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Distinct genetic structure in populations of *Chrysoperla externa* (Hagen) (Neuroptera, Chrysopidae) shown by genetic markers ISSR and COI gene

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ABSTRACT. Distinct genetic structure in populations of *Chrysoperla externa* (Hagen) (Neuroptera, Chrysopidae) shown by genetic markers ISSR and COI gene. Green lacewings are generalist predators, and the species *Chrysoperla externa* presents a great potential for use in biological control of agricultural pests due to its high predation and reproduction capacities, as well as its easy mass rearing in the laboratory. The adaptive success of a species is related to genetic variability, so that population genetic studies are extremely important in order to maximize success of the biological control. Thus, the present study used nuclear (Inter Simple Sequence Repeat – ISSR) and mitochondrial (Cytochrome Oxidase I – COI) molecular markers to estimate the genetic variability of 12 populations in the São Paulo State, Brazil, as well as the genetic relationships between populations. High levels of genetic diversity were observed for both markers, and the highest values of genetic diversity appear associated with municipalities that have the greatest areas of native vegetation. There was high haplotype sharing, and there was no correlation between the markers and the geographic distribution of the populations. The AMOVA indicated absence of genetic structure for the COI gene, suggesting that the sampled areas formed a single population unit. However, the great genetic differentiation among populations showed by ISSR demonstrates that these have been under differentiation after their expansion or may also reflect distinct dispersal behavior between males and females.

KEYWORDS. Genetic distance; green lacewings; Insecta; population structure; variability.

The family Chrysopidae is composed of approximately 1,200 species distributed in 80 genera (Freitas & Penny 2001). Larvae and adults exhibit distinct feeding habits, while adults are predators and/or consume pollen, nectar, and honeydew, all larvae are generalist predators. Larvae feed on various insects that are considered agricultural pests, such as aphids, mealybugs, and whiteflies, as well as lepidopteran eggs and larvae (Canard 2001; Freitas 2001; Papas *et al.* 2011).

Green lacewings have a wide geographic distribution, being recorded worldwide except in Antarctica (Brooks & Barnard 1990). The genus *Chrysoperla* Steinmann, 1964 is one of the most studied green lacewing genera and comprises 36 described species. Only four species of this genus occur in Brazil (Freitas 2003): *Chrysoperla defreitasi* Brooks, 1994; *Chrysoperla raimundoi* Freitas & Penny, 2001; *Chrysoperla genanigra* Freitas, 2003; and *Chrysoperla externa* (Hagen, 1861). The latter is regarded as one of the most common species in the Americas and can be found from the southern USA to Argentina. In Brazil, *C. externa* occurs throughout the country and has been observed in different environments, such as native vegetation areas and agroecosystems (Adams & Penny 1985; Belorte *et al.* 2004; Ramiro & Faria 2006; Queiroz *et al.* 2009; Costa *et al.* 2010).

The species *C. externa* is considered a potential biological control agent in management programs in Central and South America (Albuquerque *et al.* 1994), due to its high predation capacity and reproductive potential, as well as its successful mass rearing in the laboratory (Freitas 2001). Also, this species can be used in Integrated Pest Management (IPM) because it is not affected by certain insecticides (Godoy *et al.* 2004; Moura *et al.* 2009; Castilhos *et al.* 2011).

Several studies have demonstrated the high predation efficacy of pests by larvae of *C. externa* in the laboratory (Ecole *et al.* 2002; Figueira *et al.* 2002; Silva *et al.* 2002; Adriano *et al.* 2010) and in the greenhouse. For instance, Auad *et al.* (2007) observed a reduction of 40–50% of nymphs of *Bemisia tabaci* (Gennadius, 1889) biotype B in tomato. In the field, this species is found in several crops, such as cotton (Barros *et al.* 2006; Ramiro & Faria 2006), coffee (Silva *et al.* 2006), citrus (Souza & Carvalho 2002), and soybean (Belorte *et al.* 2004), where it is sometimes considered the most abundant predator.

However, it is essential for biological control success that individuals reared in the laboratory would establish in the field and be efficient in pest control. Thus, population-genetic studies are extremely important because the genetic variability of a species is directly associated with its ability to withstand different conditions when introduced into new environments (Hopper *et al.* 1993; Baker *et al.* 2003; Hufbauer & Roderick 2005). The genetic variability allows

that individuals respond differently to the variations of temperature, presence of pathogens, affecting also the foraging efficiency against target/alternative prey (Hopper *et al.* 1993). Population-genetic studies also provide valuable historical information about genetic effects caused by reduction of native areas, which would act as ecological corridors and/or genetics reservoirs (Morales *et al.* 2013).

Genetic variability and population structure may be ascertained using molecular markers, both in the nuclear and mitochondrial (mtDNA) genomes. Inter Simple Sequence Repeat (ISSR) markers have been widely used in genetic variability and population structure studies for different insect orders (Reddy *et al.* 1999; Luque *et al.* 2002; Borba *et al.* 2005; Hundsdoerfer *et al.* 2005; Hundsdoerfer & Wink 2005; Rouex *et al.* 2007; Souza *et al.* 2008; Kehlmaier & Assmann 2010; Helmi & Khafaga 2011; Taylor *et al.* 2011). In the same manner, the region of mitochondrial cytochrome oxidase subunit I gene (COI) is used in several population studies of genetic variability, population structure, phylogeny, phylogeography, and identification of various insect species of different orders (Clark *et al.* 2001; Smith-Caldas *et al.* 2001; Finn *et al.* 2006; Asokan *et al.* 2007; Vandergast *et al.* 2007; Ståhls & Savolainen 2008; Boehme *et al*. 2010; Wilson *et al.* 2010; Velonà *et al.* 2011; Nie *et al.* 2012; Henry *et al.* 2012; Sole *et al.* 2013), and some were performed with *C. externa* (Winterton & Freitas 2006; Morales & Freitas 2010; Morales *et al.* 2013).

This study aimed to analyze the genetic variability distribution in populations of *Chrysoperla externa* in agroecosystems of the São Paulo State and to compare the genetic changes in these populations by means of ISSR molecular markers and the mitochondrial COI gene. Finally, we aimed to identify the most effective ISSR markers for this species, since there are no studies performed with ISSR markers in neuropterans to this date.

MATERIAL AND METHODS

Specimen collecting. The specimens of *C. externa* were collected with the aid of entomological net and 90 individuals were collected. The samplings were performed in areas of perennial crops in 12 municipalities in the São Paulo State (Table I). These specimens were stored in absolute ethanol and identified on the basis of external morphological characteristics (Brooks & Barnard 1990).

The distance, in a straight line, between the sampled municipalities was calculated with the software Google Earth version 7.0.1.8244 (Google 2012), and a map (Fig. 1) was made with software GPS Track Maker© version 13.8 (Junior 2012).

DNA extraction. DNA was extracted from the thorax of the insects, while the other parts (head, wings, and abdomen) were stored in absolute ethanol in freezer in the *Laboratório de Biologia Molecular* at the *Departamento de Fitossanidade*, FCAV (LBM), each receiving a specific identification number (Table I). The DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega), following the protocol supplied by the manufacturer. The numbered DNA samples obtained, with the corresponding number, were stored in the same laboratory.

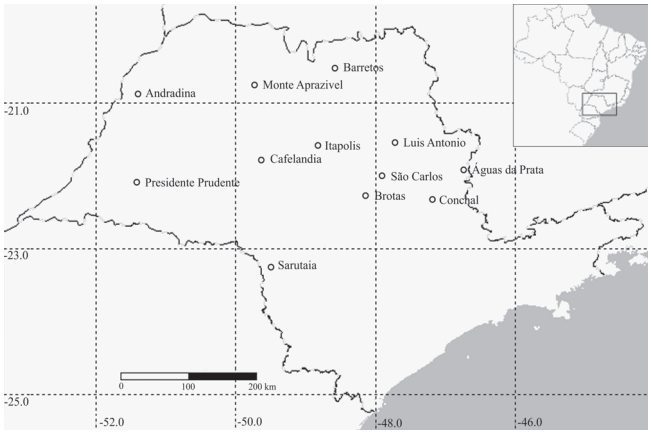

Fig. 1. A, Sampled municipalities in the São Paulo State. B, Political map of Brazil.

ISSR-PCR. Primer selection and amplification. The ISSR primers used belong to set 9 of the University of British Columbia (UBC Set#9), which contains 100 primers. To perform the amplification tests, the primers were initially grouped according to their similarity and melting temperature (T_m). Then, the annealing temperatures (T_a) were defined, and a temperature range extending from the highest T_m up to 5°C below the lowest T_m within each group of primers was established. The amplification tests were performed with three randomly selected specimens from different localities. Eight primers that generated polymorphic bands where then selected (Table II), followed by new standardization tests to optimize the concentrations of the polymerase chain reaction (PCR) components and the annealing temperature. PCRs were performed in a Mastercycler® thermal cycler (Eppendorf) in a final volume of 25 µL, consisting of 5 µL of 5X Green GoTaq® Flexi Buffer (Promega), 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of primer, 1 U of GoTaq® DNA Polymerase (Promega), and 2 µL of total DNA (~30 ng). The amplification conditions used were those described by Souza *et al.* (2008), with modifications: initial denaturation at 94°C for 5 min, followed by 35 cycles of 45 sec at 94°C, 45 sec for each of the temperatures contained within a specific range per primer (Table II), 1 min and 50 sec at 72°C, with a final extension phase of 72°C for 7 min. The PCR products were subjected to electrophoresis in 2% agarose gel, stained with ethidium bromide (1ng/mL), and visualized under ultraviolet light. The images were recorded using the Gel Doc 2000 – Gel Documentation System software (Bio-Rad).

Data Analysis. A binary matrix based on the absence/presence of DNA bands was constructed because ISSR markers are classified as dominant. The sizes of the bands were estimated based on the 1-kb molecular weight marker and considering only the easily visualized bands. Less intense and low-reproducibility bands were excluded from the analysis. POPGENE software version 1.32 (Yeh *et al.* 1999) was used to estimate the number of polymorphic loci (NPL), the per-

Table I. Sampled municipalities, geographic coordinates, type of crop at the collecting site, sampling date, and the specific identification number (vouchers) of *Chrysoperla externa*.

Municipalities	Geographic coordinates*		Crop	Collecting date	Voucher[1,2]
	Latitude	Longitude			
AP	-21:56:13	-46:43:01	coffee	VIII.07	143; 145; 146; 885-888
AN	-20:53:45	-51:22:44	pasture	VIII.06	75-78; 875-878
BA	-20:33:25	-48:34:04	citrus	VIII.06-XI.06	124; 125; 892; 895-897
BR	-22:17:02	-48:07:37	eucalypt	XII.07-V.08	898-905
CA	-21:48:10	-49:36:36	citrus	XI.06-XII.06	79; 81; 82; 1017-1021
CO	-22:19:48	-47:10:22	citrus	X.06	91-94; 908; 909; 913
IT	-21:35:45	-48:48:46	citrus	VIII.06-XI.06	148-150; 1063-1066
LA	-21:33:18	-47:42:14	citrus	I.07	103-106; 921-924
MA	-20:46:22	-49:42:50	citrus	II.05-VII.07	99-102; 927-930
PP	-22:07:33	-51:23:20	coffee/eucalypt	VIII.06-III.07	787; 788; 791-792, 794-797
SC	-22:01:04	-47:53:27	citrus	X.06	1119-1124, 1126
SA	-23:16:22	-49:28:48	citrus	VII.06	968-975

AP = Águas da Prata; AN = Andradina; BA = Barretos; BR = Brotas; CA = Cafelândia; CO = Conchal; IT = Itápolis; LA = Luís Antônio; MA = Monte Aprazível; PP = Presidente Prudente; SC = São Carlos; SA = Sarutaiá.
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[1]Vouchers 895, 94 and 104 were analyzed only for ISSR. Voucher 148 was analyzed only for COI.
[2]Sequences numbers 1017-1021, 1063-1066, 1119-1126 from Lavagnini (2011), with accession numbers HQ668472- HQ668472, HQ425564- HQ425567, HQ425613-HQ425619, respectively; and sequences numbers 787-797 from Morales *et al.* (2013).

Table II. Specific annealing temperature for ISSR primer and nucleotide sequence used for genetic studies of *C. externa*.

Primer	Sequence (5' – 3')	Annealing temperature (ºC)
UBC-809	AGA GAG AGA GAG AGA GG	44 – 46 – 48 – 50 – 52
UBC-820	GTG TGT GTG TGT GTG TC	47 – 49 – 51 – 53 – 55 – 57
UBC-836	AGA GAG AGA GAG AGA GYA	36 – 38 – 40 – 42 – 44 – 46 – 48
UBC-849	GTG TGT GTG TGT GTG TYA	47 – 49 – 51 – 53 – 55 – 57
UBC-856	ACA CAC ACA CAC ACA CYA	47 – 49 – 51 – 53 – 55 – 57
UBC-880	GGA GAG GAG AGG AGA	42 – 44 – 46 – 48
UBC-886	VDV CTC TCT CTC TCT CT	36 – 38 – 40 – 42 – 44 – 46 – 48
UBC-891	HVH TGT GTG TGT GTG TG	44 – 46 – 48 – 50 – 52

Y = (C or T); V = (A, C or G); D = (A, G or T); H = (A, C or T).

centage of polymorphic loci (P) and the Shannon genetic diversity index (I) (Lewontin 1972). Nei's diversity index (H_e) was obtained through TFPGA software version 1.3 (Miller 1997). This estimator corresponds to the expected heterozygosity corrected for populations with low sample size ($n < 50$) (Nei 1978) and was based on a Taylor expansion (Lynch & Milligan 1994). The average number of band presences per individual (BPI) and the number of private bands (PB) were calculated with FAMD software version 1.3 (Schlüter & Harris 2006). The genetic distance among populations, based on the Lynch-Milligan method, and the Analysis of Molecular Variance (AMOVA), employing Jaccard's similarity coefficient, were also obtained in the same program. The correlation between the genetic and the straight-line geographic distance was calculated in TFPGA software version 1.3 (Miller 1997) using the Mantel test (1967). The significance of the correlation between the two matrices was tested by 999 random permutations of the similarity matrix to generate a null distribution of correlation coefficients (z-values). A significant result was inferred if ⩾ 95% of the randomly generated statistics were greater than the observed value.

COI. Amplification and sequencing. The mitochondrial COI gene was amplified by PCR in a Mastercycler® thermal cycler (Eppendorf). The reaction occurred in a final volume of 25 µL, consisting of 12.5 µL of GoTaq® Colorless Master Mix (Promega), 0.4 µM of each of the primers C1-J-2183 (5'CAACATTTATTTTGATTTTTTGG3') and TL2-N-3014 (5'TCCATTGCACTAATCTGCCATATTA3') (Simon *et al.* 1994), and 2.5 µL of total DNA (~40 ng). Amplification occurred under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 40 sec at 94°C, 50 sec at 55°C, and 1 min at 72°C, with a final extension phase of 72°C for 10 min. The PCR products were subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide (1ng/mL) to confirm amplification. Each PCR product was then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the protocol supplied by the manufacturer. The products were sequenced using the same primers and amplification conditions. The sequencing reactions took place in an ABI Prism 3100 Genetic Analyzer automatic sequencer using the Big Dye™ Terminator version 3.1 (Perkin-Elmer Applied Biosystems), and the sequences were deposited in the LBM database. The haplotypes were deposited in the GenBank database under the accession numbers KJ586656 to KJ586673.

Data analysis. The sequences were read in Chromas Lite software version 2.01 (Technelysium Ltd. 2005) and aligned with BioEdit software version 7.1.3.0 (Hall 1999). Descriptive analyses were performed using DnaSP software version 5.10.01 (Librado & Rozas 2009), and the number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), average number of nucleotide differences (k), fixation index (F_{ST}), and the number of migrants (N_m) were obtained. Nucleotide composition and genetic distance between populations were calculated by MEGA version 5.01 (Tamura *et al.* 2011), the latter using the Kimura 2-parameters (K2P) correction model, which considers transitions more frequent than transversions, a fact that is observed in mtDNA (Page & Holmes 1998). For the correlation analysis with the geographic distance matrix, the Mantel test (Man-

tel 1967) was used in TFPGA version 1.3 (Miller 1997) and significant result was inferred if P ⩾ 95%. AMOVA was applied using Arlequin software version 3.5.1.3 (Excoffier & Lischer 2010). Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) neutrality tests were performed with the software DnaSP version 5.10.01 (Librado & Rozas 2009), to determine if populations follow a neutral model of evolution, with constant population size over time. A haplotype network was built using TCS version 1.12 (Clement *et al.* 2000), and the clades were nested according to the methodology of Templeton *et al*. (1987). Correlation between the nested clades and the geographic location of haplotypes was analyzed using GeoDis software version 2.6 (Posada *et al.* 2000), and the indices were tested in the phylogenetic inference key (Templeton 2004) made available by GeoDis (darwin.uvigo.es/software/geodis.html).

RESULTS

ISSR-PCR. Of the 100 primers tested, 43 produced fragments consistent or of low intensity. Twenty-seven primers were polymorphic, while 16 showed only bands of the same size. Among the polymorphic primers, 16 primers stand out which showed more intense bands: UBC-809 $(AG)_8G$, UBC-810 $(GA)_8T$, UBC-812 $(GA)_8A$, UBC-817 $(CA)_8A$, UBC-818 $(CA)_8G$, UBC-819 $(GT)_8A$, UBC-820 $(GT)_8C$, UBC-828 $(TG)_8A$, UBC-836 $(AG)_8YA$, UBC-840 $(GA)_8YT$, UBC-846 $(CA)_8RT$, UBC-849 $(GT)_8YA$, UBC-856 $(AC)_8YA$, UBC-880 $(GGAGA)_3$, UBC-886 $VDV(CT)_7$ and UBC-891 $HVH(TG)_7$.

Eight primers were selected and generated a total of 134 polymorphic fragments, of which 17 were obtained with UBC-809, 21 with UBC-820, 15 with UBC-836 and UBC-849, 22 with UBC-856, 16 with UBC-880 and UBC-891, and 12 with UBC-886 (Fig. 2).

The populations displayed, on average, 48.67 polymorphic loci and 36.32% of polymorphism. The population of Brotas showed an elevated number of bands per individual (BPI) and of private bands (PB) (Table III). The Nei's diversity index (H_e) and Shannon's diversity index (I) were, on average, 0.0952 and 0.1470, respectively, and high values were observed for the populations of Brotas, São Carlos, Barretos and Andradina (Table III).

The AMOVA indicated the presence of a genetic structure with the level of differentiation between populations (φ_{ST}) of 0.18172 (p = 0.00). The percentage of variation was 18.17% among the populations, while 81.83% was observed within populations.

The genetic distance between populations was small, ranging from 0.0586 (Monte Aprazível and Presidente Prudente) to 0.1432 (Águas da Prata and Presidente Prudente) (Table IV) and the Mantel test indicated no correlation between the genetic and geographic distance matrices (r = 0.0396; P = 63%) (Table IV).

COI. The 87 sequences, of 648 bp each, presented an average nucleotide composition of 44.3% thymine (T), 12.7% cytosine (C), 28.4% adenine (A), and 14.5% guanine (G). Twenty-four polymorphic sites (S) were obtained, resulting in 22 haplotypes (h) (Table V) and an average haplotype diversity (Hd) of 0.7952 (Table VI). The average number of nucleotide differences (k) and average nucleotide diversity (π) were low, respectively, 1.8762 and 0.0029 (Table VI).

The genetic distance between populations was very small and varied from 0.001 to 0.005 (Table VII). The number of migrants (N_m) was 28.07. The Mantel test indicated no correlation between genetic and geographic distances (r = 0.1850; P = 82%) (Table VII).

The AMOVA also showed a high similarity between the populations, indicating that 100% of the variability was observed within populations. This similarity was supported by

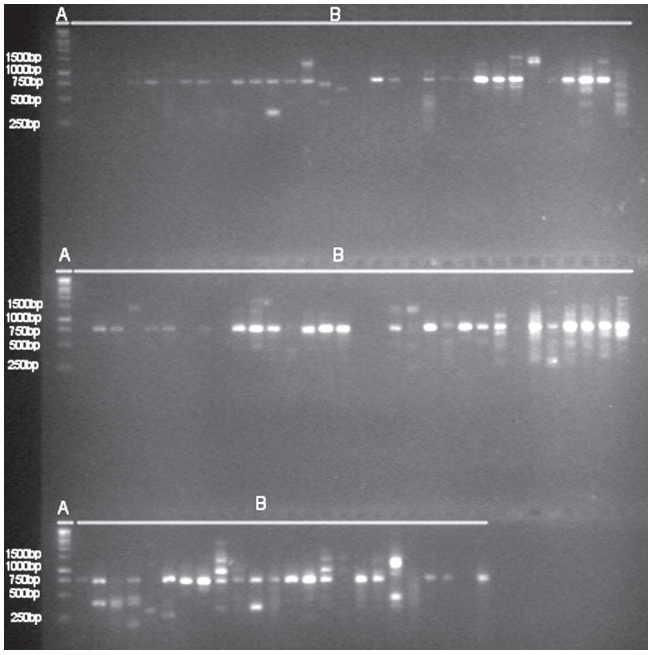

Fig. 2. Example of ISSR bands on 2% agarose gel. A, 1-kb molecular weight marker. B, Amplified bands in the populations of *Chrysoperla externa* (UBC 809 primer).

Table III. Indices of genetic diversity in *C. externa* population with ISSR genetic markers.

Municipalities	n	NPL	P (%)	BPI	PB	He	I
Águas da Prata	7	35	26.12	10.28	0	0.0937	0.1376 ± 0.2230
Andradina	8	58	43.28	13.75	1	0.1025	0.1607 ± 0.2108
Barretos	6	46	34.33	12.33	0	0.1097	0.1646 ± 0.2207
Brotas	8	91	67.91	23.62	7	0.1699	0.2658 ± 0.2213
Cafelândia	8	47	35.07	11.62	1	0.0855	0.1339 ± 0.2052
Conchal	7	38	28.36	12.86	0	0.0811	0.1222 ± 0.2106
Itápolis	6	34	25.37	12.50	0	0.0744	0.1111 ± 0.2062
Luís Antônio	8	43	32.09	13.37	1	0.0703	0.1130 ± 0.1838
Monte Aprazível	8	51	38.06	14.00	2	0.0845	0.1362 ± 0.1974
Presidente Prudente	8	45	33.58	16.87	2	0.0917	0.1403 ± 0.2186
São Carlos	7	54	40.30	16.86	1	0.1022	0.1583 ± 0.2171
Sarutaiá	8	42	31.34	11.37	1	0.0768	0.1210 ± 0.1976
Average	7.4	48.67	36.32	14.22	1.33	0.0952	0.1470 ± 0.0605
Total	89	134	100	–	16	–	–

n = number of analyzed individuals; NPL = number of polymorphic loci; P = percentage of polymorphic loci; BPI = average number of band presences per individual; PB = private bands; He = Nei's diversity index (1978); I = Shannon genetic diversity index.

Table IV. Genetic distance based on ISSR marker (below diagonal) and geographic distance (km) (above diagonal) of *C. externa* populations.

	AP	AN	BA	BR	CA	CO	IT	LA	MA	PP	SC	SA
AP		492.3	248.0	150.9	300.3	64.2	219.8	110.9	337.8	482.7	121.4	319.8
AN	0.0839		293.5	370.0	209.4	462.9	279.3	389.5	174.4	137.1	381.8	329.3
BA	0.0813	0.0786		198.4	175.3	247.1	120.8	145.7	120.9	338.3	177.5	316.2
BR	0.1033	0.1137	0.0853		162.8	98.3	103.2	91.6	234.8	335.6	38.5	176.6
CA	0.0998	0.0932	0.0735	0.0849		258.9	86.4	199.7	115.8	185.9	179.2	163.9
CO	0.0891	0.0863	0.0895	0.1028	0.0650		187.9	101.9	314.5	434.3	82.2	257.8
IT	0.1289	0.0956	0.0995	0.1341	0.0923	0.0932		114.8	132.1	272.0	105.7	197.2
LA	0.1287	0.1235	0.1015	0.1201	0.0891	0.0760	0.0681		225.7	386.0	53.6	264.1
MA	0.1316	0.1071	0.0994	0.1326	0.0847	0.0815	0.0645	0.0629		227.6	233.6	282.0
PP	0.1432	0.1205	0.1101	0.1370	0.0984	0.0916	0.0677	0.0771	0.0586		359.6	234.6
SC	0.1402	0.1098	0.0980	0.1401	0.1082	0.0907	0.0690	0.0722	0.0697	0.0711		214.6
SA	0.1282	0.1063	0.0992	0.1153	0.0936	0.0928	0.0975	0.0877	0.0857	0.0972	0.1029	

AP = Águas da Prata; AN = Andradina; BA = Barretos; BR = Brotas; CA = Cafelândia; CO = Conchal; IT = Itápolis; LA = Luís Antônio; MA = Monte Aprazível; PP = Presidente Prudente; SC = São Carlos; SA = Sarutaiá.

Table V. Haplotype distribution of the gene COI. The haplotype corresponds to the number shown in the network.

Municipalities	Haplotype
Águas da Prata	143/787 (1), 145 (1), 146 (1), 885 (1), 886 (1), 887 (1), 888 (1)
Andradina	146 (2), 885 (1), 886 (3), 887 (1), 77 (1)
Barretos	146 (3), 886 (2)
Brotas	146 (2), 885 (1), 886 (2), 887 (1), 900 (1), 901 (1)
Cafelândia	146 (2), 885 (1), 886 (5)
Conchal	146 (3), 886 (1), 908 (1), 909 (1)
Itápolis	146 (2), 885 (1), 886 (2), 1064 (1), 1065 (1)
Luís Antônio	146 (1), 886 (5), 921 (1)
Monte Aprazível	146 (2), 886 (4), 99 (1), 930 (1)
Presidente Prudente	143/787 (1), 886 (3), 887 (1), 792 (1), 794 (1), 795 (1)
São Carlos	146 (2), 886 (2), 1120 (2), 1126 (1)
Sarutaiá	146 (3), 885 (1), 886 (2), 887 (2)

Table VI. Values of genetic variability in *C. externa* populations.

Municipalities	n	S	h	Hd	k	π
Águas da Prata	7	12	7	1.00000	3.80952	0.00588
Andradina	8	5	5	0.85714	1.71429	0.00265
Barretos	5	1	2	0.60000	0.60000	0.00093
Brotas	8	8	6	0.92857	2.32143	0.00358
Cafelândia	8	4	3	0.60714	1.28571	0.00198
Conchal	6	6	4	0.80000	2.20000	0.00340
Itápolis	7	6	5	0.90476	2.00000	0.00309
Luís Antônio	7	2	3	0.52381	0.57143	0.00088
Monte Aprazível	8	3	4	0.75000	0.92857	0.00143
Presidente Prudente	8	10	6	0.89286	3.00000	0.00463
São Carlos	7	5	4	0.85714	2.47619	0.00382
Sarutaiá	8	5	4	0.82143	1.60714	0.00248
Average	7.25	2	1.83	0.79524	1.87619	0.00290
Total	87	24	22	–	–	–

n = number of analyzed sequences; S = number of polymorphic sites; h = number of haplotypes; Hd = haplotype diversity; k = average number of nucleotide differences; π = nucleotide diversity.

the non-significant F_{ST} value (F_{ST} = -0.01974; p = 0.72239 ± 0.01273), which indicated the absence of genetic structure between these populations. The observed values of Tajima's D and Fu's F_S neutrality tests were -1.83527 ($p < 0.05$) and -15.020 (p = 0.000), respectively.

Table VII. Nei's genetic distance based on mtDNA (COI) (below diagonal) and geographic distance (km) (above diagonal) of *C. externa* populations.

	AP	AN	BA	BR	CA	CO	IT	LA	MA	PP	SC	SA
AP		492.3	248.0	150.9	300.3	64.2	219.8	110.9	337.8	482.7	121.4	319.8
AN	0.004		293.5	370.0	209.4	462.9	279.3	389.5	174.4	137.1	381.8	329.3
BA	0.003	0.002		198.4	175.3	247.1	120.8	145.7	120.9	338.3	177.5	316.2
BR	0.005	0.003	0.002		162.8	98.3	103.2	91.6	234.8	335.6	38.5	176.6
CA	0.004	0.002	0.001	0.003		258.9	86.4	199.7	115.8	185.9	179.2	163.9
CO	0.004	0.003	0.002	0.003	0.003		187.9	101.9	314.5	434.3	82.2	257.8
IT	0.004	0.003	0.002	0.003	0.002	0.003		114.8	132.1	272.0	105.7	197.2
LA	0.004	0.002	0.001	0.002	0.001	0.002	0.002		225.7	386.0	53.6	264.1
MA	0.004	0.002	0.001	0.002	0.002	0.002	0.002	0.001		227.6	233.6	282.0
PP	0.005	0.003	0.003	0.004	0.003	0.004	0.004	0.003	0.003		359.6	234.6
SC	0.005	0.003	0.003	0.004	0.003	0.004	0.004	0.003	0.003	0.004		214.6
SA	0.004	0.002	0.002	0.003	0.002	0.003	0.003	0.002	0.002	0.003	0.003	

AP = Águas da Prata; AN = Andradina; BA = Barretos; BR = Brotas; CA = Cafelândia; CO = Conchal; IT = Itápolis; LA = Luís Antônio; MA = Monte Aprazível; PP = Presidente Prudente; SC = São Carlos; SA = Sarutaiá.

Nested Clade Phylogeographic Analysis (NCPA) showed no significant values for the D_c (clade distance), D_n (nested clade distance), or I-T (interior-tip distance) provided by the analysis. Therefore, it was not possible to reject the null hypothesis of no geographic association between haplotypes, a fact that was observed in the haplotype network, where both more ancestral haplotypes (centers 146 and 886) and more derivatives (centers 885 and 887) were shared by several populations (Fig. 3).

DISCUSSION

Intense bands were found using primers ISSR formed by different nucleotides sets and a high number of polymorphic bands was observed. This result demonstrates the great capacity of the technique ISSR-PCR to detect polymorphism in this species. Among the primers that showed more intense bands, 11 were described as effective for others insect orders whereas for five primers there are no reports. The primers UBC-809 and UBC-891 were utilized with Coleoptera (Souza *et al.* 2008); UBC-809 and UBC-818, with Diptera (Abbot 2001; Vaulin *et al.* 2006); UBC-809, UBC-812 and UBC-

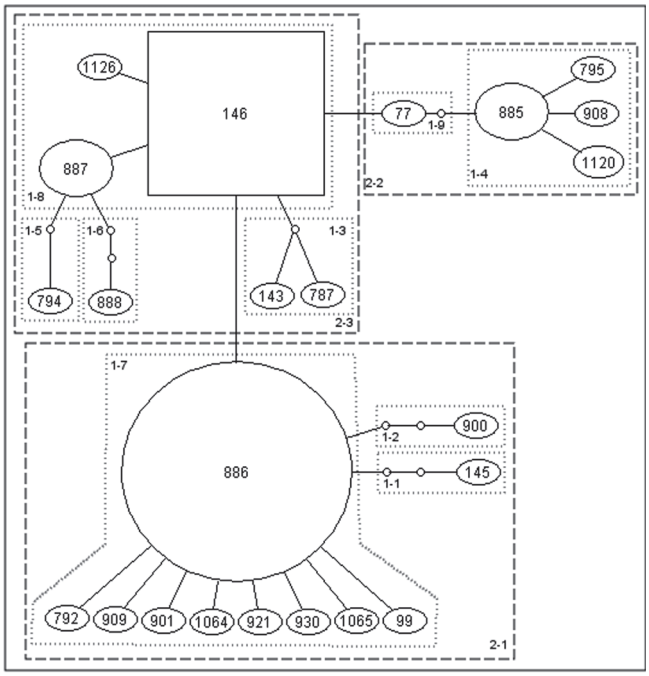

Fig. 3. Haplotype network for *Chrysoperla externa* in the São Paulo State, Brazil. Haplotypes are named and their size represents their frequency. Each solid line corresponds to a mutational change that interconnects two haplotypes that has a possibility greater than 95%. Small circles without haplotype names denote missing intermediate haplotypes.

819, with Hemiptera (Abbot 2001; Taylor *et al.* 2011); UBC-836, UBC-840, UBC-856 and UBC-880 with Hymenoptera (Borba *et al.* 2005; Nascimento 2008); and the primers UBC-809, UBC-810, UBC-812, UBC-818, UBC-836, UBC-840 and UBC-886 were reported for Lepidoptera (Kar *et al.* 2005; Pradeep *et al.* 2007; Velu *et al.* 2008).

The predominance of A-T in the nucleotide composition of COI gene is expected for the mitochondrial DNA of insects (Hoy 2003). In the present study, the number of haplotypes was 22 and similarly to what has been found by Morales & Freitas (2010) and Morales *et al.* (2013) who observed 24 haplotypes in 40 sampled individuals and 41 haplotypes in 122 sampled individuals, respectively. It is noticeable that the increase of haplotypes was not proportional to the increase in the sample size. Thus, in the present study, the number of haplotypes distributed among the populations corroborates previous studies. Also it can be concluded that the populations in the São Paulo State satisfactorily shows the variability of the species.

Few segregating sites are responsible for the different haplotypes as shown by the low average number of nucleotide differences (k = 1.837) and average nucleotide diversity (π = 0.00283).

Both markers, ISSR and COI, showed a high genetic diversity for *C. externa*, which may indicate a high adaptive capacity for these natural enemies in agroecosystems, as suggested by Reed & Frankram (2003) for different invertebrate species, because the effects of genetic drift tend to be minimized by high variability within populations.

For all parameters obtained by ISSR (NPL, P, BPI, PB, H_e and I), the highest values were observed in the municipality of Brotas, with high values for Barretos and São Carlos. The municipalities of Brotas and São Carlos also showed high values for haplotype diversity (Hd). High values of haplotype diversity were also observed by Morales & Freitas (2010) and Morales *et al.* (2013) in studies of populations of *C. externa*. Morales *et al.* (2013) observed higher values of haplotype and nucleotide diversity in regions with native vegetation (Hd = 0.962 and π = 0.00861) compared to agroecosystems (Hd = 0.866 and π = 0.00353). Among the sampled municipalities, Brotas, Barretos, and São Carlos have the largest native vegetation cover, with 10,565.21 ha, 12,148.03, and 13,030.66 ha, respectively, distributed between areas of forest, secondary forest, savanna vegetation, and white-water floodplains (Instituto Florestal 2001). The municipality of Brotas also has two conservation units, the São Carlos Ecological Station and part of the Itirapina Ecological Station. Therefore, the larger diversity associated with these municipalities may be related to the presence of native vegetation, which may constitute a reservoir of genetic diversity, as suggested by Morales *et al.* (2013), as well as the proximity between these fragments of native vegetation and the studied agroecosystems.

The genetic distance between populations was small for both markers. Low genetic distances were also observed by Wells (1994) between populations of other species of the genus *Chrysoperla*, although using enzyme markers and by Morales & Freitas (2010) for populations of *C. externa* from the municipality of Jaboticabal. These data show that the analyzed populations in our study present high genetic similarity, indicative of a short isolation period between them or the presence of enough gene flow to keep the populations homogeneous (McDermott & McDonald 1993). The presence of gene flow is confirmed by the number of migrants to COI gene (N_m = 28.07), and does not associate with geographic distance, as observed in the Mantel test for both markers. This fact is also noted in NCPA, in which all populations share haplotypes among themselves and have high genetic similarity.

The observed values of Tajima's D and Fu's Fs neutrality tests were negative, indicating an excess of rare alleles within the population and may suggest population expansion (Ramos-Onsins & Rozas 2002; Hartl & Clark 2010). The structure of the haplotype network, with star-like appearance, also confirms the hypothesis of population expansion because it indicates the presence of several similar haplotypes, with low nucleotide diversity, suggesting that most of these haplotypes have emerged recently (Ferreri *et al.* 2011).

With regard to the COI gene, AMOVA indicated the absence of genetic structure between the populations. However, the level of differentiation ($ö_{ST}$) between populations to ISSR was 0.18172 (p = 0.00) and values of 0.15 to 0.25 indicate great genetic differentiation (Wright 1978; Hartl & Clark 2010). The differences obtained between the markers regarding population structure can be attributed to the characteris-

tics of the markers. The mitochondrial COI molecular marker, due to the absence of recombination and to the lower accumulation of substitutions compared to intergenic regions of nuclear DNA, reveals the evolutionary history of populations over a wide time scale, so that recently isolated populations tend to display high genetic similarities. In contrast, SSR nuclear markers are highly polymorphic and can reveal more recent effects of reproductive isolation or low gene flow (Loxdale & Lushai 1998; Hartl & Clark 2010).

Other hypothesis for the differences of population structure may be related to a differential migratory behavior among females and males. Duelli (1984) described the strategy of "obligatory preovipository migration" in *Chrysoperla plorabunda* (Fitch, 1855) (cited as *Chrysopa carnea* (Stephens, 1836) and *Chrysoperla carnea*), in which females need to fly by two nights before mating. Males also showed migratory behavior in the first two nights after emergence, called the "adaptive dispersal flight" (Duelli 1980), however this does not prevents their mating (Duelli 1984, 2001). A similar behavior was reported by Liu *et al.* (2011) with *Chrysoperla sinica* (Tjeder, 1936), where more females than males were observed migrating. Therefore, the absence of genetic structure in COI gene may indicate that females of *C. externa* migrate in greater number or more farther than males, increasing the gene flow, once this gene shows the maternally inherited genetic material. On the other hand, ISSR marker displays the paternal inheritance too, and the genetic structure may suggest that males move less, allowing differentiation in some populations.

Thus, our results show that the *Chrysoperla externa* presents genetic features which contribute for its use in biological control, such as a high genetic variability, and great genetic similarity among populations. However, the dispersal behavior or population expansion followed by isolation of these populations may result in their genetic differentiation, as indicated by the data obtained using the ISSR markers. Thus, more studies on the migratory behavior of *C. externa* are needed, as well as temporal analyses of its genetic variability by the same markers.

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