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Genetic differentiation of geographically separated populations of the southern green stink bug *Nezara viridula* (Hemiptera: Pentatomidae)

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Abstract

Genetic variation in the southern green stink bug *Nezara viridula* (Linnaeus) from 11 geographically separated sampling locations (Slovenia, France, Greece, Italy, Madeira, Japan, Guadeloupe, Galapagos, California, Brazil and Botswana) was studied by sequencing 16S and 28S rDNA, cytochrome b and cytochrome *c* oxidase subunit I gene fragments and random amplified polymorphic DNA (RAPD) analysis. Sequencing revealed 11 distinct haplotypes clustering into lineages A, B and C. Lineage C was characteristic for a single analysed specimen from Botswana. Lineage B was detected in Japan, and it probably arose in Asia. Haplotypes of European and American specimens belonged to lineage A; specimens from France, Slovenia, Madeira and Brazil shared highly similar haplotypes (>99%) from subgroup A1, while all the specimens from Greece, California, Galapagos and Guadeloupe shared a haplotype from subgroup A2. RAPD data were more variable but consistent with mtDNA sequences, revealing the same clustering. They separated the Botswanian specimen from Japanese specimens and from a group of more closely related specimens from Europe and America. Sequence and RAPD results both support the African origin of *N. viridula*, followed by dispersal to Asia (lineage B) and, more recently, by expansion to Europe and America (lineage A). RAPD analysis revealed two highly supported subgroups in Japan, congruent with mtDNA lineages A2 and B, suggesting multiple colonization of Japan. Invariant sequences at the 28S rDNA combined with other results do not support the hypothesis that cryptic (sibling) species exist within the populations investigated in this study.

Keywords: cryptic species, dispersal, Hemiptera, heteroplasmy, insect, mitochondrial DNA, native area, pest, pseudogenes, RAPD, sequence diversity

Introduction

The southern green stink bug *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae) is one of the most important pentatomid pests of agricultural and horticultural crops worldwide (Todd, 1989; Panizzi, 1997; Panizzi *et al*., 2000). This species is highly polyphagous, feeding on plants from more than 30 families. While showing a preference for

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legumes (Todd, 1989), it is also an important pest of macadamia (Jones *et al*., 2001) and pecan nuts (Coombs, 2000) and it has re-emerged in recent years as a significant pest of cotton (Greene *et al*., 1999; Willrich *et al*., 2004). There is a growing concern that due to its high vagility (Jones, 1988), polyphagous feeding habits and its ability to move to alternative hosts (Panizzi, 1997; Panizzi *et al*., 2000), combined with global warming and changes in agricultural practices, *N. viridula*, as well as other stink bugs, will become even more important pests worldwide.

Nezara viridula is currently present throughout tropical and subtropical regions (Todd, 1989; Panizzi *et al*., 2000). The species was first described by Linnaeus in 1758 as *Cimex viridulus* from material collected '*in Indiis*' (Freeman, 1940). Based on the distribution pattern of distinct colour morphs, it was first assumed to have originated from the Indo-Malayan Region (Yukawa & Kiritani, 1965). However, in later studies, which also considered the distribution of other species of the genus and examined the distribution of specialist parasitoids, it was proposed that the area of origin of *N. viridula* was most probably the Ethiopian region (Hokkanen, 1986; Jones, 1988). In the 18th century it was already present in Africa and Mediterranean region, in Madeira, in tropical parts of Asia and in the West Indies (Hokkanen, 1986). It was first recorded in Japan in 1879 (Jones, 1988), in Australia in 1916 (Clarke, 1992), in New Zealand in 1944 (Cumber, 1949), in Hawaii in 1961 (Jones, 1995) and in California in the mid 1980s (Jones, 1988). It was not reported from Brazil prior to 1923 (A.R. Panizzi, personal communication). It is still an active invading species and newly established populations have recently been reported from the Galapagos (Henry & Wilson, 2004) and England (Barclay, 2004). It is assumed that current spread into temperate regions is made possible by global warming (Musolin & Numata, 2003, 2004).

Much of the worldwide problem with *N. viridula* may be traced to the need to understand the nature of variation within and among its populations. An important aspect of the biology of *N. viridula* that has emerged in recent years is a high level of variability in mating behaviour among geographically separated populations, which do not differ morphologically. *Nezara viridula* has a complex specific-mate recognition system (in the sense of Paterson, 1985) that involves chemical and vibrational signals (Borges *et al*., 1987; Brézot *et al*., 1994; Čokl *et al*., 2000; Miklas *et al*., 2003b). Differences in the ratio between the two main components of the male pheromone blend have been reported from various parts of the world (Aldrich *et al*., 1987, 1989, 1993; Brézot *et al*., 1994). Vibrational signals of geographically separated populations differ in their temporal characteristics (Kon *et al*., 1988; Ryan *et al*., 1996; Čokl *et al*., 2000, 2001; Miklas *et al*., 2001, 2003a; Virant-Doberlet & Čokl, 2004) and it seems that these differences are genetically determined (Virant-Doberlet *et al*., 2000). Isozyme analyses revealed consistent differences among *N. viridula* populations and clearly separated the Japanese population from populations in Slovenia, France, Guadeloupe (Lesser Antilles), Brazil and California (Meglič *et al*., 2002; unpublished data). Significant geographical variation in widely distributed species could be interpreted as an indication of incipient speciation or the presence of cryptic (sibling) species. The Hemiptera is a group in which morphologically indistinct cryptic species are particularly common, the planthopper genus *Nilaparvata* (Jones *et al*., 1996; Claridge *et al*., 1997b) and treehopper genus *Enchenopa* (Wood, 1987; Lin & Wood, 2002) being the two best documented cases. On the grounds of differences found in their sexual communication system the existence of cryptic species has been suggested within the taxon *N. viridula* (Ryan, 1996; Ryan *et al*., 1996; Jeraj & Walter, 1998). However, cross-mating studies on southern green stink bugs from geographically isolated populations have not provided sufficient evidence to resolve the status of geographically isolated populations (Jeraj & Walter, 1998; Virant-Doberlet, unpublished data).

To date, there have been no reports of DNA marker analyses of the southern green stink bug populations and there is only one other population genetic study based on random amplified polymorphic DNA (RAPD) markers published on insects from the family Pentatomidae (Sosa-Gomez *et al*., 2004). Only single nucleotide sequences of parts of the mitochondrial 16S rDNA, cytochrome oxidase subunit I (COI) and cytochrome b (cyt b) are available from a study in which *N. viridula* was used as an out-group in the phylogenetic studies of anthocorid bugs (Heteroptera: Anthocoridae) (Muraji *et al*., 2000a, b). The same three mtDNA gene fragments (16S rDNA, COI, cyt b) were analysed in the present study. In addition, we sequenced nuclear 28S rDNA fragment and performed RAPD analysis.

The main objective of this study was to examine genetic structure and differentiation of allopatric populations of *N. viridula* to provide insight into the native area and broad scale dispersal pattern and to check whether molecular data support the hypothesis that the taxon *N. viridula* comprises a complex of cryptic species.

Materials and methods

Insect material and DNA isolation

Southern green stink bugs were sampled from the following 11 countries: Slovenia, Italy, Greece, France, Madeira, Guadeloupe (Lesser Antilles), Galapagos, California, Brazil, Japan and Botswana (fig. 1). Insects were field collected between 1999 and 2003 and stored in 96% ethanol. To minimize the possibility of contamination by parasites and endosymbionts, DNA was extracted from dissected thoracic muscles with the Promega Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's protocol for isolation of genomic DNA from animal tissues (mouse tail).

Sequence analysis

The fragments of the 16S and 28S rDNA, COI and cyt b genes were amplified using previously published primers (table 1). In some individuals the COI region could not be amplified with the primers described, most probably due to mutations at the forward priming site. To generate at least a shorter product of COI (220 bp; sequence – 171 bp), the reverse primer was combined in polymerase chain reaction (PCR) with the internal primer (table 1) designed at the positions 147–169 of the COI sequences obtained in this study. PCR re-amplification was also performed for specimens from which weak PCR products were amplified. However, the majority of sequences generated by PCR re-amplification differed substantially from the 171 bp sequence obtained from the same specimen. It could be inferred that this sequence is a nuclear copy of mitochondrial COI gene

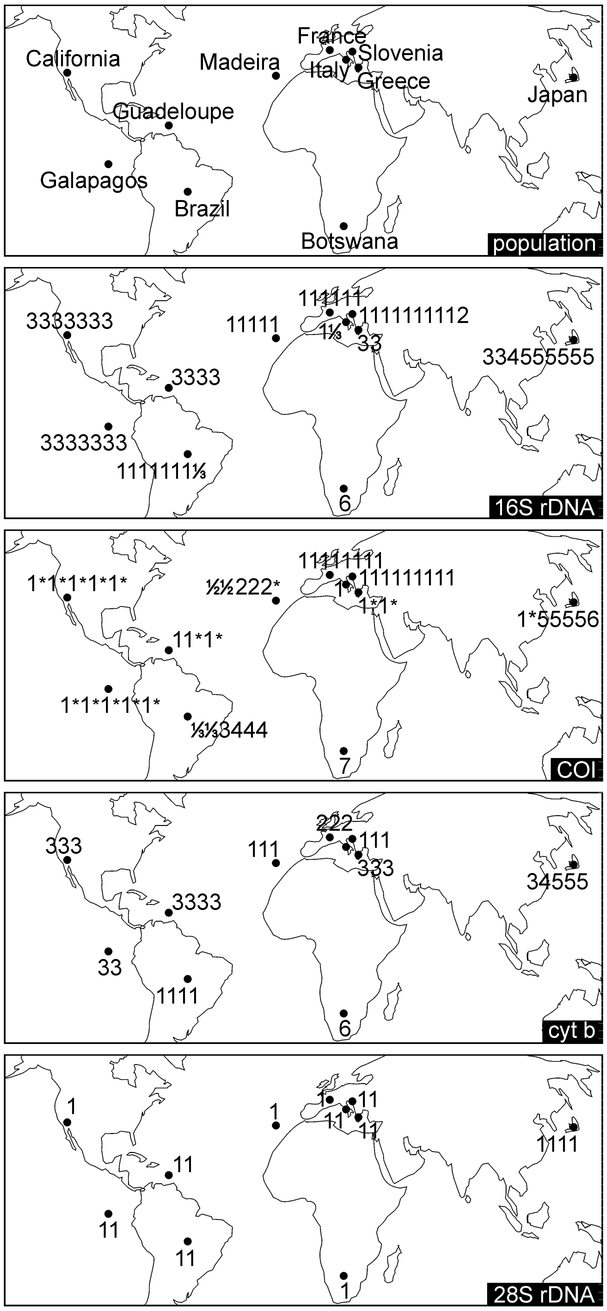


Fig. 1. Haplotype distribution at the 442-bp 16S rDNA, 348-bp COI, 411-bp cyt b and 401-bp 28S rDNA fragments in *Nezara viridula*. Distinct haplotypes were numbered and entered into the map for each specimen. * Indicates that only shorter COI fragments were sequenced; 1/3 indicates presence of two haplotypes within the same specimen (heteroplasmy).

fragment. For this reason, all such sequences were excluded from further analyses.

Amplification reactions were performed in a volume of 20 μl containing 5 pmol of each primer, 1× PCR buffer (Promega), 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Promega), and ~50 ng template DNA. PCR amplification was carried out on the GeneAmp PCR System 9700 (PE Applied Biosystems) using 5 min initial denaturation at 94°C, followed by 35 cycles of 45 s denaturation at 94°C, 45 s annealing at 60°C (16S, 28S) or 47°C (COI) or 51°C (shorter COI fragment, cyt b), 2 min extension at 72°C, and finalized by 7 min at 72°C. Purified PCR fragments were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) on the ABI PRISM 310 DNA Sequencer (PE Applied Biosystems).

Sequences were aligned using ClustalW (Higgins *et al.*, 1994). Distinct sequences were deposited in the GenBank under the Accession Nos: AY839153–AY839165 and AY839167–AY839180. DNA sequences (each fragment separately and the composite sequence) were analysed using Phylip program package ver. 3.62 (Felstenstein, 2004). Genetic distances were calculated using the Kimura two-parameter method (Kimura, 1980). A tree was constructed using the neighbour-joining (NJ) algorithm (Saitou & Nei, 1987) and visualized using TreeView program (Page, 1996). We also estimated the divergence times using the standard insect molecular clock of 2.3% pairwise divergence per million years (Brower, 1994), which is appropriate for recently diverged taxa (up to 4 million years) with low absolute amounts of sequence divergence. The congruence between the three sequence data sets (16S rDNA, COI and cyt b) was estimated with the incongruence length difference (ILD) test (Farris *et al.*, 1994) in PAUP* version 4.0b (Swofford, 2001) using a heuristic search (10 replicates) with tree bisection-reconnection (TBR) branch swapping to find the most parsimonious trees. The incongruence length difference test was done with 1000 replicates. Sequences of two other pentatomid bugs, *Piezodorus lituratus* (Fabricius) and *Rhaphigaster nebulosa* (Poda) were used as out-groups.

RAPD analysis

RAPD-PCR amplification was performed on genomic DNA extracts of 68 *N. viridula* individuals sampled from eight countries: Botswana (1), Brazil (10), California (10), France (9), Galapagos (10), Japan (10), Madeira (8) and Slovenia (10). The following 12 primers (Operon Technologies) were selected for the assay: OPD-02, OPD-03, OPD-07, OPG-03, OPI-07, OPI-16, OPO-15, OPQ-09, OPW-02, OPW-06, OPX-01 and OPX-11. Amplification reactions were performed in a volume of 20 μl containing 10 μM primer, 2.5 mM $MgCl_2$, 200 μM each dNTP (PCR Nucleotide Mix; Promega), 1 U AmpliTaq DNA polymerase, Stoffel Fragment (Applied Biosystems/Roche Molecular Systems), 1× buffer (10 mM Tris-HCl, 10 mM KCl, pH 8.3) and ~50 ng template DNA. Reactions were run on a GenAmp PCR System 9700 thermocycler (Perkin Elmer) programmed for: 5 min at 94°C, followed by 40 cycles of 94°C for 45 s, 36°C for 45 s and 72°C for 2 min; and a final extension step at 72°C for 5 min. Duplicate amplification reactions were performed on two template DNA samples per population for each primer to test the reproducibility of the banding patterns observed. A negative control without DNA template was included in each series of PCR amplifications to check for contamination. PCR products were separated electrophoretically in ethidium bromide stained 2% agarose-TBE gels.

Reproducible RAPD bands were scored as binary presence/absence data. A bootstrapped (1000 samples) phylogram was inferred from the individual pairwise-distance matrix based on Nei and Li's similarity coefficient

Table 1. Primers used for sequence analysis.

Locus	F primer (5′→ 3′) R primer (5′→ 3′)	Size (bp)	Reference
16S rDNA	CCGGTTTGAACTCAGATCATGT CGCCTGTTTAACAAAAACAT	560	Muraji & Tachikawa, 2000
COI	AGCAGGAATTTCTTCAATTTT CTGTAAATATGTGATGTGCTC	426	Muraji *et al.*, 2000b
Shorter COI	TCATTCTTGAYCCTTCAGGA CTGTAAATATGTGATGTGCTC	220	F primer – designed using the program Primer3 (Rozen & Skaletsky, 2000) R primer (Muraji *et al.*, 2000b)
cyt b	TAGGATATGTTTTACCTTGAGGACA TCCTCCTAATTTATTAGGAATTG	486	Muraji *et al.*, 2000a
28S rDNA	CCCGTCTTGAAACACGGACCAA CCACAGCGCCAGTTCTGCTTAC	551	Muraji & Tachikawa, 2000

(Nei & Li, 1979) using the neighbour-joining (NJ) algorithm (Saitou & Nei, 1987; Studier & Keppler, 1988). All calculations were carried out using the FreeTree 0.9.1.50 program (Hampl *et al.*, 2001). Principal components analysis (PCA) of the genetic distance matrix was conducted with GenAlEx 5 program (Peakall & Smouse, 2001). Bayesian analogue of F_{ST} (θ^B) was estimated via '*f* free model' in the Hickory program (Holsinger & Lewis, 2004). Non-informative priors (uniform on [0, 1]) were used for *f* and θ^B and the following sampler characteristics: burn-in of 50,000 iterations, sample run of 250,000 iterations and thin of 50. The sample from Botswana was excluded from θ^B estimation.

Results

16S rDNA

442 bp fragments of 16S rDNA were obtained and aligned for 61 southern green stink bugs. Sequence alignment revealed 29 polymorphic sites in six distinct haplotypes: Nv-16S-1 to Nv-16S-6 (table 2). The majority of specimens from European samples (France, Slovenia, Madeira and Italy) and all the specimens from Brazil shared the Nv-16S-1 haplotype, while all the specimens from Greece, California, Guadeloupe and Galapagos, and two specimens from Japan shared the Nv-16S-3 haplotype (fig. 1). Two haplotypes were detected only in Japan: Nv-16S-4 and Nv-16S-5; and a highly divergent (4.4% to 5.6%) Nv-16S-6 haplotype was identified in the specimen from Botswana.

Sequences with dual nucleotides at three positions were found in one specimen from Italy and in one from Brazil. As the positions and nucleotide variants exactly matched the combination of two common haplotypes (Nv-16S-1 and Nv-16S-3) it was assumed that both were present in these two specimens (mitochondrial heteroplasmy).

COI

Sequences of 348 bp COI fragment were obtained for 35 individuals; in other specimens the COI region could not be amplified with the primers previously described (table 1). Shorter, 171 bp fragments of the COI fragment were thus obtained (with the newly designed primer) for additional 16 specimens (see Materials and methods, sequence analysis).

Sequence alignment revealed 40 polymorphic sites in seven distinct haplotypes (Nv-COI-1 to Nv-COI-7) (table 2) including one amino acid change in the Botswanian specimen. At the COI fragment, polymorphisms were detected among specimens sharing the Nv-16S-1 haplotype; and inversely, many specimens with distinct, but closely related haplotypes, Nv-16S-1 to Nv-16S-4, shared the Nv-COI-1 haplotype (table 2). Nevertheless, the results at the COI fragment were generally in agreement with the results at the 16S rDNA fragment (fig. 1).

Several sequences with dual nucleotides were found (fig. 1) in which nucleotides exactly matched the combination of haplotypes common in the region concerned and were therefore considered as heteroplasmic. For two specimens from Madeira, it was assumed that they possessed haplotypes Nv-COI-1 and Nv-COI-2 and similarly, for two specimens from Brazil, it was assumed that they had haplotypes Nv-COI-1 and Nv-COI-3. Heteroplasmy was also detected in many European specimens, namely, sequences of European specimens showed one of the following combinations of nucleotides at positions 63 and 174: (63-G, 174-A), (63-A, 174-R) or (63-R, 174-R). All variations were at the third base of the codon and none affected the amino acid sequence. To avoid speculating on the haplotypes characteristic for the particular specimen, all the specimens from Europe were characterized with the same haplotype (Nv-COI-1; 63-G, 174-A) although the European samples most probably harboured more variation.

cyt b

411 bp cyt b fragments were sequenced for 31 southern green stink bugs. Alignment revealed 51 polymorphic sites in six distinct haplotypes (Nv-cytb-1 to Nv-cytb-6) including four amino acid changes, two in Nv-cytb-5 haplotype (identified in Japan) and two in Nv-cytb-6 haplotype (identified in Botswana). While the results were in general agreement with the results of the 16S rDNA and COI sequence analyses, the majority of French and Slovenian specimens that shared identical haplotypes at the 16S rDNA and COI fragments differed by two transversions at the cyt b (table 2).

28S rDNA

Sequences of the 401 bp 28S rDNA fragment were obtained and aligned for 20 southern green stink bugs (fig. 1). All sequences were identical; they differed however from the sequence of *Rhaphigaster nebulosa* by four nucleotides and from the sequence of *Piezodorus lituratus* by five nucleotides.

Table 2. Polymorphic sites among the composite haplotypes (16S rDNA, COI and cyt b) in *Nezara viridula*.

	NV-16S		NV-COI		NV-cytb	
Nv-1	1	tt--aatattataccaa--t-ttaaacaa	1	aatagtcgcaattccatagctcgatctcctccatttatgt	1	caatcataaacgtactatacttatttactttgtttgtcaagttccaaact
Nv-2	2	.................t...........	1	....	1	....
Nv-3	1	....	1	....	2	..t...................................t...........
Nv-4	1	....	2	........................c...............	1	....
Nv-5	1	....	3	................c.......c...............	1	....
Nv-6	3	.c.........cc........c.......	1	....	3	................t.................................
Nv-7	4	.c.........cc........c......t	1	....	4	......c.........t.................................
Nv-8	1	....	4	.......a..........a..........ct..c.c.ca.	1	....
Nv-9	5	..ca. .g....gat.c....c...t-..	5	..c..g.a...c......a.....c....ct....c.ca.	5	....t......a...a....................at..a...t.....
Nv-10	5	..ca. .g....gat.c....c...t-..	6	..c..g.at..c......a.....c....ct....c.ca.	5	....t......a...a....................at..a...t.....
Nv-11	6	acc.ttagcct..t.t..tat.att..g.	7	gc.ta.ta.gc.cttg.tatctag.tctt.ttc.a.t..c	6	tccctgctggtacct.tcgtccgcacgtcccacca.a..taccttcggtc

Positions 63 and 174, where dual nucleotides (A or G) were found in many European specimens, are underlined. See fig. 2 for lineage and subgroup designation of individual composite haplotypes.

Composite haplotype

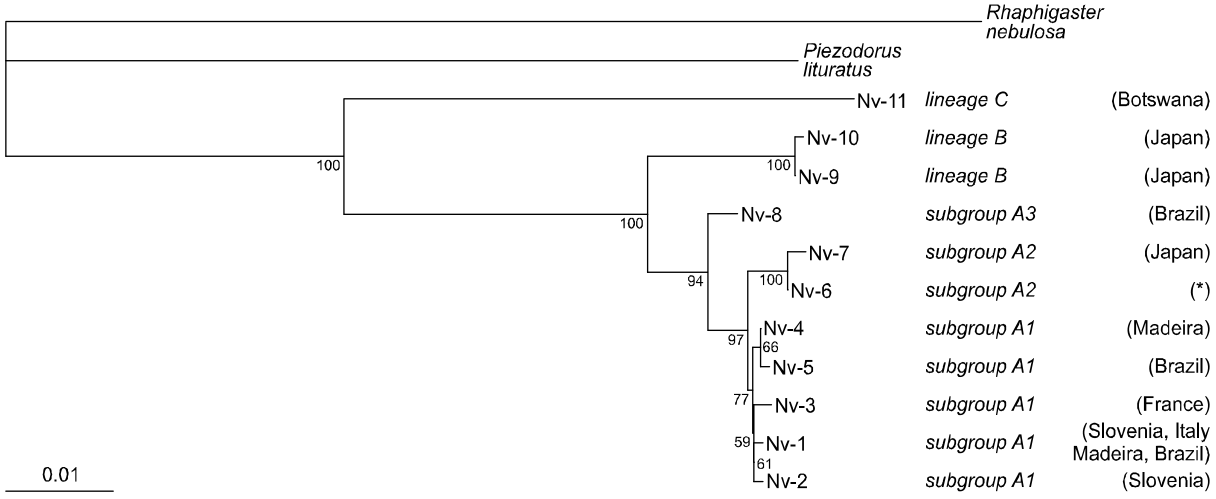
An incongruence length difference (ILD) test (Farris *et al.*, 1994) was performed in order to verify whether the three polymorphic mtDNA gene fragments (COI, 16S rDNA and cyt b) could be combined into the single sequence. The ILD test did not show significant incongruence between COI and cyt b ($P = 0.092$) and between 16S and cyt b ($P = 1$) fragments, but it revealed significant incongruence between COI and 16S rDNA ($P = 0.008$) and between all three data sets ($P = 0.002$). However, since the P values were above the critical value ($P = 0.01-0.001$) suggested by Cunningham (1997), sequence analyses were also performed with the combined data set.

Three polymorphic mtDNA gene fragments (COI, 16S rDNA and cyt b) generated a single 1201 bp sequence data set. Eleven haplotypes (Nv-1 to Nv-11) were identified, including the haplotype already deposited in the GenBank (AB021151 (COI), AB020514 (cyt b), AB020419 (16S rDNA); haplotype Nv-10 in this study). They clustered into three main lineages, designated as A, B and C (fig. 2). Lineage B was limited to Japan and lineage C to Botswana, while the specimens from lineage A were found in all of the remaining populations as well as in Japan. Lineage A was further divided into A1, A2 and A3 subgroups. Samples from France, Slovenia and Madeira shared highly similar haplotypes (> 99.7%) of the subgroup A1 (table 3). A single haplotype, Nv-6 of the subgroup A2, was present in the samples from Greece, California, Galapagos and Guadeloupe, whereas haplotypes from two subgroups (A1 and A2) were detected in Italy and Brazil. Haplotype Nv-8 (subgroup A3) was only found in three Brazilian specimens. It was identical to the haplotype Nv-1 at the 16S rDNA and cyt b, but resembled more closely the Nv-9 haplotype at the COI fragment (table 2).

RAPD data

A unique RAPD profile was generated for each individual by scoring 94 (3–16 per primer, 200–800 bp; fig. 3) polymorphic bands. Observed genetic differences among samples were largely due to frequency differences of amplified fragments rather than to complete absence or presence of particular bands: only three fragments present in the single African specimen were population-specific and another three fragments were confined to the remainder of the sample. The average Nei and Li's distance between the African representative and non-African specimens (0.57) was substantially greater than the average distance measured among the non-African individuals (0.34), and more than twice that measured among the European (Slovenia, France and Madeira) specimens (0.26). Within the Japanese sample the average distance between specimens belonging to divergent mtDNA lineages A2 (found also in European and American samples) and B was 0.39, whereas the average distance among lineage B specimens was merely 0.14 and the distance between the two lineage A2 specimens only 0.17.

The first three principal components in PCA together explained approximately 40% of variation contained in the distance matrix (fig. 4). The first component clearly separated the Japanese sample, the second component segregated the Galapagian sample and the third component strongly isolated the lone African specimen. Considering the first three PCs simultaneously, five groups could thus be



* Greece, Guadeloupe, Galapagos, California, Japan, Italy, Brazil

Fig. 2. Neighbour-joining tree showing relationship among composite haplotypes (16S rDNA, COI, cyt b) found in *Nezara viridula*. Sequences of *Piezodorus lituratus* and *Rhaphigaster nebulosa* (both from the Pentatomidae family) were used as out-groups. Bootstrap values are given at the nodes.

Table 3. Kimura two-parameter distance (%) between composite mtDNA haplotypes of *Nezara viridula*.

	Nv-1	Nv-2	Nv-3	Nv-4	Nv-5	Nv-6	Nv-7	Nv-8	Nv-9	Nv-10
Nv-2	0.2									
Nv-3	0.3	0.3								
Nv-4	0.2	0.2	0.3							
Nv-5	0.3	0.3	0.3	0.1						
Nv-6	0.7	0.7	0.8	0.7	0.8					
Nv-7	0.5	0.5	0.6	0.5	0.6	0.2				
Nv-8	0.8	0.8	0.8	0.8	0.8	1.3	1.1			
Nv-9	2.5	2.5	2.6	2.4	2.4	2.8	2.6	1.9		
Nv-10	2.6	2.6	2.7	2.4	2.5	2.9	2.7	2.0	0.1	
Nv-11	8.5	8.5	8.5	8.5	8.6	8.5	8.5	8.6	9.2	9.3

See fig. 2 for lineage and subgroup designation of individual composite haplotypes.

identified: African, two Japanese, Galapagian and combined European and mainland American group. Two Japanese specimens with mtDNA haplotypes of subgroup A2 formed a detached cluster near the aggregation of European and American specimens, suggesting the possibility of a hybrid origin. Within the latter, samples from Madeira and Brazil were closely associated while the Californian, French and Slovenian samples formed more distinct subgroups.

In the NJ tree (fig. 5) most nodes lacked significant bootstrap support, however, the overall pattern and arrangement of specimens was in basic agreement with the results of mtDNA analysis. Most individuals clustered together according to their sample affiliation, but only Galapagian (84%) and Brazilian (62%) sample-defined clades received substantial bootstrap support. A specimen from Madeira and one from France were included in the clade otherwise composed of the Slovenian sample. The specimen from Botswana was connected to the tree by the longest terminal branch but its placement was unresolved. The Japanese sample was partitioned into two distinct clades, each with significant bootstrap support (99 and 100%), matching the division of the sample observed in PCA and in mtDNA analysis: two specimens with mtDNA haplotypes of subgroup A2 were placed as a basal lineage to the clade composed of specimens from mtDNA lineage B. Dendrogram estimation performed without the two putative hybrid individuals from the Japanese sample (with mtDNA haplotypes of subgroup A2) resulted in major topological restructuring (not shown). The Botswanian specimen was shifted to a position of a basal lineage to the Japanese clade, so that three deep lineages emerged (Botswana, Japan and Europe with America) in concordance with the results obtained from the analysis of mtDNA data.

We adopted a Bayesian approach (Holsinger *et al.*, 2002; Holsinger & Wallace, 2004) to calculate θ^B, an estimate of F_{ST}. The point estimate (posterior mean) of θ^B across all samples was 0.3472 (SD 0.0237) and its 95% credible interval was [0.2991, 0.3917]. Pairwise comparisons resulted in diverse θ^B estimates ranging from 0.17 to 0.38 (table 4). Differentiation among the European (Slovenia, France and Madeira) samples was low (0.17–0.19) relative to that observed among the recently derived American (Brazil, California and Galapagos) samples (0.28–0.36). The latter was in the range of estimates obtained in comparisons of the American with

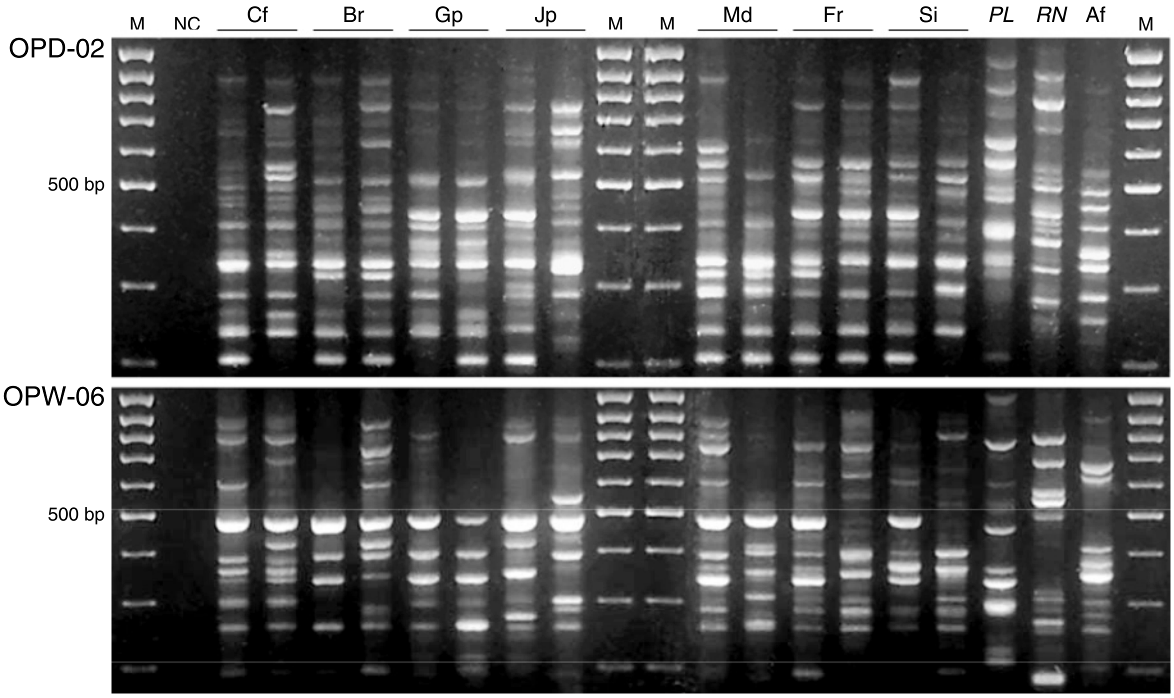


Fig. 3. Individual RAPD profiles obtained by primers OPD-02 and OPW-06 for specimens of *Nezara viridula* from California (Cf), Brazil (Br), Galapagos (Gp), Japan (Jp), Madeira (Md), Slovenia (Si) and Botswana (Af), for *Piezodorus lituratus* (PL) and for *Rhaphigaster nebulosa* (RN). M, molecular size marker (100 bp ladder); NC, negative control.

the European samples (0.22–0.38). The highest values for the pairwise estimates were observed in comparisons with either the Japanese or the Galapagian population. When the pairwise comparisons with the Japanese population were performed without two Japanese specimens with mtDNA haplotypes from subgroup A2, the values of θ^B increased on average by 0.1.

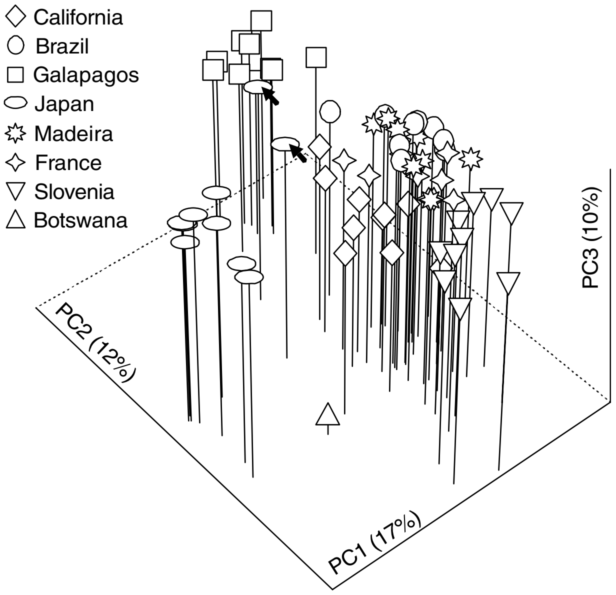


Fig. 4. Principal components (PC) plot based on RAPD data from eight allopatric samples of *Nezara viridula*. The percentage of total variation explained by each of the first three PCs is indicated next to their labels. Arrows point to the two Japanese specimens of inferred hybrid origin.

Discussion

Genetic structure and differentiation, origin and dispersal

Analyses of mitochondrial and nuclear variation both revealed the existence of three main genetic lineages in *N. viridula*. Differences between them were detected at each of the mtDNA fragments analysed. The deepest division was observed between the highly divergent African specimen and non-African samples. The extent of divergence in mtDNA haplotype and accumulation of three specific RAPD fragments demonstrate that the African population has undergone a long history separated from other populations examined. The basal phylogeographic position of the African specimen was also supported by sequences of both outgroup species (*P. lituratus* and *R. nebulosa*) which placed the root of the tree between the African haplotype (Nv-11) and all non-African haplotypes (fig. 2). The second split then occurred between the European (-American) and Japanese lineages. Biochemical markers suggest a similar conclusion, i.e. a closer relationship among European and American populations than between the Japanese and European/American populations (Meglič *et al.*, 2002; M. Virant-Doberlet *et al.*, unpublished data).

Assuming 2.3% pairwise sequence divergence per million years (Brower, 1994), the observed average 8.6% divergence between Botswana and Europe and 9.3% divergence between Botswana and Japan (table 3) suggest that the African and non-African gene pools have been separated for

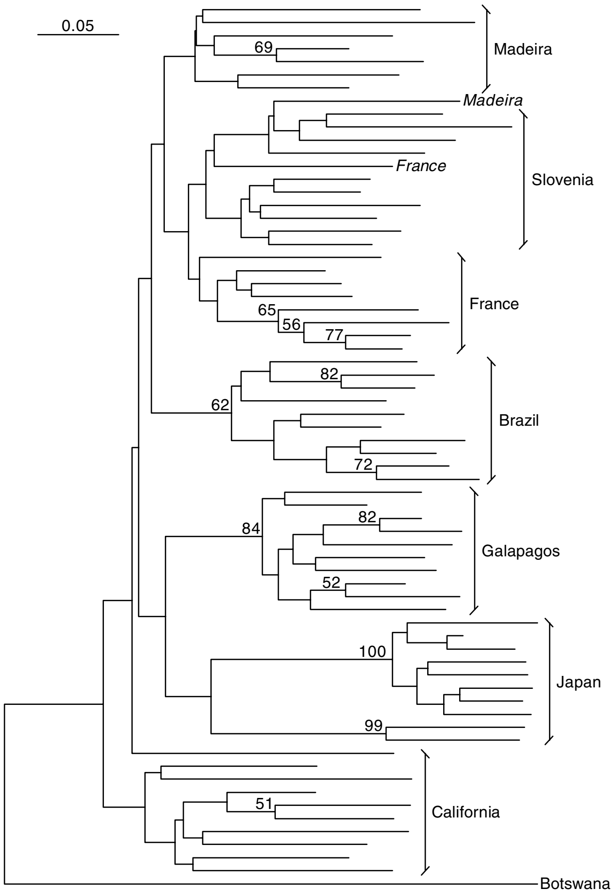


Fig. 5. Neighbour-joining tree based on individual RAPD profiles of *Nezara viridula* sampled from eight allopatric locations. Labelled in italics are the specimens from Madeira and France that clustered outside their samples' clades. Bootstrap values (1000 replications) above 50% are given at the nodes.

3.7–4 million years, and European and Far Eastern lineages for 1.2 million years. Both splits can be correlated with a vicariance event linked to climate and vegetation change. The first estimate places divergence in early to mid-Pliocene and the second in mid-Pleistocene. In early Pliocene, subtropical Africa was considerably warmer and more humid than at present. Starting in the mid-Pliocene, the continent began to experience periodically cooler, drier and seasonally contrasted climate (deMenocal, 2004). East African vegetation shifted from closed canopy to open grassland savannah and desert appeared in the north of the continent and in the Arabian peninsula, which probably separated the African and Eurasian populations of *N. viridula*. Contemporary populations inhabiting Europe and Japan most likely originated from postglacial expansion of refugial populations. During the last glacial maximum (18,000 years ago) there were three regions with potentially suitable habitat (tropical grassland and savannah) for *N. viridula* available in Central Africa, in the Indian subcontinent and in Southeast Asia (Adams & Faure, 1997). Colonization of Europe may have been facilitated by the Neolithic spread of agriculture from the Levant, which began around 10,000 years ago (Colledge *et al.*, 2004).

The presence in Japan of two deep mitochondrial lineages is strongly suggestive of a recent introduction of a European/American haplotype into the islands. The invasion of undetected genotypes into areas where a species has already been established has been termed a 'cryptic invasion' (e.g. Mun *et al.*, 2003). Traditional tree methods are constrained to describe relationships among taxa that are perfectly hierarchical and may, when applied at the intraspecific level (and using biparentally inherited markers), produce distorted representation of often reticulate evolution. Consequently, Smouse (1998) suggested using tree-free methods, such as different ordination methods, for phylogeographic analysis. Nevertheless, comparing the trees estimated with and without the suspect individuals may offer further evidence in support of their hybrid origin. McDade (1992, 1997) showed that hybrids between more distantly related parents are more likely to cause major restructuring of cladistic relationships and also that tree methods most often place hybrids as basal members of a lineage that includes one or both parents, whereas non-hierarchical ordination methods place hybrids close to one or the other of its parents. This was precisely the outcome of our analyses based on the RAPD data regarding the placement of the Japanese sample and the African specimen.

Although haplotypes from two subgroups were detected in European populations, diversity was still low, with sequence differences of $<0.8\%$ (table 3, fig. 2). RAPD data also demonstrate low level of differentiation among European populations. Interestingly, three American populations (California, Galapagos and Guadeloupe) showed the same genetic structure as the Greek sample: all samples from these four populations were fixed for the Nv-6 haplotype. Brazil was the most variable among American populations

Table 4. Point estimates (posterior means, below diagonal) and 95% credible intervals (above diagonal) of θ^B between pairs of *Nezara viridula* populations.

	Slovenia	France	Madeira	Brazil	California	Galapagos	Japan	Japan*
Slovenia		0.110–0.254	0.105–0.246	0.220–0.375	0.159–0.300	0.301–0.460	0.259–0.409	0.351–0.514
France	0.176		0.118–0.265	0.217–0.373	0.196–0.347	0.254–0.420	0.268–0.416	0.355–0.522
Madeira	0.170	0.187		0.170–0.321	0.126–0.267	0.216–0.377	0.239–0.390	0.354–0.518
Brazil	0.296	0.290	0.242		0.210–360	0.279–0.442	0.305–0.454	0.405–0.563
California	0.225	0.269	0.193	0.281		0.230–0.381	0.226–0.368	0.323–0.485
Galapagos	0.379	0.336	0.293	0.357	0.303		0.295–0.444	0.406–0.570
Japan	0.333	0.342	0.313	0.378	0.295	0.369		
Japan*	0.432	0.438	0.434	0.483	0.402	0.487		

*Two specimens with mtDNA haplotypes Nv-6 and Nv-7 omitted.

with four haplotypes, two of which were found only in Brazil. Haplotype Nv-5 is similar to the haplotype Nv-4 found in Madeira. Haplotype Nv-8 is similar to the Nv-1 haplotype at the 16S rDNA and cyt b, but resembles much more the haplotypes from group B at one segment of the COI fragment (table 2). This is probably not a consequence of recombination. Namely, all mutations were synonymous and they occurred in the hot spot region. RAPD data confirmed closer relatedness of Brazilian population to both populations from the Western Europe (Madeira and France) (fig. 4). Two distinct routes therefore appear to have lead to the colonization of the Americas. One leading from the eastern Mediterranean (Greece, Italy) to Central America and from there on to the eastern USA and the western coastal areas of South America (and possibly also to California); and another, which originated in the western Mediterranean (Iberian peninsula) and led to the eastern coastal areas of South America.

RAPD analysis revealed reduced variation within the Galapagian population, confirming a very recent colonization (up to a few decades) of the islands (Henry & Wilson, 2004). The amount of variation observed within the Californian population was greater, comparable to that observed within the European populations. *Nezara viridula* has first been recorded from this area in 1986, even though it has been long established in the rest of the USA (Jones, 1988). Therefore, the relatively high genetic diversity of Californian population is surprising and may be a consequence of polytopic immigration.

Cryptic species

Genetic distances among the European, American and Japanese populations were in the range found at the intraspecific level in insects (e.g. Brown *et al*., 1994). The maximal difference between composite mtDNA haplotypes was 2.9% and no population-specific fragments were found in RAPD analysis. Therefore we cannot, on the basis of genetic differentiation, consider these populations as distinct species.

The specimen from Botswana accumulated three specific RAPD fragments and numerous nucleotide substitutions (table 2); sequence differences between Nv-11 and other haplotypes ranged from 8.5 to 9.3%, which is more typical of interspecific comparisons in insects (Funk, 1999). Due to the highly distinct mtDNA haplotype and RAPD profile, the specimen from Botswana may represent a distinct species, but further sampling within Africa will be necessary to ascertain its taxonomic status.

Recent studies of mating behaviour in *N. viridula* from allopatric populations revealed substantial differences in chemical and vibrational signals (Aldrich *et al*., 1993; Virant-Doberlet & Čokl, 2004). Males of *N. viridula* show preferences for female vibrational signals from their own population (Miklas *et al*., 2001, 2003a, b) and recent evidence indicates that differences in composition of male pheromone blend may have functional significance for female behaviour (Miklas, 2002). Mating asymmetry has thus far only been recorded between Australian and Slovenian populations, which have significantly different vibrational signals (Ryan, 1996; Jeraj & Walter, 1998). Similar mating asymmetry was observed in Japan, between *N. viridula* and its sympatric congeneric *N. antennata* Scott, which also show complete post-mating isolation (Kon *et al*., 1994). Under laboratory conditions, individuals of *N. viridula* from allopatric populations usually readily interbreed, however, in the case of Brazilian and Slovenian populations, reproductive performance of interpopulation hybrids is greatly reduced (M. Virant-Doberlet, personal observation).

Taken together, these findings provide evidence of differentiation among existing allopatric populations. However, it is necessary to keep in mind that prezygotic reproductive isolation does not necessarily correlate with measurable genetic distance (Butlin & Tregenza, 1998; Walter, 2003) and that differences in mating signals do not necessarily have any role in maintaining reproductive isolation (Claridge & Morgan, 1993; Walter, 2003). In the majority of insects that are reproducing sexually, specific-mate recognition system (in the sense of Paterson, 1985), which is unique for each species and results in assortative mating and reproductive isolation, is probably the most useful character for understanding patterns of biodiversity in the field (Claridge *et al*., 1997a; Walter, 2003).

Heteroplasmy and nuclear integration of mtDNA fragments

The present study revealed many sequences with dual peaks at variable nucleotide positions. We interpreted them as heteroplasmy or as the integration of the mitochondrial COI gene fragment into the nuclear DNA. All assumptions were made in accordance with the findings on heteroplasmy and nuclear integrations in other species, including insects (Zhang & Hewitt, 1996; Frey & Frey, 2004). It seems that the level of heteroplasmy in *N. viridula* is quite high in all populations containing more than one haplotype. This is suggestive of the level of paternal leakage of mitochondrial DNA that may not be a negligible factor in the mtDNA-based phylogeographic studies in this species.

In further studies of COI gene in *N. viridula* special precautions regarding the primer selection will be needed also due to the COI pseudogene observed in many specimens from America, Europe and Japan, especially in samples showing haplotypes from lineage A2.

Conclusions

The present study has provided the first set of genetic data for *N. viridula* which should serve as a foundation and reference point for more in-depth studies. It lends further evidence in support of the previously proposed African origin of *N. viridula* and presents evidence of pre-Pleistocenic dispersal into Asia and postglacial (re)colonization of Eurasian and American continents. However, detailed inferences about the historical range, dispersal pattern and existence of cryptic species will require application of high resolution molecular markers in conjunction with more comprehensive sampling of populations, particularly in areas of the postulated Pleistocene refugia in Central Africa and South Asia.

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