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Population genetics of *Chrysoperla externa* (Neuroptera: Chrysopidae) and implications for biological control

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(With 3 figures)

Abstract

Green lacewings are insects with great potential to be use in the biological control of agricultural pests, but relatively few studies have attempted to understand the genetic structure of these agents, especially those of predatory insects. The purpose of this study was to characterize genetically populations of *C. externa* using sequences of subunit I of the cytochrome oxidase, a mitochondrial gene, and examine the population structure of this species in sampled areas in São Paulo state. The results indicate high genetic diversity but no genetic structure, detected by AMOVA analysis, and high levels of haplotype sharing in the network. These genetic patterns could be a consequence of environmental homogeneity provided by agroecosystem (citrus orchard), allowing gene flow among populations. Probably there is a unique population in the area sampled that could be used as a population (genetic) source for mass-reared and posterior release in these farms.

Keywords: COI gene, green lacewings, mitochondrial DNA, population structure.

Genética de populações de *Chrysoperla externa* (Neuroptera: Chrysopidae) e implicações para o controle biológico

Resumo

Crisopídeos são insetos com grande potencial para uso em controle biológico de pragas agrícolas, mas relativamente poucos estudos têm tentado compreender a estrutura genética destes agentes, especialmente no caso de insetos predadores. O objetivo deste trabalho foi caracterizar geneticamente populações de *C. externa* utilizando sequências da subunidade I do gene mitocondrial citocromo oxidase e avaliar a estruturação populacional desta espécie em áreas amostras no estado de São Paulo. Os resultados indicaram elevada diversidade genética e nenhuma estruturação genética, detectada pela AMOVA, além de elevado compartilhamento na rede haplotípica. Este padrão genético poderia ser uma consequência da homogeneidade ambiental favorecida pelos agroecossistemas (citricultura), permitindo fluxo gênico entre as populações. Provavelmente há uma única população, do ponto de vista genético, na área amostrada que poderia ser utilizada em criações massais e em liberações nas fazendas desta região.

Palavras-chave: gene COI, crisopídeos, DNA mitocondrial, estrutura populacional.

1. Introduction

The approaches involved in biological control programs are closely related with ecological concepts, especially about prey-predator population cycles (Hufbauer and Roderick, 2005) and food-web ecology (Straub et al., 2008). Some traits involved in these ecological processes could be modified by the admixture of different genetically structured populations, once insects should be locally adapted (Hufbauer and Roderick, 2005), these admixture could affect their genetic diversity, which can have positive and negative results in the suppression of a prey (Straub et al., 2008).

The genetic structure and diversity of a species population can be assessed by molecular markers (Schrey et al., 2005; Smith, 2005; Ito et al., 2011; Muirhead et al., 2012), allowing the evaluation of gene flow, migration rates, isolation among populations, and other microevolutionary processes related with genetic differentiation (for a review see Hufbauer and Roderick, 2005). These points can elucidate approaches

[1] *In memoriam*

for biological control releases increasing the chances of success. Even releases of native insects should have criteria; they should be mass-reared from local collected populations, so they will have adaptive traits for the local environment, due to the genetic similarity with population source (Ito et al., 2011).

Field experiments using volatile plant compounds and synthetic sex pheromone of aphids were effective in attracting native species of green lacewings. The purpose of these experiments was to attract high numbers of green lacewings to the area, thus increasing the biological control of pests (Koczor et al., 2010). This research had two interesting aspects: 1) *Chrysopa pallens* and *Chrysopa formosa*, whose adults are also predators, were attracted in high number in the traps containing the synthetic aphids sex pheromone and 2) adults of the *carnea* complex, which only the larvae are predators, were attracted in high number by volatiles plant compounds, once they feed on pollen and nectar (Koczor et al., 2010). This relationship between species and volatiles compounds plays a role in the search for prey/food, and can possibly be observed among populations of the same species that present genetic structure. The admixture of such populations could negatively affect their capability in the search for prey/food, and therefore, the biological control becomes less effective.

Chrysoperla externa (Hagen, 1861) (Neuroptera: Chrysopidae) is one of four species in the genus *Chrysoperla* that occur in Brazil (Freitas, 2003). These species have a wide geographical distribution, from the south of the United States to the south of South America (Adams and Penny, 1985), and can be found in different types of environments, including agroecosystems (Canard et al., 1984).

The larvae of *C. externa* are generalist predators, feeding on insects considered to be agricultural pests, aphids, scale insects (Monophlebidae, Pseudococcidae, Eriococcidae, Coccidae, Diaspididae), spittlebug (Cercopidae, Cicadellidae, Membracidae, Fulgoridae), whiteflies (Aleyrodidae), psyllids (Psyllidae), thrips (Thysanoptera), eggs and larvae of Lepidoptera, mites (Tetranychidae, Eriophyidae), beetles, Diptera and others insects of Neuroptera order (Freitas, 2002). As a result, green lacewings have been studied to be used in biological control programs and research regarding population structure, using molecular markers, could help in determining which population to be released for pest control. Genetic studies with *Thanasimus dubius* (Coleoptera: Cleridae), with sequences of mitochondrial COI gene, showed two main groups structured in north and south along its geographical distribution, with high gene flow within groups. It is possible that prey pheromone to attract mates also attracts the predator, due to this adaptive trait of *T. dubius*, the exchange of these natural enemies between the north and south region should be avoided, happening only in the region where there is genetic similarity among individuals (Schrey et al., 2005).

Some molecular studies have been carried out with *C. externa* populations, indicating high genetic diversity, but low genetic distances among populations due to widespread haplotype sharing, suggesting lack of genetic structure (Morales and Freitas, 2010; Morales et al., 2013). Although, when green lacewings populations of natural areas and agroecosystems are compared, significant genetic structure has been observed among groups, but not within groups (Morales et al., 2013). However, few studies related to the population structure of predator insects have been conducted despite their ecological and economic importance (Schrey et al., 2005).

C. externa populations naturally occur in citrus orchards (Souza and Carvalho, 2002), allowing the use of this native species for biological control instead of an exotic species, that can cause environmental damage and extinction of non-target insects (Howarth, 1991; Ito et al., 2011). There are many practices involving this action, 1) habitat management to attract and maintain populations of *C. externa* in the area (Fiedler et al., 2008), this one maybe requires more knowledge about ecology (such as plants which can provide nectar, pollen and/or shelter for these insects) than about genetic structure; 2) inoculative and inundative releases, in these cases the genetic studies on population structure are essential.

The orange and its sub-products have relative influence over the economy, Brazil produces 60% of the world's orange juice (Brasil, 2012), and the main orange producer is São Paulo State, responsible for 73% of the national production (São Paulo, 2012).

Considering few studies regarding genetic patterns of *C. externa* populations and economy relevance of citrus orchards, the purpose of this study is to evaluate the genetic diversity and population structure of *C. externa* naturally found in citrus orchards in São Paulo State. Genetic analysis can determine if genetic structure is present in these populations or if they represent a single population. This information can help in the planning of biological control programs in this type of environment (citrus orchard) and which population would be chosen in case of mass-reared insects.

2. Material and Methods

Specimens were collected from citrus orchards using entomological nets at twelve locations across São Paulo State, Brazil, resulting in 114 samples of green lacewings (Table 1, Figure 1).Only the thorax was used for DNA extraction, and the head, wings and abdomen of each specimen received a voucher identification number and were stored in ethanol in the entomological collection of the Laboratório de Biologia Molecular do Departamento de Fitossanidade, Campus Jaboticabal-SP, FCAV-UNESP, Brazil.

2.1. Total DNA extraction, polymerase chain reaction (PCR) and sequencing

DNA was extracted from a single individual preserved in ethanol using a Wizard™ Genomic DNA Purification Kit (Promega) supplemented with Proteinase K following the manufacturer's protocols with a few minor changes. The homogenized individuals were incubated at 55°C for 3 hours. A fragment of the mitochondrial gene

encoding the first subunit of cytochrome oxidase (COI) was amplified via PCR using the primers C1-J-2183 (5' CAACATTTATTTTGATTTTTTGG 3') and TL2–N-3014 (5' TCCATTGCACTAATCTGCCATATTA 3') (Simon et al., 1994). PCR was performed in volumes of 25 µL containing 2.0µL 10X buffer, 1.5mM $MgCl_2$, 0.4µM dNTPs, 0.4µM of each primer and 1.5 units Taq polymerase (Promega). The thermal cycling conditions were: initial denaturation at 94°C (2 min), 35 cycles at 94°C (40 sec), 55°C (50 sec) and 72°C (1 sec), and a final annealing step at 72°C (10 min). The PCR products were checked for DNA amplification on 1% agarose gels. The reaction products were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega) following the manufacturer's protocols. Purified PCR products were sequenced with BigDye™ Terminator version 3.1 (Perkin-Elmer Applied Biosystems) using the same conditions and primers employed in the PCR. Subsequently, the fragments were washed several times with 75% isopropanol, followed by centrifugation. Sequences were read using an ABI Prism 3100 Genetic Analyzer.

Table 1. Samples of *Chrysoperla externa* populations, used for genetic analysis, collected in São Paulo State, Brazil.

LC	GL	Date of collection	GC	N
CA	Cafelândia	01-XII-2006	21°48'45"S; 49°33'45"W	10
PI	Pirajuí	21-XI-2006	22°00'01"S; 49°27'07"W	10
IA	Iacanga	13-XI-2006	21°53'38"S; 49°01'43"W	10
BA	Bariri	12-VI-2006	22°04'28"S; 48°44'26"W	10
LU	Lucianópolis	02-X-2006	22°26'10"S; 49°31'10"W	10
IT	Itápolis	03-VIII-2006	21°35'46"S; 48°48'48"W	10
NE	Nova Europa	11-VI-2006	21°46'42"S; 48°33'33"W	10
BE	Boa Esperança do Sul	11-X-2006	21°59'42"S; 48°23'30"W	10
DE	Descalvado	11-X-2006	21°54'14"S; 47°37'12"W	09
SE	Santa Ernestina	04-X-2006	21°28'02"S; 48°23'20"W	10
AG	Agudos	16-X-2006	22°28'09"S; 48°59'22"W	06
SC	São Carlos	25-X-2006	22°00'55"S; 47°53'28"W	09

LC: Locality code; GL: Geographical localities; GC: Geographical Coordinates; N: sample size.

2.2. Data analysis

The sequences were visualized with Chromas v.2.01 © (1998-2005) Technelysium Pty Ltd and were aligned using BioEdit v. 7.0.9.0 (Hall, 1999).

Measures of mitochondrial DNA (mtDNA) diversity, such as the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), polymorphic sites (S) and average number of pairwise nucleotide differences (k), were calculated with DnaSP v.5 (Librado and Rozas, 2009). The genetic distances for all of the individuals and each population were estimated with MEGA v.4 (Tamura et al., 2007). The haplotype network was inferred based on the statistical parsimony approach implemented in TCS v.1.21 (Clement et al., 2000).

Inferences regarding changes in population size were made using Arlequin v. 3.5 (Excoffier and Lischer 2010).

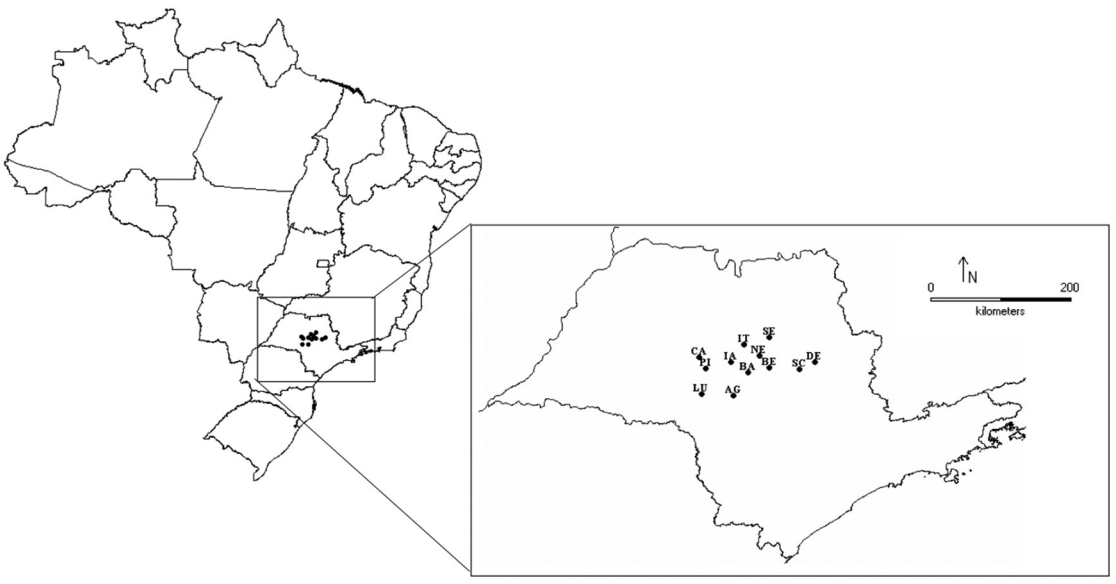

Figure 1. Brazilian map with *Chrysoperla externa* populations sampled in São Paulo State (detailed). The locality codes are the same of Table 1.

These included mismatch distribution analysis (Rogers and Harpending, 1992) and two neutrality tests, Tajima's D test (Tajima, 1989) and Fu's Fs test (Fu, 1997). All analyses proposed are appropriate for demographic inferences; however, neutrality tests provide more statistical support for detecting demographic events than mismatch distribution analysis (Ramos-Onsins and Rozas, 2002).

Arlequin v. 3.5 (Excoffier and Lischer 2010) was used to conduct an Analysis of Molecular Variance (AMOVA) to assess population genetic structure, and the results were tested for statistical significance ($p<0.05$) with 16,000 permutations. This analysis estimates how intra- and interspecific variability is partitioned among several hierarchical levels: Φst – covariance within populations; Φsc – covariance among populations within groups; Φct – covariance among groups. The pairwise FST among populations was calculated with 16,000 permutations in Arlequin v. 3.5 (Excoffier and Lischer 2010), and results were considered significant when $p<0.05$. The effective number of migrants per generation (Nm) was obtained using FST values with the formula FST=1/2Nm+1.

3. Results

3.1. Molecular diversity indices and haplotype network analysis

A 649-bp fragment of the mitochondrial COI gene was used to analyze the 114 individuals (accession numbers HQ425564 - HQ425621; HQ668468 - HQ668523). The fragments analyzed had 35 polymorphic sites (S) and a nucleotide diversity (π) of 0.0035±0.002. Thirty-six haplotypes (h) were observed with a haplotype diversity (Hd) of 0.86, implying that the haplotypes were very different from each other. Despite this high haplotypic diversity, the average genetic distance among individuals was low, 0.004. Molecular diversity indices among each population are shown in Table 2.

The haplotype network (Figure 2) was star-like, and haplotypes were shared across different localities (Table 3). According to the parsimony criterion, two haplotypes were considered the most ancestral, 01 and 04, because they were more interior in the network and were the most frequent among the individuals sampled. Among them, haplotype 04 is more ancestral because its outgroup weight is the highest, 0.163, this parameter corresponds to the probability of this haplotype being the root or outgroup of network (Figure 2) (Castelloe and Templeton, 1994).

3.2. Neutrality tests and demographic inferences

All tests performed allowed us to infer that the populations analyzed were subject to demographic events. The neutrality tests were significant; Tajima's D was –1.99553 (p=0.003) and Fu's Fs was –27.0672 (p=0.0). Both of these negative and significant values indicate population expansion (Tajima, 1989; Fu, 1997). Although less powerful, the unimodal curve found with the mismatch distribution provided the same inference of neutrality tests,

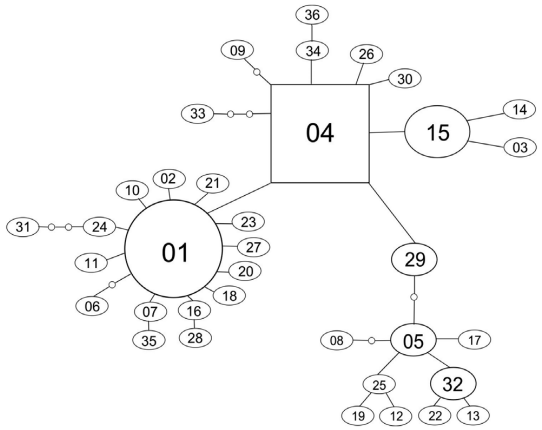

Figure 2. Haplotype network unrooted with sequences of mitochondrial COI gene obtained from *Chrysoperla externa* populations. Haplotypes are named as in Table 3. Each solid line represents one mutational change that interconnects two haplotypes that has a possibility greater than 95% level. Small circles without haplotype names denote missing intermediate haplotypes.

Table 2. Molecular diversity indices of mitochondrial COI gene for each *Chrysoperla externa* population.

	N	S	h	Hd	π	k
Cafelândia	10	07	05	0.7556	0.0027	1.733
Pirajuí	10	04	04	0.7778	0.0020	1.288
Iacanga	10	15	09	0.9778	0.0064	4.133
Bariri	10	09	06	0.7778	0.0030	1.955
Lucianópolis	10	08	05	0.8000	0.0023	1.933
Itápolis	10	08	06	0.8444	0.0039	2.577
Nova Europa	10	08	07	0.9111	0.0035	2.266
Boa Esperança do Sul	10	06	07	0.9111	0.0026	1.711
Descalvado	09	10	06	0.9167	0.0045	2.888
Santa Ernestina	10	10	08	0.9333	0.0036	2.311
Agudos	06	04	04	0.8667	0.0027	1.733
São Carlos	09	07	06	0.9167	0.0039	2.555

N: sample size; S: polymorphic sites; h: number of haplotypes; Hd: haplotype diversity; π: nucleotide diversity; k: average number of pairwise nucleotide differences.

a population expansion (Figure 3). The star-like structure of the haplotype network also corroborated these results (Figure 2).

3.3. Population genetic structure

Global AMOVA did not indicate genetic structure among populations (Φst=0.00114, p=0.44), and the analysis shows that 99.89% of the genetic variation is within populations, whereas only 0.11% is among populations. Due to this result, hierarchical AMOVA was not performed, but pairwise FSTs (Table 4) among the twelve populations sampled was calculated to detect the level of genetic proximity. The results of the pairwise FST test indicate that some populations are very closely related genetically (Table 4), so they were considered a single population unit due to the lower and non-significant results of this test.

The effective number of migrants per generation (Nm) was estimated for the twelve populations (Table 4), indicating intense gene flow, which agrees with the absence of genetic structure found in the Global AMOVA. One of the lowest values for Nm found was between Pirajuí and São Carlos populations, Nm=2.51, which coincide with the highest value for FST, 0.166 (Table 4).

Table 3. List of mitochondrial COI gene haplotypes shared in *Chrysoperla externa* populations in each geographical locality sampled.

Localities Codes	Haplotype
CA	1(05); 2(01); 3(01); 4(02); 5(01)
PI	1(03);4(04); 6(01); 7(02)
IA	1(01); 4(02); 8(01); 9(01); 10(01); 11(01); 12(01); 13(01); 14(01)
BA	1(05); 11(01); 15(01); 16(01); 17(01); 18(01)
LU	1(03); 4(04); 15(01); 19(01); 20(01)
IT	1(04); 4(01); 5(01); 15(02); 21(01); 22(01)
NE	1(03); 4(01); 15(02); 23(01); 24(01); 25(01); 26(01)
BE	1(02); 4(03); 23(01); 27(01); 28(01); 29(01); 30(01)
DE	1(02); 4(02); 15(03); 23(01); 31(01); 32(01)
SE	1(01); 3(01); 4(03); 5(01); 33(01); 34(01)
AG	1(01); 4(02); 15(02); 35(01)
SC	1(02); 4(02); 15(01); 32(01); 34(01); 36(01)

The number in parentheses refers to the frequency of the given haplotype in the sampled area. The localities codes (LC) are the same of Table 1.

4. Discussion

The potential use of *C. externa* in the biological control of agricultural pests requires a genetic study on natural enemies' populations for planning their release with the purpose of admixture only populations with some genetic

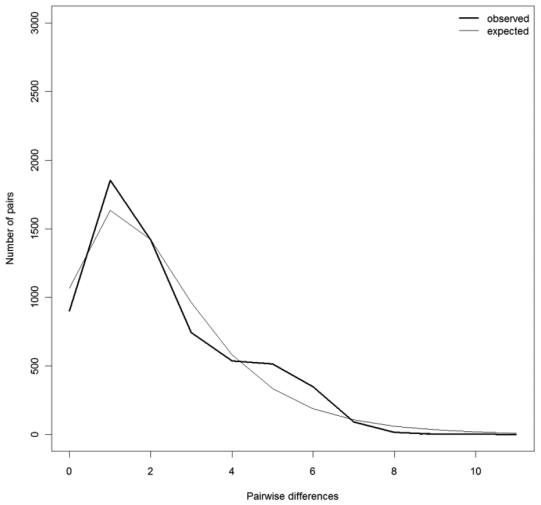

Figure 3. Graphics obtained with mismatch distribution analysis realized with sequences of mitochondrial COI gene of *Chrysoperla externa*.

Table 4. Genetic distances (FST distances) and effective number of migrants (Nm), below and above diagonal, respectively, for twelve populations of *Chrysoperla externa* sampled in São Paulo State, Brazil.

	BE	DE	SE	SC	NE	IT	BA	CA	PI	LU	AG	IA
BE	-	1.83	11.40	8.27	14.38	24.30	124.50	14.01	0.00	14.78	38.96	12.32
DE	–0.375	-	20.50	31.75	6.52	6.91	71.92	10.91	18.73	9.30	6.20	18.35
SE	0.042	–0.025	-	500.5	99.50	249.50	2.90	8.60	3.73	45.95	7.35	500.50
SC	0.057	–0.016	–0.001	-	11.70	38.96	3.70	5.45	2.51	13.80	12.32	19.73
NE	–0.036	–0.083	0.005	0.041	-	7.44	33.83	8.31	41.16	7.54	6.83	83.83
IT	–0.021	–0.078	0.002	–0.013	–0.072	-	24.30	9.30	14.65	9.76	9.60	1.44
BA	0.004	–0.007	0.147*	0.119	–0.015	–0.021	-	10.91	44.95	27.30	8.30	7.07
CA	–0.037	–0.048	0.055	0.084	–0.064	–0.057	–0.048	-	10.70	9.76	13.00	17.74
PI	0.000	0.026	0.118*	0.166*	0.012	0.033	0.011	–0.049	-	99.5	18.73	4.13
LU	–0.035	–0.057	–0.011	0.035	–0.071	–0.054	0.018	–0.054	0.005	-	8.70	125.50
AG	–0.013	–0.088	–0.073	0.039	–0.079	–0.055	0.057	–0.040	0.026	–0.061	-	125.50
IA	0.039	–0.028	–0.001	–0.026	–0.006	–0.036	0.066	–0.029	0.108*	–0.004	0.004	-

The localities codes are the same of Table 1. *indicates significant values with p<0.05 (p-values not shown).

similarity. The observation of high genetic diversity and low genetic distance, evidenced by high haplotype sharing among populations, is an important issue regarding the population dynamics of *C. externa,* highlighting the intense gene flow, as shown in Table 4. The different farms with citrus orchards sampled in this study form a unique habitat, allowing the dispersion of populations as being considered a suitable habitat for their survival. This is corroborated by the results from the neutrality tests and mismatch distribution, suggesting demographic events of population expansion *i.e.*, representing colonization events of these areas over time. Agreeing with this hypothesis, data set shows high haplotype diversity among populations (Table 2), in contrast with low nucleotide diversity, which suggest a rapid demographic expansion from a small effective population size (Avise, 2000).

The global AMOVA results showed no genetic structure, in agreement with the inference of high gene flow observed from the Nm parameter (Table 4). These populations most likely represent subpopulations of a major genetic unit across the geographic area in the countryside of São Paulo State in which citrus is cultivated. Such inference is congruent with the genetic analysis of *C. externa* populations sampled in native areas and agroecosystems, and the same pattern of genetic structure has been found in the latter (Morales et al., 2013).

Possible explanations for this genetic similarity is that citrus orchards favor the establishment and maintenance of genetic variability in *C. externa* populations, there is abundant food and large areas of citrus orchards forming corridors used by these insects in the countryside of São Paulo State. All tests conducted to detect demographic events support the hypothesis of corridor use, and the AMOVA test and Nm values (Table 4) infer high gene flow among the sampled populations. Even the Tietê River does not seem to act as a barrier to gene flow. In fact, some populations occur on different sides of the river, these values are too low and not significant to constitute genetic structure. On the other hand, this genetic similarity could be due to a shared polymorphism among populations that was present before population expansion or any differentiation that is too recent to be detected by these analyses. However, due to intense anthropic interference and extensive citrus orchard corridors, the hypothesis of high gene flow to explain the lack of genetic variation across large geographic distances is the most acceptable. The observed genetic similarity shows that *C. externa* constitutes a single population in the sampled area and that the insects found here can be used as a population (genetic) source for other citrus orchards with the same profile. Although there is no management of these populations (introgression of new individuals), the molecular diversity indices and haplotype network (Figure 2) indicate that high levels of genetic variability remain, and what is essential for successful biological control of agricultural pests.

The haplotype network is in agreement with the results of the other tests. The congruent results from the statistical analyses strengthen the inferences made regarding the population dynamics of *C. externa*.

Analyses with mitochondrial sequences have identified groups/clades within species, which agrees with geographical range or ecological traits. Genetic analysis with *Pilophorus typicus* (Hemiptera: Miridae) populations, a predatory bug as *C. externa* larvae, indicate that they are genetically structured forming two main clades (Ito et al., 2011). This is probably due to ecological or behavioral features of these insects instead of geographical conditions, once Japan archipelago presents small territorial proportions in relation to São Paulo State, promoting genetic homogeneity among populations through gene flow. Another case of genetically structured populations was observed between north and south populations along geographical range of *Thanasimus dubius* (Coleoptera: Cleridae), also a predatory bug. The genetic divergence found should be due to differences in sex pheromone of its prey and not exclusively the geographic distances (Schrey et al., 2005).

C. externa populations analyzed in this study did not show the same pattern. There are two possible reasons, not mutually exclusive: 1) the anthropic action is so strong that already erased genetic structure that existed before the agroecosystems and/or 2) recent colonization of this species in agroecosystems, so that one molecular marker, such as mitochondrial, is inappropriate to detect the recent history of this species in this environment. Probably, its evolutionary history is as recent as the history of agriculture in Brazil, so it should be used molecular markers with different modes of inheritance, as nuclear genes, for multi-locus analysis.

Considering these results, it is possible to conclude that the populations of *C. externa* sampled in the countryside of São Paulo State constitute a single population, with high dispersion and gene flow, most likely favored by the homogeneity of agroecosystems. For these farms, the mass-reared green lacewings for biological control releases could come from the same population source, which could be collected in any locality sampled in this work, due to the genetic identity among *C. externa* populations. Beyond biological control success, this practice avoids genetic introgression and extinction of non-target insects.

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