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ISOZYME VARIATION IN THE SOUTHERN GREEN STINK BUG *NEZARA VIRIDULA* (L.) (HETEROPTERA: PENTATOMIDAE)

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Abstract The southern green stink bug *Nezara viridula* (Heteroptera: Pentatomidae) is a cosmopolitan species with a still uncertain geographical origin. The ancestral home of this species is supposed to be Africa and/or the Mediterranean; presumably it was spread world-wide during the last two centuries by 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: glucosephosphate isomerase (GPI), isocitrate dehydrogenase (IDH) malate dehydrogenase (MDH), malic enzyme (ME), mannosephosphate isomerase (MPI) and phosphoglucomutase (PGM). Results indicate that in some enzyme systems such as PGM and MPI there are consistent differences among the tested populations.

KEY WORDS: *Nezara viridula*, Pentatomidae, isozymes

Izvleček RAZNOVRSTNOST IZOENCIMOV PRI STENICI VRSTE *NEZARA VIRIDULA* (L.) (HETEROPTERA: PENTATOMIDAE)

Stenica vrste *Nezara viridula* (Heteroptera: Pentatomidae) je kozmopolitska vrsta, katere geografski izvor je še vedno nejasen. Verjetno izvira iz Afrike in/ali sredozemske regije in se je s trgovanjem in širjenjem kmetijstva v zadnjih 250 letih

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razširila po vsem svetu. Stenice te vrste, ki jih najdemo na različnih celinah, se med seboj morfološko ne razlikujejo, vendar obstajajo v paritvenem vedenju stenic iz geografsko ločenih populacij očitne razlike. S pomočjo vodoravne škrobne elektroforeze smo želeli preveriti uporabnost biokemičnih markerjev za določanje genske raznolikosti geografsko ločenih populacij stenice vrste *N. viridula*. Testirali smo populacije iz Slovenije, Francije, Zahodne Indije (Guadeloupe) in Brazilije. Domnevne polimorfne alele smo našli pri naslednjih encimskih sistemih: glukozefosfatizomeraza (GPI), izocitratdehidrogenaza (IDH) malatdehidrogenaza (MDH), malični encim (ME), manozefosfatizomeraza (MPI) in fosfoglukomutaza (PGM). Rezultati kažejo, da za nekatere encimske sisteme (PGM in MPI) obstajajo med populacijami značilne razlike.

KLJUČNE BESEDE: *Nezara viridula*, Pentatomidae, izoencimi

Introduction

The southern green stink bug *Nezara viridula* (L.) (Heteroptera. Pentatomidae) is a cosmopolitan pest today present on all continents except the Arctic and Antarctica. Its geographical origin is still uncertain. Kiritani (1970) thought it was in SE Asia (Kiritani 1970); however, according to the most recent investigations, it was most likely to be eastern and/or northern Africa and/or the Mediterranean (Hokkanen, 1986, Jones, 1988). During the last two and half centuries it was spread world-wide by human trade and agriculture. The species was first described by Linnaeus in 1758 as *Cimex viridulus* from material collected in India and it was found as early as 1789 in America, in the West Indies. In 1916 *N. viridula* was first recorded in Australia, in 1944 in New Zealand, in 1961 on Hawaii and as late as in the beginning of eighties of the last century in California and Brazil (Hokkanen, 1986, Todd, 1989). Slovenia falls into the area of potential endemicity of this species. *Nezara* is very common along the Slovenian coast of the Adriatic sea and spreads up to the edge of Kraški Rob (Gogala & Gogala, 1989) and other parts influenced by the Mediterranean climate.

In recent years more detailed studies of mating behaviour revealed substantial differences among populations of *N. viridula* on different continents. The mating behaviour of *Nezara* has been divided into two phases: long-range mate location and short-range courtship that results in mating (Todd, 1989) and involves an interplay between chemical and vibrational communications. Long-range mate location is mediated by male sex pheromones. Analysis of sex pheromones of males from different continents revealed geographical variations in the ratio between *cis* and *trans* isomer of bisabolene epoxide in the blend. Aldrich and coworkers (1987) described several pheromone strains. During the premating period both males and females emit species and sex specific vibratory signals, called songs (Čokl et al., 1972). The vibratory songs of existing geographically isolated populations differ in their temporal

parameters, such as duration of the signal and their repetition frequency (Ryan et al., 1996, Čokl et al., 2000).

Current knowledge of mating behaviour and signals used in sexual communication of *N. viridula* and observed asymmetrical mating between individuals from certain populations (Jeraj & Walter, 1998) suggest this taxon might comprise a complex of cryptic (sibling) species. Southern green stink bugs from different continents do not differ morphologically and all gathered evidence on behaviour and hybridisation between geographically isolated populations still provide insufficient data to determine the status of populations on different continents. During the last thirty years biochemical and molecular markers proved to be a quick and successful method for identification of sibling species (Menken & Raijman, 1996, Loxdale & Lushai, 1998). It is therefore surprising that in the literature there is almost no information on markers (isozymes and DNA markers like RAPD, mtDNA, microsatellite markes) in studies of phytophagus stink bugs. Especially since the family Pentatomidae with over 4000 species is one of the largest within Heteroptera and many species are ecologically and economically important insect pests (Panizzi, 1997). To our knowledge, the only genetic studies were done as part of a Ph.D. of Garrouste (1995) and Ryan (1996).

The aim of the present study was to determine the suitability of biochemical markers for assessment of genetic variation between geographically isolated populations of *N. viridula* on different continents.

Materials and Methods

Experimental Animals

Horizontal starch gel electrophoresis was used to assess the biochemical genetic variation within and among populations (May, 1980; Pasteur, 1988). Adult southern green stink bugs from following countries were used: Slovenia, France, French West Indies (Guadeloupe), and Brazil. Tested individuals from Slovenia were collected as adults near the town of Izola on the Adriatic coast. Bugs from France and the French West Indies were F1 generation of bugs collected in the wild and then randomly mated in 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 bean plants and bean pods (*Phaseolus vulgaris*).

Sample Preparation

Thoracic muscles were dissected out, immediately frozen, and stored at -70°C. Preliminary tests showed that the enzyme activity did not differ between fresh and frozen tissue. Muscles from individual bugs were homogenized separately in 1.5 ml Eppendorf vials in 250 ml of extraction buffer (50 mM TRIS-HCl, pH 7.5, 5% sucrose, 14 mM mercaptoethanol). The samples were centrifuged for 2 min. at 10000

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cycles/s and stored in dried ice until run time. A small amount of supernatant was absorbed on wicks (0.5 x 1 cm, filter paper Schleider and Schull No. 470) and immediately loaded onto a gel. On the same gel, we always combined samples from 2 populations since this facilitated the comparison. On each gel an individual from Guadeloupe was used as the reference sample.

Preparing, Running Gels and Staining

Gels were prepared using Sigma potato starch (S-4501) and buffers described by May (1980), Clayton & Tretiak (1972) and Selander et al.(1971): R - pH 8.5 (gel), pH 8.1 (electrode); 4 - pH 6.7 (gel), pH 6.3 (electrode); A - pH 7.1 (gel), ph 7 (electrode); 9 - pH 8 (gel), pH 7.8 (electrode); C - pH 6.1 (gel), pH 6.1 (electrode). Gels (11% starch in buffer) was poured into 20 x 20 x 0.8 cm forms, covered with plastic foil and cooled overnight at room temperature. Before use, gels were cooled to 4°C in a refrigerator. Electrophoresis was performed at 4 - 5°C inside a large glass-door refrigerator. Running conditions at this temperature were 75 90 mA for 4 - 5 h. Wicks were removed after 30 min.

Gels were horizontaly sliced to 1 mm thick slices and each slice was stained for a different enzyme system. In the literature there are no data for enzyme activity and polymorphic enzyme systems for bugs from the family Pentatomidae. 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). Histochemical staining followed standard techniques described by Allendorf et al. (1977), Brewer (1970), Shaw & Prasad (1970) and Meglič & Staub (1996). All staining reactions were conducted in the dark at room temperature except for GPI, MPI, and PGM which were incubated at 37 °C.

Data Analysis

The initial 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.

Each band was designated by its migration distance towards the 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 of 100 and the relative distances in mm of other bands were determined. Genetic nomenclature follows the one described by Richmond (1972) and modified by Meglič & Staub (1996). When possible, we calculated the frequencies of putative alleles for each tested population.

Results

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. Polymorphic banding patterns and the frequency of putative alleles observed in six enzyme systems allowed the comparison among bugs from tested populations (Tables 2, 3).

Allelic interpretation of zymograms was possible only for PGM. This enzyme appeared to be monomeric (Fig. 1, Table 2). All tested individuals from the French West Indies and Brazil were homozygotes for one allele.

Allelic interpretation was not possible for MPI. This enzyme is apparently composed of more than one polypetide chain; however without hybrids we can not interpret the banding pattern. No conclusion can yet be drawn on the polymeric status of the remaining enzyme systems, GPI, IDH, MDH and ME.

Tab. 1: Activity of buffer/enzyme combinations screened in preliminary tests. 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 (1980), Clayton & Tretiak (1972) , Selander and coworkes (1971)

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)	++	/	++	<u>+++</u>	++
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)	+++	<u>+++</u>	+++	+++	+++
Isocitrate dehydrogenase (IDH, E.C. 1.1.1.42)	++	++	<u>++</u>	++	++
Malate dehydrogenase (MDH, E.C. 1.1.1.37)	++	+	<u>++</u>	++	++
Malic enzyme (ME, E.C. 1.1.1.40)	++	/	<u>++</u>	++	++
Mannosephosphate isomerase (MPI, E.C. 5.3.1.8)	<u>++</u>	+	+	+	++
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

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Fig. 1: Starch gel electrophoretic patterns of PGM for individual *N. viridula* bugs from Slovenia (SLO) and French West Indies (FWI). * reference sample

Tab. 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	Allele	SLO	FRA	FWI	BRA
N		*100*	*80*	*100*	*80*
PGM	97	0.46	0.19		
	100	0.54	0.81	1	1

Discussion

Despite the increasing application of molecular techniques, enzyme electrophoresis is still the most widely used technique in insect population genetics because it provides a rapid and cost-effective approach (Menken & Raijmann, 1996). Protein electrophoresis detects the changes in genomic DNA indirectly, through differences in a net charge on a protein which depends on the amino acid sequence. Mutations in a protein-coding gene are reflected in changed amino acid sequences and consequently in a different net charge of the protein. A major drawback of gel electrophoresis is its relative insensitivity since it is estimated that it detects only around one-third of amino acid substitutions (Pasteur et al., 1988). Nevertheless, we found differences in isozyme staining pattern between geographically isolated populations of *N. viridula* for all the chosen enzyme systems: GPI, IDH, MDH, ME, MPI, PGM.

Enzyme systems which, due to their activity, polymorphic staining pattern and consistent scoring, were suitable for analysis of genetic variability of *N. viridula* are among systems which were also polymophic in other insects. In Heteroptera there are relatively few studies of genetic variation within and between populations in comparison with the number of studies in Auchenorrhyncha. Most studies were done on water striders (Gerridae) (for example: Sperling & Spence, 1990: Preciosi & Fairbarn, 1992) and bugs from the genus *Triatoma* (Reduviidae) which in South

America are the vectors of Chagas disease (Dujardin & Tibayrenc, 1985; Garcia et al., 1995; Costa et al., 1997). Isozyme polymorphism was also studied in the family Lygaeidae (Sillén-Tulberg, 1983) and Nabidae (Grasela & Steiner, 1993). The results of the studies on two members of the family Pentatomidae were never published. In his PhD work on a pentatomide bug *Oebalus poecilus* from Guayana, Garrouste (1995) described seven polymorphic enzyme systems: CK1, CK2, EST, HK, MDH, PGI and PGM. Ryan (1996) in his PhD work assessed different Australian populations of *N. viridula*. He described nine polymorphic enzyme systems: ME, PGM, 6-PGD, IDH, AK, ESTa, ESTb, HK, ALDH, although he did not find any significant and consistent differences between populations.

The results of the present study are not directly comparable with his. He used whole insects, a different extraction buffer and electrophoresis on cellulose acetate plates, and the presentation of the results is incomplete, without figures and relative migration distances of isozymes. Starch gel electrophoresis is more sensitive than on cellulose acetate plates (Menken & Raijmann, 1996). Nevertheless, according to his results, we could assume that in the Australian populations the third allele for PGM is present.

The results of the present study indicate there is evidence of genetic differentiation among geographically isolated populations of *N. viridula*. However, it is not yet possible to determine the taxonomic status of populations on different continents. With hybridisation of bugs from particular populations and studying the inheritance of putative alleles and loci we could in the future determine more precisely the extent of genetic variation between populations of *N. viridula* on different continents. The observed differences also warrant the development and use of more specific genetic markers such as RAPD, RFLP, microsatellite markers, and analysis of mitochondrial DNA in order to clarify whether the taxon *N. viridula* consists of at least two unrecognized sibling species. Although it is difficult to define allopatric populations as distinct species, we could, with assessment of several other populations, use biochemical and molecular data 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 allele in populations which were presumably displaced from one continent to the other (French West Indies, Brazil), while it seems that in Australian populations the third allele is present, but not yet found in European populations.

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

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				+

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