DIVERSITY OF THE SOUTHERN GREEN STINK BUG *NEZARA VIRIDULA* (L.) (HETEROPTERA: PENTATOMIDAE)

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ABSTRACT

The southern green stink bug *Nezara viridula* (L.) (Heteroptera: Pentatomidae) is a global pest of considerable ecological, agricultural and economical interest. The ancestral home of this species is supposed to be Africa and/or Mediterranean and presumably it was spread worldwide during the last two centuries with human trade and agriculture. Bugs found today on different continents do not differ morphologically, however there are substantial differences in their mating behaviour. We used horizontal starch gel electrophoresis to determine the suitability of biochemical markers for assessment of genetic variation between geographically isolated populations of *N. viridula*. The initial survey of populations from Slovenia, France, French West Indies and Brazil resulted in the resolution of polymorphic banding patterns within the following enzyme systems: GPI, IDH, MDH, ME, MPI and PGM. Results indicate there are consistent differences among tested populations.

KEY WORDS: Nezara viridula, biochemical genetic variation, diversity

Manuscript received December 5, 2001.
Accepted for publication December 20, 2001.

JOURNAL Central European Agriculture ISSN 1332-9049

IZVLEČEK

Stenica vrste *Nezara viridula* (L.) (zelena smrdljivka) (Heteroptera: Pentatomidae) je kozmopolitska vrsta, ki je zaradi svoje zmožnosti preseljevanja, množičnega pojavljanja in velike polifagije v svetu eden ekološko in ekonomsko pomembnejših rastlinskih škodljivcev. Geografski izvor te vrste je še vedno nejasen, predvidevajo, da izvira iz Afrike ali/in mediteranske regije. Človek naj bi jo s trgovanjem in širjenjem kmetijstva šele v zadnjih 200 letih razširil po vsem svetu. V zadnjih letih se je pojavila potreba po razjasnitvi taksonomskega statusa geografsko ločenih populacij, ker je možno, da takson *N. viridula* vsebuje kompleks prikritih vrst dvojčic. Stenice iz geografsko ločenih populacij se med seboj ne razlikujejo, najnovejše raziskave ekologije in paritvenega vedenja zelene smrdljivke pa so pokazale, da obstajajo med populacijami na različnih kontinentih očitne razlike. S pomočjo vodoravne škrobne elektroforeze smo želeli preveriti uporabnost biokemičnih markerjev za določanje genske raznolikosti geografsko ločenih populacij stenice vrste *N. viridula*. Iz odraslih stenic smo izolirali oprsne mišice in jih takoj po izolaciji shranili na -70°C do začetka analize. Analizirali smo vzorce iz vsake žuželke posebej. Testirali smo populacije iz Slovenije, Francije, Zahodne Indije (Guadeluope) in Brazilije. Testirali smo več kombinacij pufrov in encimskih sistemov in glede na aktivnost encimov, polimorfizem alelov in ponovljivost izoencimskega vzorca, smo za analizo izbrali naslednjih šest encimskih sistemov: glukozefosfat izomeraza (GPI), izocitrat dehidrogenaza (IDH), malat dehidrogenaza (MDH), malični encim (ME), manozefosfat izomeraza (MPI) in fosfoglukomutaza (PGM) (Tabela 1). Rezultati kažejo, da s pomočjo encimske elektroforeze lahko ločimo med pripadniki geografsko ločenih populacij zelene smrdljivke in da obstajajo med populacijami značilne razlike (Slika 2; Tabeli 2, 3). Razlike med populacijami, ki smo jih dobili na osnovi biokemičnih markerjev, opravičujejo tudi uporabo drugih bolj specifičnih metod določanja genske raznolikosti (RAPD, mikrosatelitni markerji in analiza mitohondrijske DNA). Čeprav je težko definirati alopatrične populacije kot ločene vrste, lahko iz podatkov o genetski strukturi populacij na podlagi biokemičnih in molekularnih markerjev lahko sklepamo vsaj o poteku kolonizacije in preseljevanju populacij.

INTRODUCTION

Phytophagus stink bugs (Pentatomidae) are one of the largest families within Heteroptera with over 4000 described species. Among the several pentatomid pests of legume crops, the southern green stink bug *Nezara viridula* (Picture 1) is the most important.

This species feeds on plant species in more than 30 families, with preference for legumes and brassicas [17]. It feeds on all parts of a plant, including stems, leaf veins, growing shoots, immature fruits, seeds and even flowers. Because of its vagility, massive occurrence and extremely polyphagus feeding habits, *N. viridula* is one of important pests of agricultural crops in the world. The high damage caused on soybean, bean, rice, wheat, cotton and tomato fields as well as in macadamia and pecan orchards is globally economically important and it was predicted as cropping patterns of susceptible cultivated plants change, *N. viridula* will still continue to become increasingly important pest world-wide [13].

Nezara presently occurs throughout the tropical and subtropical regions all around the world and it is still spreading to new areas [13, 23]. The geographical origin of *N. viridula* is still unknown, however the most likely origin is eastern Africa and/or Mediterranean [11, 13]. During the last two centuries it was spread worldwide through human trade and agriculture.

Picture. 1: Southern green stink bug *Nezara viridula*. Couple mating on a bean.

Efficient pest control of *N. viridula* is nowadays achieved by the use of high doses of insecticides applied over wide areas. This species has long been a target for biological control, mainly through the introduction of parasitic wasps and flies [5, 13]. However, recently the degree of success in many parts of the world has been seriously questioned [6, 12].

The status of geographically isolated populations of *N. viridula* around the world requires clarification since the evidence suggests that this taxon might comprise a complex of cryptic (sibling) species. Green stink bugs found today on different continents do not differ morphologically. However, in recent years more detailed studies revealed substantial differences among populations on different continents. For example, analysis of male sex pheromone from different continents revealed geographical variations in the ratio between *cis* and *trans* isomere of bisabolene epoxide in the pheromone blend and several pheromone strains have been described [1]. Furthermore, species and sex specific vibrational signals (songs) of existing geographically isolated populations differ in their temporal characteristics [8] and these differences can be attributable to genetic factor [24].

The aim of the following study was to determine the suitability of biochemical markers for assessment of

genetic variation between geographically isolated populations of *N. viridula.*

MATERIALS AND METHODS

Horizontal starch gel elctrophoresis was used to assess the biochemical genetic variation within and among populations [14, 18]. Adult bugs from populations from following countries were used: Slovenia, France, French West Indies (Guadeloupe) and Brazil. Tested individuals from Slovenia were collected as adults near town Izola on Adriatic coast. Bugs from France and French West Indies were F1 generation of bugs collected in the wild and then randomly mated in the laboratory culture. Green stink bugs from Brazil originated from the colony maintained at CNPSo EMBRAPA in Londrina. Larvae and adults were fed on a diet of raw peanuts (*Arachis hypogaea*), sunflower seeds (*Helianthus annuus*) and growing green been plants and bean pods (*Phaseolus vulgaris*).

Thoracic muscles were dissected out and and immediately frozen and stored at -70 °C. Preliminary tests showed the activity of enzymes does not differ between fresh and frozen muscle tissue. Muscles from individual bugs were homogenized separately in 1.5 ml Eppendorf vials in addition of 250 µl of extraction buffer (50 mM TRIS-HCl, pH 7.5, 5% sucrose, 14 mM mercaptoethanol). Small amount of supernatant was absorbed on wicks and immediately loaded on a gel. On the same gel we always combined samples from two populations since this facilitated the comparison. On each gel an individual from French West Indies was used as a reference sample.

Gels were prepared using Sigma potato starch (S-4501) and buffers described by May [14], Clayton & Tretiak [7] and Selander and coworkers [21]. For initial screening a limited sample of 10 randomly chosen individuals from Slovenia and French West Indies were used in order to determine the enzyme/buffer combinations (Table 1).

Table 1: Activity of buffer/enzyme combinations screened in preliminary tests.

Enzyme system	Buffer system*				
	4	R	A	9	C
Adenylate kinase (AK, E.C. 2.7.4.3)	0	0	0	0	0
Alcohol dehydrogenase (ADH, E.C. 1.1.1.1)	0	0	0	0	0
Esterase (EST, E.C. 3.1.1.1)	+	+	+	+	+
6-phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.43)	+	0	++	++	+
Phosphoglucomutase (PGM, E.C. 2.7.5.1)	++	/	++	+++	++
Fructose -1,6-diphosphatase (FDP, E.C. 3.1.3.11)	0	0	+	0	+
Glycerate dehydrogenase (G2DH, E.C. 1.1.1.29)	0	0	0	0	0
Glucosephosphate dehydrogenase (GPI, E.C. 5.3.1.9)	+++	+++	+++	+++	+++
Isocitrate dehydrogenase (IDH, E.C. 1.1.1.42)	++	++	++	++	++
Malate dehydrogenase (MDH, E.C. 1.1.1.37)	++	+	++	++	++
Malic enzyme (ME, E.C. 1.1.1.40)	++	/	++	++	++
Mannosephosphate isomerase (MPI, E.C. 5.3.1.8)	++	+	+	+	++
Sorbitol dehydrogenase (SDH, E.C. 1.1.1.14)	0	0	0	0	0
Shikimate dehydrogenase (SKDH, E.C. 1.1.1.25)	0	0	0	0	0

Activity: 0 - no activity, + - low activity, ++ - strong actvity, +++ - very strong activity, / - not tested.; Buffer/enzyme combinations used in the study are underlined.

* Buffer systems described by May [14], Clayton & Tretiak [7], Selander and coworkers [21].

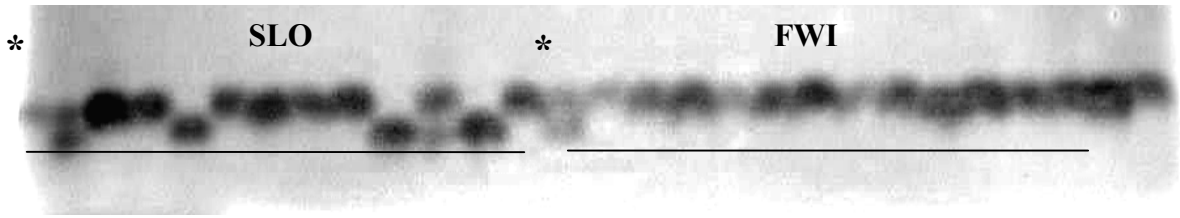
Histochemical staining followed standard techniques [2, 3, 15, 22]. The initial screening of enzyme/buffer combinations resulted in the resolution of the polymorphic banding patterns withing the following enzyme systems: glucosephosphate isomerase (GPI), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), mannosephosphate isomerase (MPI), phosphoglucomutase (PGM).

Each band was designated by its migration distance towards anode relative to the migration distance of the reference sample. The migration distance of the reference and of the most common band was assigned a value 100 and relative distances (in mm) of other bands were determined. Genetic nomenclature follows the one described by Richmond [19] and modified by Meglič & Staub [15]. When possible, we calculated frequencies of putative allels for each tested population.

RESULTS AND DISCUSSION

The screening of enzyme/buffer combinations resulted in the resolution of the polymorphic banding patterns withing the following enzyme systems: GPI, IDH, MDH, ME, MPI, PGM. MPI and PGM showed the most polymorphic pattern and allowed consistent scoring (Picture 2). Polymorphic banding patterns and frequency of putative alleles observed in six enzyme systems allowed for comparison among bugs from tested populations (Tables 2, 3).

Picture 2: Starch gel electrophoretic patterns of PGM for individual *N. viridula* bugs from Slovenia (SLO) and French West Indies (FWI).



* reference sample

The results of the present study indicate there is evidence of genetic differentiation among geographically isolated populations of *N. viridula* based on isozyme analysis. Isozyme banding patterns observed in six enzyme systems used in the present study and comparison of the putative allele frequencies indicate there are consistent differences among populations, although it is not yet possible to determine the taxonomic status of populations on different continets. However, since isozyme electrophoresis detects only a minority of amino acid substitutions [18], the observed differences warrant the development and use of more specific genetic markers like RAPD, microsatellite markers and analysis of mitochondrial DNA in order to clarify whether taxon *N. viridula* contains unrecognized sibling species.

Table 2: Putative allele frequencies for geographically isolated populations of *N. viridula*. SLO- Slovenia, FRA - France, FWI -French West Indies, BRA - Brazil. *N* - number of the bugs tested.

Enzyme	Allel	SLO	FRA	FWI	BRA
N		100	80	100	80
PGM	97	0.46	0.19	-	-
	100	0.54	0.81	1	1

Table 3: Isozyme banding patterns for GPI, IDH, MDH, ME, MPI for geographically isolated populations of *N. viridula*. SLO- Slovenia, FRA - France, FWI - French West Indies, BRA - Brazil.

Enzyme	Band	SLO	FRA	FWI	BRA
N		100	80	100	80
GPI	85	+	-	-	-
	97	+	+	-	+
	100	+	+	+	+
	105	+	+	+	+
IDH	100	+	+	+	+
	103	+	-	+	-
	117	-	+	-	-
	120	+	+	+	+
	124	+	+	+	+
MDH	97	-	-	-	+
	100	+	+	+	+
	103	-	-	-	+
	105	+	+	+	+
	107	-	-	-	+
	108	+	+	+	+
	110	-	+	-	+
ME	97	-	+	-	-
	100	+	+	+	+
	103	+	+	+	+
	105	+	-	+	+
MPI	90	-	+	-	-
	93	-	-	-	-
	95	-	+	+	+
	97	+	-	+	-
	99	+	-	-	-
	100	+	+	+	+
	103	+	-	-	-
	105	-	-	-	+

+: band present; -: band not detected, *N*: number of tested bugs

Biological control of this pest by imported parasitic wasps and flies in several cases proved less successful than anticipated [6, 12]. One of the possible explanations for the inexplicabale variability of the success of parasitoids in controlling *N. viridula* may be the existence of cryptic (sibling) species within the taxon *N. viridula*, since parasitoids may be extremely specific in their host relationship. Allozyme data at the moment tentatively support this hypothesis.

The ecological view of modern agricultural systems requires an understanding of the nature of the variation within and among populations of pests [4]. Despite the increasing application of molecular techniques, enzyme electrophoresis is still the most widely used technique and provides the most cost-efficient approach in agricultural entomology. There is an increasing need for simple and relatively cheap techniques for pest species identification and the analysis of intraspecific variability in agricultural entomology [16]. Phytophagus stink bugs (Pentatomidae) are important pests of many economically important crops, feeding mostly on seeds and immature fruits. The lack of data

on biochemical and genetic markers for members of this family is therefore surprising. Garrouste [10] described seven polymorphic enzyme systems in *Oebalus poecilus* from Guayana (CK1, CK2, EST, HK, MDH, PGI, PGM). Ryan [20] assessed different Australian opulations of *N. viridula* and he described nine polymorphic enzyme systems (ME, PGM, 6.PGD, IDH, AK, ESTa, ESTb, HK, ALD), but he didn't find any differences among tested populations.

Progress in applied entomology is frequently hampered by taxonomic problems [9]. Many groups of animal contain high levels of cryptic diversity, insects being one of the most spectacular examples. Usually sibling species are morphologically indistinguishable from one another and cannot be separated by traditional taxonomic methods. The presence of sibling species is important for biological control strategies since control programmes designed for one species can be ineffective against another. In recent years it increasingly became clear that understanding the genetic structure and ecological features of local populations can be important for effective pest management. In principle the biological control involves importation and release of biological control agents of the same geographic origin as the pest species. Practitioners often fail to appreciate the fact that pest species may have different geographic origins and consequently they may belong to different reproductive populations (even if they do have the same name taxonomically). *N. viridula* as a cosmopolitan species presumably originating from the same ancestral population is one of the most suitable models for studying the nature of variation within and among populations of pests. This species is important pest in Africa, Asia, Australia and America and in recent years becomes increasingly more important also in Mediterranean region and it is expected that due to the global warming it will spread towards North. Although it is difficult to define allopatric populations as distinct species, biochemical and molecular data can be used at least to deduce patterns of colonizations and migrations of populations. For example, in *Nezara*, in some enzyme systems (e.g. PGM) we observed the loss of one ellele in populations which were presumably displaced from one continent to the other.

ACKNOWLEDGEMENTS

We are grateful to Dr. A. R. Panizzi who kindly sent us eggs of southern green stink bugs from Brazil and Viktor Triler for technical help. Work was financed by Slovenian Ministry of Education, Science and Sport (grants: 0105-503, 0401-501, Z4-3273).

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