyzed populations. Geographic distances between samples were calculated with GPS data of Table 1 transformed into UTM-data (metric) and plotted against the genetic distances (uncorrected "*p*") calculated in PAUP 4.0b10 with the set of sequences used for constructing the gene-trees (Table 2). Between the Tallahassee samples, in which no detailed GPS-data were available, a geographic distance of 4 m was assumed.

RESULTS

Sequencing of both gene fragments resulted in general in 1493 base pairs used as characters in the subsequent phylogenetic analysis. Among the variable characters, 213 were parsimony-uninformative and 249 were parsimony-informative. Table 2 shows the absolute and *p*-distance between each sequence. Among the *Pogonomyrmex badius* samples there were 14 variable amino acids found among a total of 497.

Population pairings were identical with both sequences (COI, CytB) separately or together and either Neighbor-Joining (Fig. 1) or Maximum Parsimony analysis. We therefore show a tree based on both genes (Fig. 1). The populations of Fort

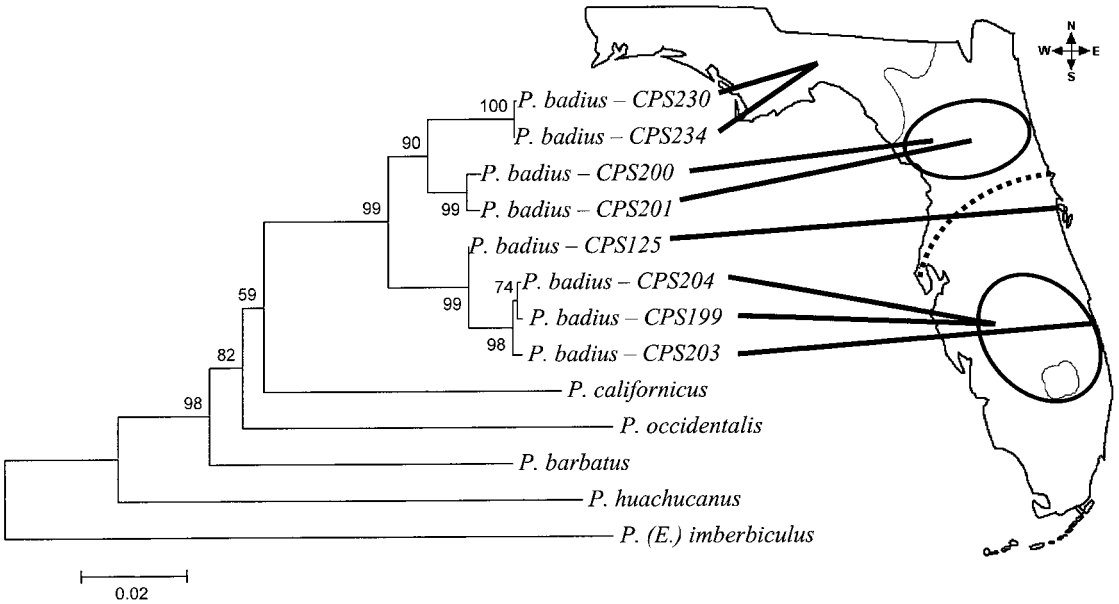

Fig. 1. Unrooted neighbor-joining tree of 1493-bp sequences (Cytochrome c Oxidase I and Cytochrome Oxidase b) of *Pogonomyrmex* spec., created with MEGA 2.1 [Distance method: Nucleotide: Kimura 2-parameter (Pairwise distance); Gaps/Missing Data: pairwise deletion; No. of bootstrap Reps: 100,000; SBL = 0.52206366] connected to the collection places of *Pogonomyrmex badius* on a contour of Florida, with circles/bows indicating hypothetical boundaries of genetically separated lineages (see text); numbers at branches indicate bootstrap replicates over 50%.

Pierce and Lake Placid grouped together, as did those from Withlacoochee and Ocala. The separation of the Fort Pierce/Lake Placid populations from the Titusville population (CPS125) was not supported in the MP analysis, and we considered them as one mitochondrial lineage. Moreover, the characters used for the Titusville population are based on the shortest of all sequences, as only one of the primer pairs yielded a sequence out of the single worker available. We justify the inclusion of this sample into the data because omitting it did not change the pattern shown in Fig. 1, and it provided additional information about the putative range of the southern mtDNA-lineage. The grouping of the Withlacoochee/Ocala population lineage together with the Tallahassee population was well supported by high bootstrap values. Because our sampling was very limited, however, the dotted line in Fig. 1 should be seen as preliminary.

Genetic distance showed a positive linear correlation with geographic distances between all population samples (Fig. 2; $n = 28$, $R^2 = 0.259$, $t = 3.011$, $P = 0.00573$). By excluding the Titusville population because of their limited genetic information, this correlation became even stronger ($n = 21$, $R^2 = 0.498$, $t = 4.341$, $P = 0.00035$). To prevent a bias of those populations where two samples were available (Tallahassee, Lake Placid) compared to those with only one, we included only one of them (CPS204 and CPS234) and reanalyzed the data. This procedure did not increase the significance of correlation, but increased the R^2 value ($n = 10$, $R^2 = 0.793$, $t = 5.540$, $P = 0.000547$). This effect is mainly due to an exponential increase in genetic variability with distances over 100 km (62.14 mi).

Discussion

Our analysis of mitochondrial DNA of *Pogonomyrmex badius* populations in Florida yielded three distinct lineages: (1) a southern lineage including samples from Lake Placid to Titusville, (2) a middle lineage including the Withlacoochee and Ocala populations, and (3) the northern lineage of the Tallahassee populations. The Withlacoochee and Ocala populations probably are more closely related to the Tallahassee populations. This view is further supported by a positive correlation of genetic with geographic distances.

The gene-tree and significant correlation of genetic and geographic distances suggest an ancient North to South movement of *P. badius* during its colonization of Florida with either very limited gene-flow back north, or less likely, higher mutation/fixation rates at the border of the expanding populations (Edmonds et al. 2004). Over all, the genetic relationships between the different population samples of *P. badius* might reflect past paleo-climatic events with a long lasting separation of populations withdrawn to protected "island" areas, like those found in the Lake Wales

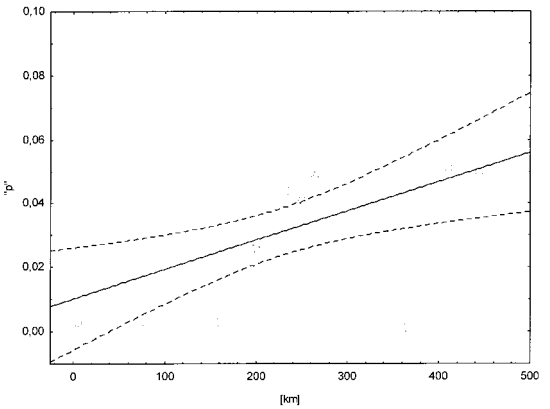

Fig. 2. Plot showing the positive correlation for calculated geographic [km] and genetic ("*p*") distances between *Pogonomyrmex badius* population samples sequenced for this study ($P < 0.006$, see text). The linear regression line is defined as "*p*" = 0.0102 + 9.176-5 [km], and accompanied by the corresponding 95% confidence interval (dotted line).

Ridge (Deyrup & Trager 1986). After the Pleistocene these populations might have come into secondary contact. This contact, however, did not lead to a sufficient flow and intermixing of the maternally inherited mitochondrial DNA before the *P. badius* populations were once again separated by anthropogenic devastation of their habitats, which was re-enforced by new and more competitive invasive species (Whitcomb et al. 1972; Deyrup et al. 2000; Cherry 2001).

This might be explained by the unique mating behavior of *P. badius* sexuals, and therefore dispersal of the gene carrying 'units' of this species. Mating behavior of *P. badius* is highly promiscuous (Page 1986; Crozier & Pamilo 1996; Rheindt et al. 2004) and makes gene flow between populations probable. However, there is also some indication for inbreeding, as females were reported to mate on their native nests, probably even with their brothers (Van Pelt 1953; M. Deyrup, Archbold Biological Field Station, Lake Placid/FL, pers. comm.). Additionally, no data are available on the dispersal of *P. badius* females, e.g., no reports on huge mating swarms similar to other *Pogonomyrmex* species (Hölldobler 1976). Rheindt (2003) showed that differences exist in allele frequencies of microsatellites (nuclear DNA) between three of the *P. badius* populations analyzed in this study (Withlacoochee, Ocala, Lake Placid). This was a first indication of either restricted gene flow between populations or rapid diversification in these distinct populations. As *P. badius* populations seem to show significant inbreeding (Rheindt 2003), restricted gene flow is likely between them. This is further corroborated by our analysis of mitochondrial DNA variations obtained from six different localities in Florida. Our analyses show clearly separated lineages and an increase of genetic distances with geographic distance.

A more fine-scaled mapping of *P. badius* populations will be needed to separate exactly the geographic range of the different mtDNA-lineages. However, we found at least three lineages which showed inter-population sequence variations that are normally found between *Pogonomyrmex* species (Strehl, unpublished data). Such phylogenetically distinct lineages, which are restricted in their geographical distributions might be characterized as Evolutionary Stable Units (ESUs), warranting protection because they may contain significant components of the evolutionary history of a species (Moritz 1994; Bulgin et al. 2003). This is of special interest because *Pogonomyrmex badius* is considered to represent an endemic ant genus of Florida (Deyrup & Trager 1986). It is also the only representative of the genus *Pogonomyrmex* east of Mississippi and the only North American *Pogonomyrmex* species with a substantial worker polymorphism (Taber 1990). Therefore, to protect the genetic diversity of the probably endangered ant species *P. badius* it is important to clarify the population structure and determine the range and extent of ESUs in Florida.

Acknowledgments

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