Folia biologica (Kraków), vol. **56** (2008), No 3-4
doi:10.3409/fb.56_3-4.153-157

Polymorphism of the Genus *Isophya* (Orthoptera, Phaneropteridae, Barbitistinae) Revealed by RAPD

Beata GRZYWACZ and Elżbieta WARCHAŁOWSKA-ŚLIWA

Accepted April 22, 2008

GRZYWACZ B., WARCHAŁOWSKA-ŚLIWA E. 2008. Polymorphism of the genus *Isophya* (Orthoptera, Phaneropteridae, Barbitistinae) revealed by RAPD. Folia biol. (Kraków) **56**: 153-157.

The random amplified polymorphic DNA (RAPD-PCR) method was used to study the genetic polymorphism of 20 species of the genus *Isophya*. Each primer amplified a different set of DNA fragments, all oligonucleotides failed to generate any specific diagnostic band that could lead to the identification of *Isophya* species, and none of the amplified fragments were present in all species. RAPD markers detected a high level of polymorphism in all species. The data were in most cases not congruent with morphological subdivision to the species group and cytotaxonomic studies. The genetic lineages of *Isophya* seem to be in discordance with relationships proposed by systematists.

Key words: Orthoptera, Phaneropteridae, *Isophya*, genetic variability, RAPD-PCR.

Beata GRZYWACZ, Elżbieta WARCHAŁOWSKA-ŚLIWA, Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.
E-mail: grzywacz@isez.pan.krakow.pl
warchalowska@isez.pan.krakow.pl

The bush-cricket of the genus *Isophya* Brunner von Wattenwyl, 1882 is one of the largest genera of the subfamily Phaneropterinae in the Palearctic region (OTTE *et al*. 2004), with about 45 species occurring in Europe (HELLER *et al*. 1998). The intrageneric relationships are poorly understood and thus the groups of related species within the genus are still not defined (SEVGILI *et al*. 2006). Moreover, this genus includes a large number of morphologically similar species (BEY-BIENKO 1954; HARZ 1969). Most taxonomic studies of *Isophya* species are based on classical morphological features and identification is oftentimes extremely difficult (ORCI *et al*. 2005). However, species are grouped according to their morphology into the *Isophya costata*-group, *I. pavelli*-group, *I. schneideri*-group, *I. modesta*-group, *I. pirenea*-group, *I. kraussii*-group, and *I. strauberi*-group.

Chromosomal analysis of 25 species/subspecies of the genus *Isophya* demonstrated their karyotypic evolution. The most remarkable changes seen in the sex chromosomes appeared in 19 species as inversions of the ancient acrocentric X chromosome. This feature could probably help in resolving the taxonomy of the genus *Isophya.* Karyotypic differentiation has been less rapid in autosomes than in sex chromosomes. Interspecific autosomal differentiation has involved the distribution and quantity of C-heterochromatin and the number of NORs in this genus (WARCHAŁOWSKA-ŚLIWA *et al*. 2008).

RAPD (random amplified polymorphic DNA) technique is a quick, easy and inexpensive method for the analysis of differentiation of DNA and for screening genetic similarities and differences in whole genomes (HADRYS *et al*. 1992; WILLIAMS *et al*. 1990). This technique has been used extensively for detection of genetic diversity in a large number of insect-species (e.g. BLACK *et al.* 1992; HUNT & PAGE 1992; WILLIAMS *et al*. 1994; DOWDY & MC GAUGHEY 1996; PEARSON *et al*. 2002). In Orthoptera RAPD molecular markers have been used to study genetic differentiation between populations of the grasshopper *Sinipta dalmani* (SESARINI & REMIS 2007) and besides other techniques (allozymes, RFLP, DNA sequences), for the study of genetic diversity in the presumably morphologically cryptic species of *Laupala* crickets (PARSONS & SHAW 2001). To date, in the genus *Isophya* genetic variation in the whole genome was carried out only in the Hungarian species *I. kraussi* using the allozyme technique. The results indicated a high level of differentiation among geographically distant populations (PECSENYE *et al*. 2003).

The aim of the present study was the analysis of genetic variability of twenty species of *Isophya* (Orthoptera: Tettigonoidea) by random amplified polymorphic DNA (RAPD).

Material and Methods

The studied material consists of 21 species, including *Poecilimon affinis* as an outgroup. Adults and nymphs were collected in 2000-2007 from different areas, for example from Bulgaria, Greece, Macedonia, Poland and Russia. Individuals were conserved in 96% alcohol and frozen until DNA extraction. Species name, collection localities, and sample sizes are given in Table 1.

DNA was extracted from one leg of each of 46 adults using the QIAamp™ DNA Dneasy Tissue Kit (Qiagen™ Germany), according to the manufacturer's protocol. The purified DNA was stored at -20C until analysis. The DNA concentration was determined by comparison to a molecular weight marker XIV ™ (Roche ™, France) on 2% agarose gels followed by ethidium bromide staining and visualized in UV light.

The RAPD analysis was performed according to the method described by STOECK & SCHMIDT (1998), using Taq polymerase Fermentas (Lithuania). The RAPD reaction on DNA of the same individual was repeated three times. As a result of this procedure identical band patterns were obtained, indicating the reliability of the method. RAPD

Table 1

Data concerning the studied *Isophya* species

Species-group	Species	Collection localities and year	Number of individuals
I. pyrenaea	*Isophya altaica* Bey-Bienko, 1926	Russia, Altai Mts, Cherge, 2006	2
	I. obtusa Brunner von Wattenwyl, 1882	Bulgaria, Stara Planina Mts, Pleven Lodge, 24.05.2006	2
	I. camptoxypha (Fieber, 1854)	Poland, Polonina Carynska, 24.07.2007	2
I. pavelli	*I. Rectipennis* Brunner von Wattenwyl, 1882	Bulgaria, N Black Sea coast, Sofia University Botanical Garden, 20.06.2006	2
	I. rammei Peshev, 1981	Bulgaria, Strandzha Mts, Tsarnogorovo locality S of Malko Turnovo, 29.06.2006	2
I. costata	*I. modestior*	Serbia, Novi Sad vicinity, Frushka Gora Mt, 10.06.2007	2
I. kraussii	*I. pienensis* Maran, 1954	Poland, Bieszczady, Połonina Caryńska, 24.07.2007	2
I. modesta	*I. pravdini adamovici* Peshev, 1985	Bulgaria, E Stara Planina Mts, Karandila locality above Sliven, 17.06. 2006	3
	I. modesta longicaudata Ramme, 195	Bulgaria, N Black Sea coast, Bolata locality N of Kaliakra cape, 21.06.2006	2
	I. plevnensis Peshev, 1985	Bulgaria, C Danubian Plane, Iskar, 24.06.2006	3
	I. miksici Peshev, 1985	Bulgaria, W Stara Planina Mts, Vracanska Mt., near Pushevitsa Lodge, 24.06.2006	2
	I. andreevae Peshev, 1981	Bulgaria, Rila Mts, Eleshnitsa Lodge, 13.06 2006	3
	I. tosevski Pavicevic, 1983	Macedonia, Doiran Lake, 5.-6.05.2005	1
	I. rhodopensis Ramme, 1951	Bulgaria, Smoljan, 15.06. 2006	3
	I. petkovi Peshev, 1959	Bulgaria, E Rodopi Mts, Perpericon historic site near Murgovo vill., 15.06, 2006	3
	I. kisi Peshev, 1981	Bulgaria, N Pirin Mts., Bansko – Gotse Delchev Lodge, 14.06.2005	3
	I. bureschi Peshev, 1959	Bulgaria, N Pirin Mts.; 1.Bansko - Gotse Delchev Lodge, 14.06.2006; 2. N Pirin Mts, above Bansko, 8.08.2006	3
	I. leonorae Kaltenbach, 1965	Greece, Drama, Makedonia, near Elatia, 23.07.2004	1
I. straubei	*I. hospodar* (Saussure, 1898)	Bulgaria, E Rodopi Mts, Dolni Glavanak village, 25.04.2005	1
I. schneideri	*I. speciosa*	Bulgaria, 1. C Stara Planina Mts, Pleven Lodge, 23..05.2005; 2. Predbalkan Range, VelikoTurnovo, 29.05.2006	3
	Poecilimon affinis	Bulgaria, Rilsky Monastyr, 06.2006	1

Table 2

Primers used in studies of the genus *Isophya*

Name of primer	Sequence (5' to 3')
S4	GGACTGGAGT
S8	GTCCACACGG
S18	CCACAGCAGT
S20	GGACCCTTAC
S83	GAGCCCTCCA
S97	ACGACCGACA
S379	CACAGGCGGA
R06	GTAGCCATGG
R07	AACGTACGCG
R010	CTAGGTCTGC

PCR products were separated by electrophoresis on 2% agarose gels with ethidium bromide in TBE (Tris-borate-EDTA) at 50 V for 3 h. The specific bands were detected using fluorescent or silver tags and UV transillumination.

Ten 10-oligonucleotide primers, used in other studies of orthopteran species (JIANG & LU 2003; LI & ZHENG 2003), were initially screened using 1-3 samples of DNA from each species. Primers were synthesized in the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences. Their sequences are given in Table 2. The Bio1D++ software (Vilbert Lourmat, France) was used to calculate interspecific relationship on the basis of the similarity of DNA band patterns obtained with the RAPD method according to the NEI & LI (1979) similarity coefficients i.e. $S = 2N_{AB} / (N_A + N_B)$ where: N_{AB} is the number of shared bands in both individuals A and B; N_A and N_B are the number of bands in individual A and B. Dendrograms were constructed on the basis of the similarity values in the matrix using the unweighted pair group match average (UPGMA). The UPGMA algorithm is a phenetic distance method (NEI 1987; PAGE & HOLMES 1998; GRAUR & LI 2000) employing a sequential clustering algorithm. The results of DNA electrophoresis were entered into the database as 0 (absence of a band) and 1 (presence of a band).

Results and Discussion

This is the first report on the use of RAPD markers for the study of genetic variation in species of the genus *Isophya*. Ten different decamer primers possessing 60% G+C content were screened. Out of these 10 primers, four primers did not amplify at all or produced highly inconsistent amplification products and were excluded from further analyses. Six primers generated reproducible profiles, namely: S4, S8, S20, S83, S379 and R06. They produced a series of bands ranging from 4-12 in the molecular weight range of approximately 200-1400bp, having different intensities. All polymorphic bands were scored, the size of which ranged between 500 and 1000 bp. Figures 1a and 1b show examples of the reproducibility of RAPD profiles in the species revealed by primers S83 and R06. After generation of clear, reproducible bands, their patterns were analyzed in order to characterize DNA polymorphism in *Isophya*. The results show that band patters are different in each species.

The average homology coefficient according to NEI & LI (1979) for primer S4 was 0.29 minimum, for S8 – 0.22, for S20 – 0.25, for S83 – 0.29, for S379 – 0.40 and for R06 – 0.40. The maximum for each primer was 1.00. The dendrograms were generated from the RAPD data for each primer and are shown in Figures 2a,b. No distinct clusters can be identified.

The RAPD profile of each species was unique in terms of numbers and positions of bands. The species were tentatively grouped on the basis of band patterns and compared with groups of species

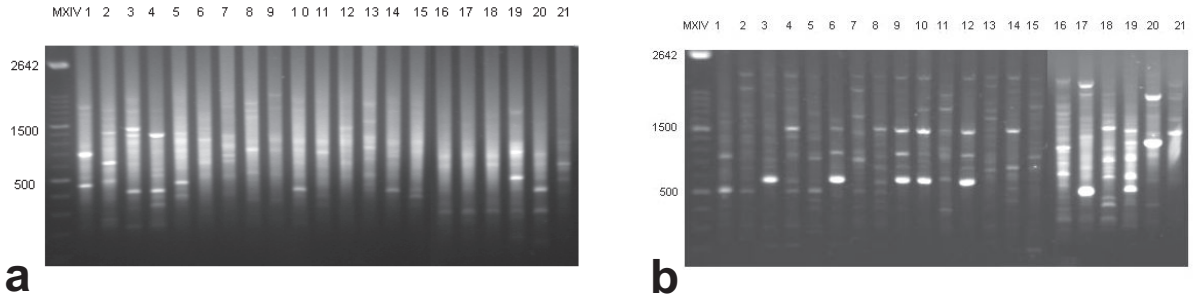
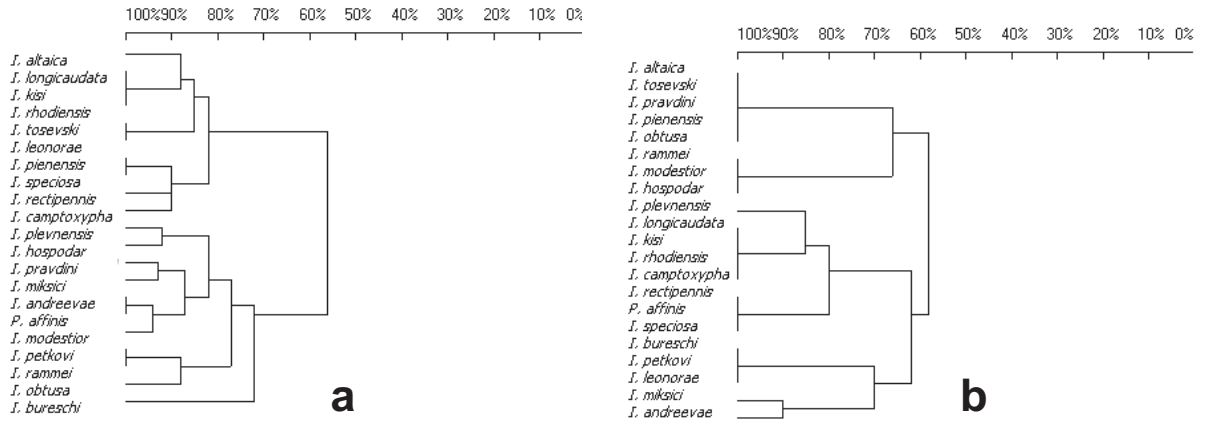


Fig 1. RAPD band pattern of *Isophya* species on an agarose gel revealed by primers (a) R06 and (b) S83. Lane 1 – molecular weight marker XIV (Roche, France); Lane 1 – *Isophya altaica,* 2 – *I. plevnensis,* 3 – *I. tosevski,* 4 – *I. pravdini,* 5 – *I. pienensis,* 6 – *I. leonorae,* 7 – *I. miksici,* 8 – *I. longicaudata,* 9 – *I. kisi,* 10 – *I. petkovi,* 11 – *I. rectipennis,* 12 – *I. rhodopensis,* 13 – *I. andreevae,* 14 – *I. obtusa,* 15 – *I. rammei,* 16 – *Poecilimon affinis,* 17 – *I. modestior,* 18 – *I. speciosa,* 19 – *I. camptoxypha,* 20 – *I. hospodar,* 21 – *I. bureschi.*



Figs 2. The diagrams of the cluster analysis of RAPD pattern similarity matrix of the studied *Isophya* species revealed by primers (a) R06 and (b) S83. The UPGMA method was used for analysis.

identified on the basis of morphology and cytotaxonomy (Table 3). Groups delineated on the basis of morphological traits do not completely coincide with divisions based on chromosome similarity, as in, for example, the *Isophya modesta*-group (WARCHAŁOWSKA-ŚLIWA *et al*. 2008)**.** It seems that the genetic lineages of *Isophya* are in discordance with relationships proposed by systematists.

Each primer amplified a different set of DNA fragments, all oligonucleotides failed to generate any diagnostic band that could lead to the identification of *Isophya* species. A high level of polymorphism among the 20 studied species was detected. None of the amplified fragments was present in all the species. It was very difficult to assess which primer was specific to a species or more frequent in some species than in others.

In tettigonids, genetic differentiation was examined using RAPD markers within *Ephippiger* species, the variability between presumed subspecies and song races of *Ephippiger ephippiger* was measured and compared with *E. terrestris* and *E. provincinalis*. The largest genetic distances within *E. ephippiger* populations were almost congruent with the variation in song pattern (RITCHIE *et al.* 1997). RAPD markers helped in detecting genetic variation also in other insect groups, for example in Lepidoptera they explained the genetic relationships between two sub-

Table 3

Groups of *Isophya* species according to different band patterns revealed by the applied primers

No	Species	Primers					
		S4	S8	S20	S83	S379	R06
1	*I. rhodopensis*	*I. rhodopensis*	*I. rhodopensis*	*I. rhodopensis*	*I. rhodopensis*	*I. rhodopensis*	*I. rhodopensis*
2	*I. kisi*	*I. kisi*		*I. kisi*	*I. kisi*	*I. kisi*	*I. kisi*
3	*I. modesta longicaudata*	*I. modesta longicaudata*			*I. modesta longicaudata*	*I. modesta longicaudata*	*I. modesta longicaudata*
4	*I. tosevski*	*I. tosevski*	*I. tosevski*		*I. tosevski*		
5	*I. pravdini*	*I. pravdini*	*I. pravdini*		*I. pravdini*	*I.pravdini*	
6	*I. plevnensis*		*I. plevnensis*				
7	*I. leonorae*		*I. leonorae*				
8	*I. bureschi*		*I. bureschi*	*I. bureschi*			
9	*I. andreevea*			*I. andreevea*		*I. andreevea*	
10	*I. petkovi*			*I. petkovi*			
11	*I. miksici*						
12		*I. camptoxypha*			*I. camptoxypha*		
13		*I. altaica*			*I. altaica*		
14		*I. pienensis*	*I. pienensis*		*I. pienensis*		
15		*I. obtusa*	*I. obtusa*		*I. obtusa*	*I. obtusa*	
16			*I. rammei*			*I. rammei*	
17						*I. hospodar*	
18						*I. speciosa*	
19						*I. modestior*	

species of the genus *Oleria.* Not only was a high level of polymorphism detected between the two studied subspecies, but also within each subspecies (GALLUSSER *et al*. 2004). The same technique was used as a genetic marker in species determination of cryptic species of *Anopheles* (Diptera) (WILKERSON *et al*. 1995; NADDAF DEZFOULI *et al*. 2002).

The RAPD method revealed the occurrence of high polymorphism among the *Isophya* species. However, there were no unique bands or band patterns specific for a particular species or group of species in the genus *Isophya*. No correlation exists between geographical origin of the studied species and similarity of their band patterns. The obtained data were in most cases incongruent with morphological subdivisions and cytotaxonomical data. Further studies are necessary for a better understanding of genetic relationships within the genus *Isophya.*

Acknowledgements

We thank Klaus-Gerhard HELLER from Magdeburg, Germany and Dragan CHOBANOV from the Department of Taxonomy, Faunistics and Zoogeography, Institute of Zoology, Bulgarian Academy of Sciences for collecting and identifying many of the specimens used in this study.

References

BEY-BIENKO G. Y. 1954. Tettigonioidea. Phaneropterinae. (In: Fauna of the USSR, Orthoptera, vol.2, Moscow, Leningrad): 1-376. (In Russian)

BLACK W. C., DUTEAU N. M., PUTERKA G. J., NECHOLS J. R., PETTORINI J. M. 1992. Use of the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids. Bull. Ent. Res. **82**: 151-159.

DOWDY A. K., MC GAUGHEY W. H. 1996. Using random amplified polymorphic DNA to differentiate strains of the Indianmeal moth (Lepidoptera: Pyralidae). Environ. Entomol. **25**: 396-400.

GALLUSER S., GUADAGNUOLO R., RAHIER M. 2004. Genetic (RAPD) diversity between *Oleria onega agarista* and *Oleria onega* ssp. (Ithomiinae, Nymphalidae, Lepidoptera) in north-eastern Peru. Genetica **121**: 65-74.

GRAUR D., LI W-H. 2000. Fundamentals of Molecular Evolution. Sinauer Associates Sunderland, MA.

HADRYS H., BALICK M., SCHIERWATER B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. **1**: 55-63

HARZ K. 1969. Die Orthopteran Europeas, The Orthoptera of Europe. I. Ser. Entomologica. W. Junk ed., The Hague. Pp.750.

HUNT G. J., PAGE J. R. 1992. Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey bee. Theor. Appl. Genet. **85**: 15-20.

JIANG G., LU G. 2003. Analysis of the genetic variations and phylogenetic relationship among three species of grasshopper genus *Hieroglyphus* (Orthoptera: Acridoidea: Catantopidae) using RAPD Markers. Entomotaxonomia **25**: 88-90.

LI K., ZHENG Z. 2003. RAPD study of the genus *Teleogryllus* (Orthoptera: Grylloidea: Gryllidae) from China. Entomotaxonomia, vol. 25, no.1 March: 9-13.

NADDAF DEZFOULI S. R., OSHAGHI M. A., VATANDOST H., DJAVADIAN E., TELMADEREI Z., ASSMAR M. 2002. Use of random amplified polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) and ITS2 PCR assays for differentiation of populations and putative sibiling species of *Anopheles fluviatilis* (Diptera: Culicidae) in Iran. Iranian J. Publ. Health **31**: 133-137.

NEI M., LI W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**: 5269-5273.

ORCI K. M., NAGY B., SZOVENYI G., RACZ I. A., VARGA Z. 2005. A comparative study on the song and morphology of *Isophya stysi* Cejchan, 1958 and *Isophya modestior* Brunner von Wattenwyl, 1882 (Orthoptera, Tettigoniidae). Zoologischer Anzeiger **244**: 31-42.

OTTE D., EADES D.C., NASKRECKI P. 2004. Orthoptera Species File Online (version 2.1.). Http://osf2x.orthoptera.org/osf2.2/OSF2X2Frameset.htm. 10 March 2004.

PAGE R. D. M., HOLMES E. C. 1998. Molecular Evolution. A Phylogenetic Approach. Blackwell Science Ltd. Oxford. Pp. 346

PEARSON C. V. M., ROGERS A. D., SHEADER M. 2002. The genetic structure of the rare lagoonal sea anemone, *Nematostella vectensis Sthephenson* (Cnidaria: Anthozoa) in the United Kingdom based on RAPD analysis. Mol. Ecol. **11**: 2285-2293.

PARSONS Y. M., SHAW K. L. 2001. Species boundaries and genetic diversity among Hawaiian crickets of the genus *Laupala* identified using Amplified Fragment Length Polymorphism. Mol. Ecol. **10**: 1765-1772.

PECSENYE K., VADKERIT E., VARGA Z. 2003. Temporal and spatial pattern of genetic differentiation in *Isophya kraussii* (Orhoptera: Tettigonoidea) in NE Hungary. Acta Zool. Acad. Scien. Hungaricae **49**: 167-178.

RITCHIE M. G., RACEY S. N., GLEASON J. M., WOLFF K. 1997. Variability of the bushcricket *Ephippiger ephippiger*: RAPDs and song races. Heredity **79**: 286-294.

SESARINI C., REMIS M. I. 2007. Molecular and morphometric variation in chromosomally differentiated populations of the grasshopper *Sinipta dalmani* (Orthoptera: Acrididae). Genetica doi: 10.1007/s10709-007-9213-y. Http://www.springerlink.com/content/100267/?Content+Status=Accepted&sort=p_Online Date&sortorder=desc&v=expanded&o=20

SEVGILI H., CIPLAK B., HELLER K-G., DEMIRSOY A. 2006. Morphology, bioacoustics and phylogeography of the *Isophya* major group (Orthoptera: Tetigoniidae: Phaneropterinae): A species complex occurring in Anatolia and Cyprus. Eur. J. Entomol. **103**: 657-675.

SIMMONS M. P., ZHANG L-B., WEBB C. T., MÜLLER K. 2007. A penalty of using anonymous dominant markers (AFLPs, ISSRs, and RAPDs) for phylogenetic inference. Mol. Phylogen. Evol. **42**: 528-542.

STOECK T., SCHMIDT H. J. 1998. Fast and accurate identification of European species of the *Paramecium aurelia* complex by RAPD-fingerprints. Microbiol. Ecology **35**: 311-317.

WARCHAŁOWSKA-ŚLIWA E., CHOBANOV D. P., GRZYWACZ B., MARYANSKA-NADACHOWSKA A. 2008. Taxonomy of the genus *Isophya* (Orthoptera, Phaneropteridae, Barbitistinae): comparison of karyological and morphological data. Folia biol. (Kraków) **56**: 227-241.

WILKERSON R. C., GAFFIGAN T. V., LIMA J. B. 1995. Identification of species related to *Anopheles* (*Nyssorhynchus) albitarsis* by Random Amplified Polymorphic DNA – Polymerase Chain Reaction (Diptera: Culicidae). Mem. Inst. Oswaldo Cruz, Rio de Janeiro **90**: 721-732.

WILLIAMS C. L., GOLDSON S. L., BAIRD D. B., BULLOCK D. W. 1994. Geographical origin of an introduced insect pest, *Listronotus bonariens* (Kuschel), determined by RAPD analysis. Heredity **72**: 412-419.

WILLIAMS J. G. K., KUBELIK A. R., LIVIAK K. J., RAFALSKI J. A., TINGEY S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. **18**: 6531-6535.