Genetic Diversity of Plum Curculio (Coleoptera: Curculionidae) Among Geographical Populations in the Eastern United States

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ABSTRACT This study investigated the genetic diversity among plum curculio, *Conotrachelus nenuphar* (Herbst), populations from 11 different geographic locations by using partial sequences of mitochondrial cytochrome oxidase I (mtCOI) gene. Polymerase chain reaction (PCR) products were 863 bp. In total, 23 unique haplotypes were identified from 50 tested samples. Haplotypes G ($n = 5$), L ($n = 12$), and T ($n = 13$) made up 60% of the 50 samples. Two distinct major clades were found in the phylogenetic analyses. Northern and southern clades were strongly supported (100/83 (MP/ML) and 100/62, respectively) by bootstraps. The mid-southern subclade within the southern clade was also moderately supported by bootstraps (73/57, MP/ML). The results of analyses of molecular variance (AMOVA) are consistent with phylogenetic analysis. The results suggested that the northern and the southern clades correspond with northern strain and southern strain of plum curculio. The three-group model of AMOVA test may suggest that there is a transitional group existing between northern and southern groups. In this study, the mtCOI sequence was highly informative as a molecular marker in that it could distinguish *C. nenuphar* from northern and southern geographic locations in the eastern United States.

KEY WORDS *Conotrachelus nenuphar*, strains, mtCOI, polymorphism, phylogeography

There are two strains of plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae): a northern univoltine strain and a southern multivoltine strain (Chapman 1938). The strains are morphologically indistinguishable, but they differ phenologically. Moreover, the two strains are reproductive incompatible (Padula and Smith 1971) and may due to infected by different symbiotic bacteria strains (McClanan et al. 2004a). The northern univoltine strain has an obligatory diapause and the southern multivoltine strain commonly has at least two generations per year (Bobb 1952). The multivoltine strain has been reported in many states, e.g., Virginia, North Carolina, Georgia, Missouri, Illinois, and Florida (Leiby and Gill 1923, Snapp 1930, Chandler 1932, Bobb 1952, Sarai 1969, Calkins 1976). However, these weevils may have been moved by human activity and spread to other places over time. For example, an isolated univoltine population was found in northern Utah in the early 1980s (Alston and Stark 2000, Alston et al. 2005).

Unlike the first generation, which attacks fruit early in the season, the second generation may be present as larvae in the fruit at harvest, posing a fruit export concern. Some states and foreign countries that do not have plum curculio have refused to import fruit from those areas where the multivoltine strain of plum curculio exists. Chapman (1938) presented an approximate distribution map of the two strains in which the mid-Atlantic region is the convergence area of the two strains, with Virginia having both strains. The distribution line is approximately the Appalachian Mountains in the mid-Atlantic area (Chapman 1938). Occurrence of the multivoltine strain may not only impose trade barriers, but a late brood would also complicate pest control. Therefore, development of a biochemical method to distinguish the two morphologically identical strains and clarify the distribution is necessary for integrated pest management decision-making to control the second peak in the eastern North America and to understand intensively their population ecology.

Only one previous study has determined molecular markers for plum curculio strain analysis. McClanan et al. (2004b) analyzed the difference between a univoltine and multivoltine strain (collected in Massachusetts and Georgia, respectively) by using a random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) assay. The study found that four of 15 primers (OPE-01, OPE-03, OPE-04, and OPE-07) tested could be used to distinguish the two strains of plum curculio.

Besides RAPD-PCR assay, mitochondria are found in most eukaryotic cells. Mitochondria have their own

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Table 1. Population, geographic source, coordinates, haplotype and genetic diversity for plum curculio mtCOI gene sequences

No.	Location	Coordinates	Acronym	Sample size	Haplotype (no. samples)	h	π
1	Amherst, MA	42° 22'N/72° 31'W	MA	5	A(1), B(1), E(1), G(2)	0.900	0.0021
2	Geneva, NY	42° 51'N/76° 58'W	NY	6	C(1), D(1), F(1), G(3)	0.900	0.0019
3	Bridgeton, NJ	39° 25'N/75° 14'W	NJ or NJa	6	T(5), U(1)	0.333	0.0004
4	Chatsworth, NJ	39° 49'N/74° 32'W	NJb	3	R(1), T(2)	0.667	0.0008
5	Kearneysville, WV	39° 22'N/77° 54'W	WV	8	L(1), M(1), P(1), Q(1), T(4)	0.786	0.0051
6	Washington, VA	38° 41'N/78° 08'W	Ra	3	S(1), T(2)	0.667	0.0008
7	Troutville, VA	37° 24'N/79° 52'W	Bo	2	L(2)	0.000	0.0000
8	Blacksburg, VA	37° 13'N/80° 24'W	BL	2	V(1), W(1)	1.000	0.0046
9	Whitethorne, VA	37° 12'N/80° 13'W	Ke	2	L(1), N(1)	1.000	0.0012
10	Byron, GA	32° 39'N/83° 45'W	GA	4	K(1), L(2), O(1)	0.833	0.0012
11	Quincy, FL	30° 35'N/84° 34'W	FL	9	H(1), I(1), J(1), L(6)	0.583	0.0010

h, haplotype diversity; π, nucleotide diversity.

All 50 mtCOI sequences were deposited into GenBank (accession numbers from EF373709 to EF373758, sample name: NYM2, NYF1, NYF2, MA4, MA31, Bo3, Bo4, FL1, FL2, FL3, FLF4, FLM1, FLM2, GAM1, GAu1, KeF2, WVM3, NJa5, NJF2, NJF4, NJM1, NJM4, NJb11, NJb12, Ra4, Ra5, WVp1, Wvp2, WVu1, WVu3, NY2, NY3, MA5, WVF1, NJF3, Ra3, BLF2, FLF2, FL4, NJb1, WVM1, WVF3, KeF1, BLF1, MAF2, FLF1, NYM1, MAF1, GAP1, GAF2, respectively).

DNA and are thought to have originated from free-living bacteria, which were closely related to α-proteobacteria (Boussau et al. 2004). Sequence data of mitochondrial DNA (mtDNA) have become a proven standard for many phylogenetic studies (Caterino et al. 2000). Many mitochondrial genes have been studied, and subunit I of the cytochrome oxidase gene (COI) has become a standard for phylogenetic inference of many insect groups (Lunt et al. 1996). The mtCOI gene sequences were used as markers to construct a phylogenetic tree for different geographic populations [sweet potato whitefly (*Bemisia tabaci* Gennadius), Frohlich et al. 1999; sweat bee (*Halictus rubicundus* Christ), Soucy and Danforth 2002; pinyon pine beetle (*Ips confusus* LeConte), Cognato et al. 2003; southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber), Szalanski and Owens 2003; *Lysiphlebus testaceipes* (Cresson), Shufran et al. 2004; *Bemisia tabaci*, Berry et al. 2004; dragonfly (*Libellula quadrimaculata* L.), Artiss 2004; and *Anopheles flavirostris* (Ludlow), Torres et al. 2006). The mtCOI gene seems a reliable molecular marker of intraspecific polymorphism of those insects. Therefore, we examined genetic diversity among plum curculio populations in different geographic locations by using partial sequences of the mtCOI gene.

Materials and Methods

Plum Curculio Populations. Plum curculios were collected from 11 geographical locations that included the northern and southern parts of their distribution range in the eastern United States. Collection sites, coordinates, and sample sizes are listed in Table 1. Larvae were collected from infested fruit by using the following procedure. Infested fruit were placed in 60- by 60-cm wooden frame racks with screen (6- by 6-mm grid) floors and fitted with a collection tray to catch the dropping plum curculio larvae under the floors. Larvae were placed in 450-ml glass jars containing moistened 50:50 potting soil: vermiculite. Each jar received around 100 larvae, and soil moisture (185 ml of water to 850 ml of mixture) was maintained throughout the pupation period (≈3 wk). The newly emerged adults were collected and maintained separately. The adults were maintained under laboratory conditions by using the procedures of T. Leskey (unpublished). Adults were placed into a plastic box (34 by 26 by 8.5 cm) with organza covered ventilated top. Approximately 25 green thinning apples (≈3 cm in diameter) were used as food and oviposition sites. The plastic box was held in an incubator chamber at 25°C and 60–80% RH. Photoperiod was controlled at 14:10 (L:D) h per day. Plum curculio adults were placed in 300 μl of TE (Tris-HCl and EDTA) buffer and then frozen at −80°C until processed.

DNA Extraction. The DNA extraction protocol was modified from a protocol developed for *Drosophila melanogaster* Meigen by Ashburner (1989). After a standard phenol-chloroform exaction, RNA was removed by adding 1.5 μl of RNase cocktail (Ambion, Austin, TX) to each sample and incubated for 1 h at 37°C (or overnight at 4°C). The samples were column-purified (Microcon Centrifugal Filter Devices, Millipore Corporation, Billerica, MA) to remove RNA fragments. Aliquots of 10 μl of each sample were electrophoresed on a 0.8% agarose gel containing ethidium bromide to visually assess the quality of the DNA. Samples also were tested using a GeneQuant DNA Calculator (Pharmacia Biotech, Piscataway, NJ) spectrophotometer to estimate the concentration of DNA calculated from absorbance at 260 nm.

Polymerase Chain Reaction. PCR was carried out in a total volume of 20 μl containing 600 nM mtCOI primers, C1-J-2195 MTD-10 (5′-TTGATTTTTTGGTCATCCAGAAGT-3′) and L2-N-3014 MTD-12 (5′ TCCAATGCACTAATCTGCCATATTA-3′) (Simon et al. 1994), 1 μl of weevil DNA, 2.5 mM $MgCl_2$, 150 μM dNTP mix (Applied Biosystems, Foster City, CA), 2 μl of 10× PCR buffer, 0.75 U of AmpliTaq Gold (Applied Biosystems) DNA polymerase, and 12.7 μl of distilled water. Amplification was completed with the following cycling profile on a Eppendorf Mastercycler (Eppendorf North America, New York, NY): 95°C for 2 min (denaturation) and then 30 cycles of 95°C for 1 min (denaturation), 52°C for 1 min (primer anneal-

ing), 72°C for 1 min (amplification), final extension 72°C for 5 min and held at 4°C. A total of 10 μl of PCR product was run on a 0.8% agarose gel containing ethidium bromide to estimate the size of the amplified DNA fragment. Images of stained gels were photographed and stored electronically using a gel photodocumentation system.

Cloning and DNA Sequencing. Fragments amplified with mtCOI gene primers were prepared for cloning as follows using a TA-cloning kit (TOPO-TA Cloning, Invitrogen): 1–2 μl of the PCR products was directly ligated into pCR2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The vector was transformed into bacterial cells (Top 10 component cell, Invitrogen). The cells that tested positive after the transformation were cultured overnight in 3 ml of Luria-Bertani medium containing 100 mg/ml ampicillin. Plasmids containing the PCR product were extracted from bacterial transformants by using a plasmid mini-prep kit (Wizard Plus SV Minipreps DNA Purification System, Promega, Madison, WI). The purified plasmids were sent to Davis Sequencing (Davis, CA) or Virginia Bioinformatics Institute (Blacksburg, VA). DNA sequencing was carried out using an ABI 3730 automated sequencer (Applied Biosystems) in both locations. T7 or M13 Reverse or both sequencing primers were used to sequence those plasmids. Two to nine individuals for the mtCOI gene were sequenced from each population.

Data Analysis. Sequences were chosen for phylogenetic analysis by comparing all individuals from each geographical location, and each different sequence represented was included in the analysis. The exactly identical sequences from different individuals were combined to one sequence to represent a haplotype (Table 1). All sequence data were analyzed using Chromas 1.45 (Conor McCarthy, School of Health Science, Griffith University, Southport, Queensland, Australia). Sequences were aligned using CLUSTAL W (Thompson et al. 1994). Pairwise nucleotide distances, base homogeneity chi-square test, and phylogenetic tree construction were estimated for mtCOI sequences by using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0 b10 software package (Swofford 2002). One mtCOI sequence from same family, *Curculio hilgendorfi* (Harold) (AB177150), was used as an outgroup to root the trees.

Phylogenetic analyses were conducted by using neighbor joining distance (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. To generate NJ trees, 1,000 bootstrapping replications were performed at a 50% majority-rule consensus tree limit. For parsimony analysis, bootstrapping was performed with PAUP by using the heuristic search option for 1,000 replications at a 50% confidence limit with a single stepwise addition and tree-bisection-reconnection (TBR) branch sweeping. For likelihood analysis, best-fit model (GTR+I model) was selected by using Modeltest 3.7 (Posada and Crandall 1998). Phylogenetic trees were constructed using ML optimality criteria with configuration following model test results. Bootstrapping was performed using heuristic search option for 500 replications with a single stepwise addition and TBR branch sweeping and without a molecular clock assumption. Moreover, a parsimony network was drawn using the program TCS 1.21 (Clement et al. 2000).

Analyses of molecular variance (AMOVAs) were conducted to assess genetic differentiation within and between geographical regions. All analyses were performed using the program Arlequin 3.1 (Excoffier et al. 2005), and significance tests in all cases were conducted using 10,000 permutations. The haplotype diversity was based on the formula $h = (1 - \Sigma xi^2)\, n/(n-1)$, where xi is the frequency of a haplotype and n is the sample size (Nei and Tajima 1981).

Results

COI Sequence Variation. In total, 50 samples were sequenced for partial mtCOI gene (Table 1). PCR products for the COI gene were 863 bp. In total, 23 unique haplotypes were identified in the 50 tested samples from 11 populations (Table 1). Haplotype G included five samples from two populations: three from NY (population acronyms are listed in Table 1) and two from MA; haplotype L ($n = 12$) were from five different populations: two from Bo, six from FL, two from GA, one from Ke and one from WV; Haplotype T had the most samples ($n = 13$) from four populations: five from NJa, two from NJb, two from Ra, and four from WV. The G, L, and T haplotypes included 10, 24, and 26% individuals, respectively, in 50 samples. The three haplotypes in total made up 60% of 50 individuals. The 20 remaining individuals represented one haplotype in each. Although the number of samples in each population was uneven, the three haplotypes occurred most frequently in most populations (eight of 11 populations). BL was the only one population with no G, L, or T haplotypes. Five of six (83.3%) samples in NJa population were haplotype T, which was the greatest frequency of one haplotype in any one population, which has more one haplotype. The average number of haplotypes in a geographic population was 3.1. However, population Bo was represented by only one haplotype and five haplotypes were represented in population WV (Table 1).

The haplotype diversity (h) and nucleotide diversity (π) of each population were shown in Table 1. The nucleotide diversity ranged from 0 to 0.0051. WV and BL have a higher π value among all populations. The average of nucleotide frequencies was A = 0.31604, C = 0.17386, G = 0.14756, and T = 0.36254. Therefore, the nucleotide frequencies were biased toward A + T, averaging 67.86%. The bias was consistent with other insect mitochondrial genes (Simon et al. 1994, Frati et al. 1997, Artiss 2004). No significant differences were detected between populations in terms of base composition in the base homogeneity chi-square test ($\chi^2 = 1.289$, df = 66, $P = 1.000$).

Nucleotide Distances between Haplotypes. The results are listed in Table 2. The nucleotide distance between haplotypes ranged from 0.12 to 4.87%. The greatest difference was between haplotypes NY3 (D)

Table 2. Uncorrected ("p") pairwise distance matrix for mtCOI sequences between 23 plum curculio haplotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1 GAF2 (K)	—																						
2 GAP1 (O)	0.00232	—																					
3 FLF2 (I)	0.00348	0.00348	—																				
4 WVF1 (P)	0.00348	0.00348	0.00463	—																			
5 (L)	0.00116	0.00116	0.00232	0.00232	—																		
6 FL4 (H)	0.00232	0.00232	0.00348	0.00348	0.00116	—																	
7 WVF3 (M)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	—																
8 FLF1 (J)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	0.00232	—															
9 KeF1 (N)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	0.00232	0.00232	—														
10 WVM1 (Q)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	0.00232	0.00232	0.00232	—													
11 BLF1 (V)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	—												
12 NJb1 (R)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	0.00232	—											
13 Ra3 (S)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	0.00232	0.00232	—										
14 NJF3 (U)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	0.00232	0.00232	0.00232	—									
15 (T)	0.00811	0.00811	0.00927	0.00927	0.00695	0.00811	0.00811	0.00811	0.00811	0.00811	0.00116	0.00116	0.00116	0.00116	—								
16 BLF2 (W)	0.00463	0.00463	0.00579	0.00579	0.00348	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00348	—							
17 MAF1 (A)	0.04519	0.04519	0.04635	0.04635	0.04403	0.04519	0.04519	0.04519	0.04519	0.04287	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	—						
18 MA5 (B)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04519	0.04519	0.04519	0.04519	0.04403	0.04056	0.00232	—					
19 NYM1 (C)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	0.00463	0.00232	—				
20 MAF2 (E)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	0.00463	0.00232	0.00232	—			
21 NY2 (F)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	0.00463	0.00232	0.00232	0.00232	—		
22 NY3 (D)	0.04403	0.04403	0.04519	0.04519	0.04287	0.04403	0.04403	0.04403	0.04403	0.04171	0.04867	0.04867	0.04867	0.04867	0.04751	0.04403	0.00579	0.00348	0.00348	0.00348	0.00348	—	
23 (G)	0.04171	0.04171	0.04287	0.04287	0.04056	0.04171	0.04171	0.04171	0.04171	0.0394	0.04635	0.04635	0.04635	0.04635	0.04519	0.04171	0.00348	0.00116	0.00116	0.00116	0.00116	0.00232	—

Different haplotypes are labeled by sample name and name of haplotype included in parentheses. Haplotype G includes samples NYF1, NYF2, NYM2, MA4, and MA31; Haplotype L includes samples Bo3, Bo4, FL1, FL2, FL3, FLF4, FLM1, FLM2, GAM1, GAu1, KeF2, WVM3; Haplotype T includes samples NJb11, NJb12, NJa5, NJF2, NJF4, NJM1, NJM4, Ra4, Ra5, WVp1, WVP2, WVu1, and WVu3.

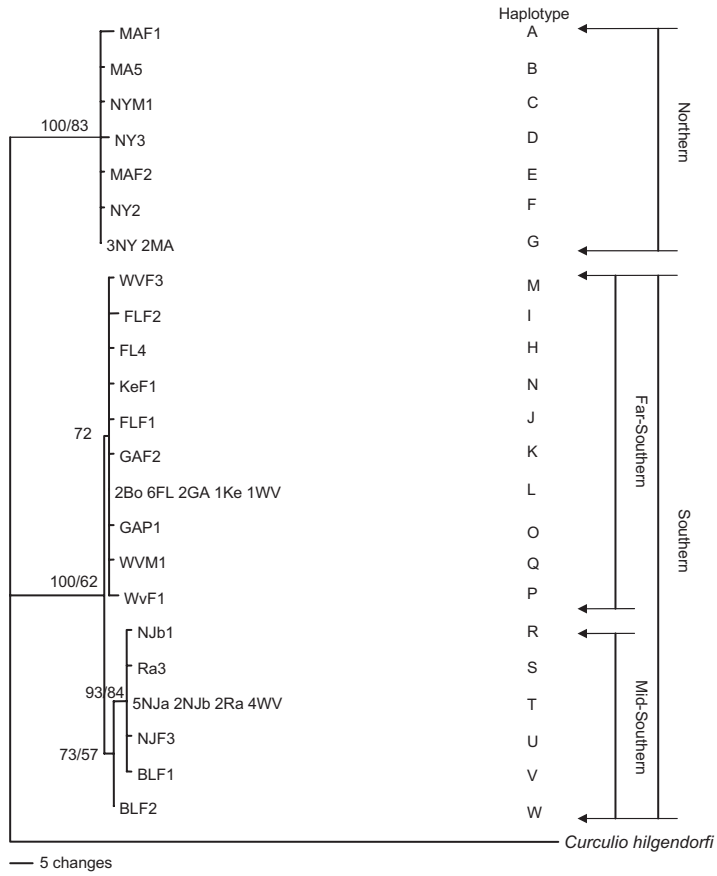

Fig. 1. Maximum parsimony tree based on mtCOI sequences of plum curculio. Bootstrap percentages of 1,000 replications are shown around the branches. Branch lengths are proportional to inferred nucleotide changes. The individual names refer to locations. Outgroups is *C. hilgendorfi*.

versus FLF2 (I), WVF1 (P), (L), or FL4 (H). Distances between the geographical population groups (northern group: NY and MA; southern group: other eight populations) ranged from 4.17 to 4.87%. Within the northern group, distances ranged from 0.12 to 0.58% and within the southern group, distances ranged from 0.12 to 1.04% (Table 2). Distances between the two subgroups ranged from 0.46 to 1.04%. Distances between the most common haplotypes were compared: G versus L = 4.06%, G versus T = 4.52%, and L versus T = 0.70%.

Phylogenetic Analysis. Bootstrap values of 70% or greater were considered as strong, 50–70% as moderate, and below 50% as weak in describing trees (Hillis and Bull 1993). MP analysis indicated that 133 nucleotide sites were uninformative and 42 sites were informative to their parsimony. Equally weighted parsimony analysis of the data set produced a single tree of 327 steps (Fig. 1). The MP tree received similar strong support at most of the same nodes as the NJ tree (data not shown). Clearly, both 100% bootstraps supported the two major clades: northern and southern. Within the southern clade, there was strong support (73%) for the mid-southern clade being a separate subclade. Additionally, strong (93%) support showed that haplotypes R, S, T, U, and V separated with haplotype W within the mid-southern subclade. The far-southern subclade was also strongly supported (72%) in the MP tree.

For ML analysis, a tree (data not shown) that was similar with the MP tree was constructed using the ML method, but bootstrap support differed somewhat. The two distinct major clades were 83% (northern clade) and 62% (southern clade). The mid-southern subclade within the southern clade was moderately supported (57%). The same structure shown by MP and ML analyses within the mid-southern group was strongly supported (84%). The far-southern subclade was not resolved in ML tree, but those 10 haplotypes, from H to Q, were still separated with the mid-southern subclade (Fig. 1).

The two major clades and the major subclades within the southern clade received strong bootstrap support in all analyses (NJ, MP, and ML). Therefore, we assigned the 50 samples of 23 haplotypes into three groups according to their major members' geographical locations (described in MP tree results).

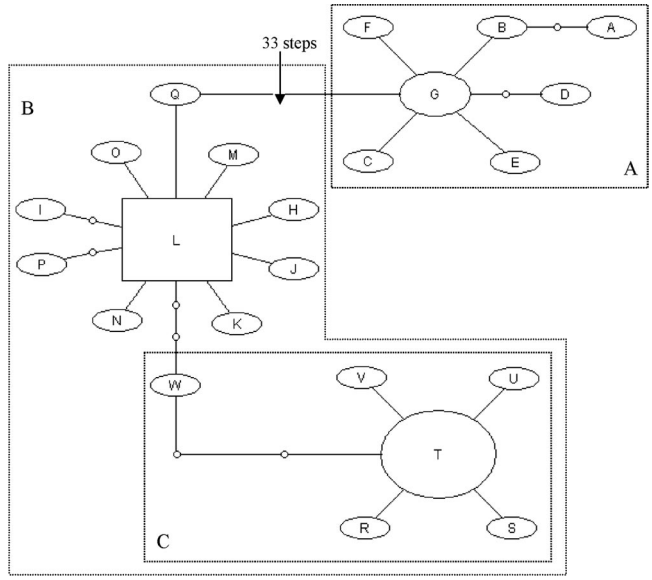

Fig. 2. Statistical parsimony network of the 23 mtCOI haplotypes detected in plum curculio. Small circles between haplotypes indicate nucleotide changes, one change per circle. There are 33 step changes between Box A and B. Box A (A, B, C, D, E, F, and G) indicates haplotypes harbored in northern region, Box B (all other haplotypes) indicates haplotypes harbored in southern region and Box C (R, S, T, U, V, and W) indicates haplotypes harbored in mid-southern region, respectively.

The phylogenetic relationships among the haplotypes also were revealed in the parsimony network (Fig. 2). All haplotypes were distinguished from each other by one or two mutational steps (except between Q and G, 33 steps). Three groups were detected among the 23 haplotypes in the network. One was haplotype G and its closely related haplotypes (A, B, C, D, E, and F); the second was haplotype L and its diverged haplotypes (H, I, J, K, M, N, O, P, and Q); and the third was haplotype T and its related haplotypes (R, S, U, V, and W). The haplotypes in first group have a long chain to change to groups 2 and 3. Therefore, the latter two groups have a close relationship and could be considered as a larger group. Figure 3 presents a distribution map of mtCOI haplotype of plum curculio geographical populations in the eastern United States.

Haplotype Diversity and Population Differentiation. The results of the AMOVA are presented in Table 3. AMOVA was used to compare three hypotheses about hierarchical structuring among the eastern Unites States plum curculio populations (Table 3). The first model had two groups: northern (MA and NY) and southern (all other populations). The second model had three groups: northern (MA and NY), mid-southern (NJa, NJb, Ra, and BL), and southern (WV, Bo, Ke, GA, and FL) populations. The third model also had three groups, northern (Ma and NY), mid-Atlantic (NJa, NJb, Ra, BL, WV, Bo, and Ke), and southern (GA and FL) populations. According to among-group variance component test results, the second and third models were statistically significant, but the second model explained the highest among group variance and is consistent with our phylogenetic analysis.

Discussion

Molecular methods have been used to differentiate genetic variants based on genetic polymorphisms in many insects. The mtCOI sequence used as a molecular marker reveals moderate to high degrees of divergence among phylogeographic populations and is an informative molecular marker for discerning genetic differences between haplotypes of many insects, e.g., *H. rubicundus* (Soucy and Danforth 2002), *I. confusus* (Cognato et al. 2003), migratory *L. quadrimaculata* (Artiss 2004), *B. tabaci* (Frohlich et al. 1999, Berry et al. 2004), and *L. testaceipes* (Shufran et al. 2004).

This is the first study on mitochondrial molecular markers in plum curculio. In this study, the mtCOI sequence was highly informative as a molecular marker in that it can distinguish *C. nenuphar* collected from northern and southern geographic locations in the eastern United States. The mtCOI sequence analyses carried out for the first time for plum curculio populations suggested that two distinct major clades. In all three kinds of phylogenetic analyses, northern and southern clades were strongly supported (100/83 [MP/ML] and 100/62, respectively) by bootstraps (Fig. 1). The individuals from NY and MA populations were of the univoltine biotype and corresponded with the northern clade in phylogenetic analyses. The individuals from GA and FL were clearly of the multivoltine biotype and were within the southern clade. Therefore, the results of phylogenetic analyses suggest that the northern clade and the southern clade corresponded with the northern strain and southern strain of plum curculio. However, the status of voltinism in several populations is still unclear. In the

Fig. 3. Distribution map of mtCOI haplotype of plum curculio geographical populations in the eastern United States. Colored dots indicate different haplotype group. Stars indicate geographic locations. The blue lines indicate the northern-most boundary (Maine) of plum curculio's range in the United States, and the southern-most boundary (tip of Florida). The black line is the western-most boundary of the plum curculio's range. The red line is the line set by Chapman (1938) delineating the ranges of the univoltine strain (above the red line) and the multivoltine strain (below the red line).

Table 3. Results of analysis of molecular variance (AMOVA) of mtCOI sequence data from populations of plum curculio

Source of variation	df	Sum of squares	Variance components	% variation	*P* value
Among pop	10	7.839	0.10131	22.71	
Within populations	38	13.100	0.34474	77.29	<0.0001
Among groups[a]	1	1.731	0.06154	12.69	0.0884
Among populations within groups	9	6.108	0.07871	16.23	0.0003
Within populations	38	13.100	0.34474	71.08	<0.0001
Among groups[b]	2	3.99	0.09810	20.62	0.0048
Among populations within groups	8	3.849	0.03295	6.93	0.0003
Within populations	38	13.100	0.34474	72.46	<0.0001
Among groups[c]	2	3.923	0.09425	19.81	0.0041
Among populations within groups	8	3.915	0.03670	7.72	0.0004
Within populations	38	13.100	0.34474	72.47	<0.0001

[a] Two groups: (MA, NY) vs. (NJa, NJb, Ra, BL, WV, Bo, Ke, GA, FL).
[b] Three groups (MA, NY) vs. (NJa, NJb, Ra, BL) vs. (WV, Bo, Ke, GA, FL).
[c] Three groups (MA, NY) vs. (NJa, NJb, Ra, BL, WV, Bo, Ke) vs. (GA, FL).

mid-Atlantic area, two NJ populations, Ra and BL, and half of the samples from WV populations belonged to a mid-southern subclade. FL, GA, Ke, Bo, and another half of samples from WV populations belonged to a far-southern subclade. Of eight samples from the WV population, 50% of the individuals were haplotype T and were placed in the far-southern group; another 50% have L, M, P, and Q haplotypes and were placed in the mid-southern group. T haplotype individuals can continuously reproduce in lab conditions (T. Leskey, personal communication), i.e., they represent a multivoltine strain. The voltinism of another four individuals is still unclear to date. From our laboratory observations, the summer generation of NJ, Ke, Bo, and BL populations can reproduce under laboratory conditions after feeding for various periods. There is no evidence for a second generation in the Ra population because of limited samples. Therefore, we believe that NJ, Ke, Bo, and BL populations have the reproductive potential for a summer generation, but more laboratory observation and field work are required to confirm their reproductive situations.

Moreover, the sequence divergence within the southern clade was low (0.46 to 1.04% between the

two subclades). In 863 bp, four to nine nucleotides (nt) differed, but within the far-southern group only one to three nucleotides differed and one to four nucleotides differed within the mid-southern group. The low diversity was not surprising, compared with other studies. Sequence difference among haplotypes ranged from 0.25 to 1.6% in another coleopteran, pinyon pine beetle (Cognato et al. 2003). However, there were up to 17.1% pairwise divergences in a whitefly (Frohlich et al. 1999). The sequence divergences are dependent on the length of the COI gene fragment selected and on the species. According to phylogenetic analyses, the mid-southern subclade was strongly supported (73/57, MP/ML). The far-southern subclade was not resolved very well in ML trees, possibly because of their very close genetic distances within the southern clade. To fully resolve this subclade, combining COI and COII gene data to enlarge the informative sites could be a possible method.

The sample size was still small. So far, 50 individuals were tested for mtCOI gene analysis, with only two samples each from three populations. The uneven sample sizes may have caused a bias in the frequency of haplotypes. From AMOVA results, most of the variation (77.29%) resulted from difference within populations. The large variation within population also suggested that the sample size has to be enlarged in future studies. According to our results, samples from northern strain plum curculio should be increased because only two northern geographic populations were included in this study. The status of subclades within the northern clade is still ambiguous. More samples could clarify the haplotype distribution status within the northern clade.

In this study, one sample from South Carolina had no PCR product by using mtCOI primers. This is the only sample using ethanol preservation. All other samples were alive until just before they were put into a −80°C freezer. The DNA extraction protocol may have to change somewhat to adapt the different preservation conditions because it is not easy to keep specimens alive in most situations.

To date, there are few molecular marker studies in species closely related to plum curculio. Using a Basic local Alignment and Search Tool (BLAST) search (Altschul et al. 1997) in the website of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), the available mtCOI sequence is *C. hilgendorfi*, which is in the same family as plum curculio but not in the same genus. The available information of these closely related species may help in studies of the evolutionary relationships for plum curculio.

We tested three different group models in AMOVA test. The second model has the highest among group variance and is consistent with the results of our phylogenetic tree and parsimony network. The three-group model may suggest that there is a transitional group existing between northern and southern groups. According to McClanan et al. (2004a) and our other study (X.Z. et al., unpublished), the northern group of plum curculios is infected by two closely related *Wolbachia* strains, the far-southern group is infected with a third *Wolbachia* strain, and the mid-Atlantic region is the convergence area. Mitochondrial DNA analysis indicated a closer relation of the mid-southern group to the far-southern group than to the northern group. However, *Wolbachia* symbionts in the mid-southern group are more closely related to those in the northern group than to those in far-southern group. Voltinism of some populations in the mid-Atlantic group is still unclear. The voltinism, *Wolbachia* infection distribution, and reproductive compatibility among populations within the mid-Atlantic region need further study to better understand plum curculio population ecology and phylogeography.

Plum curculio is a key pest of many tree fruits in North America. Knowledge of the strain distribution status is important to the management of this pest. The phylogenetic analysis based on mtCOI gene sequence could serve as a potential means to differentiate the northern and the southern plum curculio strains. This study would enrich our phylogeographical information about the pest from a molecular view and could provide an ecological and economic benefit to fruit production in the mid-Atlantic area, e.g., some cover sprays could be reduced if there is no second generation exists. In future studies, increasing sample size, testing more northern strain weevils; and inclusion of the COII gene or other molecular markers to enlarge the informative sites to more fully understand the evolution of plum curculio strains should be considered.

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