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Molecular phylogenetics of the beet webworm *Loxostege sticticalis* (L.) (Pyraloidea, Crambidae) as inferred from 18S ribosomal RNA gene sequence

Молекулярная филогения лугового мотылька *Loxostege sticticalis* (L.) (Pyraloidea, Crambidae), основанная на нуклеотидной последовательности гена 18S рибосомальной РНК

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Ключевые слова: луговой мотылёк, Crambidae, молекулярная филогения, 18S рРНК.

Abstract. The beet webworm *Loxostege sticticalis* (L.) is a dangerous agricultural pest with high migratory and outbreak capacities. Its taxonomy at the generic level has been changed several times, the two most acknowledged genera being *Loxostege* and *Pyrausta*. 18S ribosomal RNA gene fragments of *Loxostege sticticalis*, *Pyrausta despicata* and *P. sanguinalis* were sequenced and these were compared to the respective sequences of other members of the Crambidae family accessible at Genbank. As a result, *L. sticticalis* showed a 99.6 % sequence identity to *P. despicata* and *P. sanguinalis*, the latter two species being 100 % identical to each other at the aligned sequence region. *Ostrinia furnacalis* and *Chilo suppressalis* displayed sequence similarity of 99.1–99.5 % and occupied a basal position in the phylogeneric tree in relation to the three species under study. These data support the modern taxonomy of these taxa at the generic level, allowing one to set boundaries between members of the genera *Pyrausta* and *Loxostege* at molecular phylogenetic level.

Резюме. Луговой мотылёк *Loxostege sticticalis* (L.) — опасный сельскохозяйственный вредитель, характеризующийся способностью к миграциям на дальние расстояния и вспышками массового размножения. Таксономия этого вида на родовом уровне неоднократно менялась; в настоящее время он известен как представитель родов *Loxostege* или *Pyrausta*. В настоящей работе проведено секвенирование фрагмента гена 18S рибосомальной РНК *Loxostege sticticalis*, *Pyrausta despicata* and *P. sanguinalis*, и сравнение его с соответствующими последовательностями других представителей Crambidae, доступными в Генбанке. В результате, *L. sticticalis* продемонстрировал сходство на 99,6 % с *P. despicata* и *P. sanguinalis*, которые, в свою очередь, показали 100 % сходство друг с другом на данном участке элайнмента. *Ostrinia furnacalis* и *Chilo suppressalis* проявили сходство сиквенсов на 99,1–99,5 % и базальное положение на филогенетическом дереве по отношению к трём исследованным видам. Полученные данные соответствуют современным представлениям о родовой принадлежности данных таксонов, позволяя провести границы между представителями родов *Loxostege* и *Pyrausta* на молекулярно-филогенетическом уровне.

Introduction

Correct species identification and higher level systematics are important for all kinds of studies of living organisms, and understanding taxonomy in terms of phylogenetic relations, as inferred from Darwin studies and further elaborated by Haeckel [Mayr, Bock, 2002], is given high priority in modern biological research. For a long period, traditional systematics was based mainly upon morphological, developmental and ecological features [Michener et al., 1970], and in many taxa of both micro- and macroorganisms including Lepidoptera [Wilson, 2010], taxonomic considerations were problematic and doubtful due to numerous reasons, such as lack of proper morphological criteria. Introduction of nucleic acid sequence analysis has revolutionized biological studies in many ways, and one great implication is that for systematics [Schofield, Galvão, 2009; Budd et al., 2010; Nadler, de León, 2011; Chen et al., 2012]. Efforts of numerous research teams are aimed at phylogenetic reconstruction of major phylogenetic clades of Lepidoptera, providing new insights into its taxonomy, evolution and ecology [Hajibabaei et al., 2006; Linares et al., 2009; Hebert et al., 2010; Prado et al., 2011].

The beet webworm *Loxostege sticticalis* (L.) (Pyraloidea, Crambidae) is an important outbreak pest, causing serious damage to the crops such as soybean, sugar beet, alfalfa and sunflower in Eurasia, including Northern China and steppe zones of European and Asian parts of Russia [Chen et al., 2008; Frolov et al., 2008]. Initially described as *Pyralis sticticalis* (Linnaeus, 1761), it was later attributed by different authors to the genera of *Botys* Latreille, 1802, *Loxostege* Hübner, 1825, *Margaritia* Stephens, 1827 and *Phlyctaenodes* Hampson, 1899. The combination *Loxostege sticticalis* is the one most widely accepted by modern taxonomic summaries [Karsholt, Razowski, 1996; Heppner, 1998; Goater et al., 2005; de Jong, 2011]. Some species of the genus *Loxostege* were occasionally attributed to the genera of *Boreophila* Guenée, 1844 and *Cosmocreon* Warren, 1892, which were later synonymized with *Pyrausta* Schrank, 1802 by Hampson [1899] alongside with *Botys* and many other genera. This seems to be a possible source for another taxonomic combination, namely *Pyrausta sticticalis*. The latter name is exploited most often in Soviet and Russian literature [Tribel, 1976; Omelyuta, Nadvornaya, 1980; Frolov et al., 2008] and in Biological encyclopedia published in Moscow, *L. sticticalis* is referred to as an obsolete taxon name, substituted by *P. sticticalis* in 1950-ies [Gilyarov, 1989]. It can also be found in publications by European authors [Tunca, Kilinçer, 2009], as well as in some international internet resources such as FAO website (http://aims.fao.org/aos/agrovoc/c_30326). The authorship of this combination, however, could not be tracked using the available literature, public search engines and open databases. Some other examples of complicated historical records for taxonomic positions of Crambidae are *Pyrausta despicata* (Scopoli, 1763), synonym of *Pyralis cespitalis* Denis, Schiffermüller, 1775, and *Ostrinia furnacalis* (Guenée, 1854), synonym of *Botys furnacalis* Guenée, 1854, *Botys damoalis* Walker, 1859, *B. salentialis* Snellen, 1880, *Spilodes kodzukalis* Matsumura, 1897, *Pyrausta polygoni* Dyar, 1905 and *P. vastatrix* Schultze, 1908. It can be seen from the examples above that *Loxostege sticticalis*, *Pyrausta despicata* and *Ostrinia furnacalis* have been occasionally united by the frames of one genus. The phylogenetics of *Loxostege* species and its relation to *Pyrausta* and *Ostrinia* species remains unknown. In the present paper, we sequenced 18S ribosomal RNA (rRNA) gene fragments of *Loxostege sticticalis*, *Pyrausta despicata* and *P. sanguinalis*, and compared them with the respective sequences of other members of Crambidae family accessible at Genbank.

Materials and methods

Adult moths of *L. sticticalis*, *P. despicata* and *P. sanguinalis* were caught by net at the meadows in Slavyansk District, Krasnodar Territory, in June 2011 (Fig. 1). Dried moth cadavers were stored frozen for 18 months prior to analysis. For genomic DNA extraction, moth samples were homogenized with a plastic pestle in 100 µl lysis buffer, containing 2 % CTAB, 1.4 MNaCl, 100mM EDTA, 100mMTris-Cl (pH 8.0). After homogenization, 500 µl lysisbuffer with 0.2 % β-mercaptoethanol and 10 µl proteinase K (20 mg mL^{-1}) were added to the samples and incubated for 3 hrs at 65 °C. DNA was further extracted with phenol-chloroform, precipitated with isopropanol and washed with 70° ethanol [Sambrook et al., 1989]. Dried DNA pellets were re-suspended in 50 µl of deionized molecular grade water. PCR was run using a Bio-Rad C1000 DNA amplifier in 20 µl volume containing 10 µl DNA template, PCR buffer, 0.25 mMdNTPs; 1 U Taq-polymerase (Sileks, Russia), and 0.5 pMol each of forward and reverse primers (Evrogen, Russia). The primers used were rc18H and 18L [Wiegmann et al., 1999]. The PCR conditions consisted of an initial denaturation step (95 °C for 3 min), 30 amplification cycles (denaturation at 95 °C for 30 sec; annealing at 54 °C for 30 sec, elongation at 72 °C for 30–60 sec)

Fig. 1. Adults of *Loxostege sticticalis* (A), *Pyrausta despicata* (B) and *Pyrausta sanguinalis* (C). Photos by Yuri Tokarev.
Рис. 1. Имаго *Loxostege sticticalis* (A), *Pyrausta despicata* (B) и *Pyrausta sanguinalis* (C). Фотографии Юрия Токарева.

and a final extension step (72 °C for 5 min). The PCR products were separated by agarose gel electrophoresis. The gel regions containing specific products were excised, melted with 3M GITC buffer at 60 °C and mixed with an aliquot of Glass Milk. The Glass Milk particles were precipitated by centrifugation and subsequently washed with a washing buffer (10mM Tris, pH 7.5, 50mM NaCl, 25 mM EDTA and 50 % ethanol) and 96° ethanol. The amplicons were eluted by resuspending the Glass Milk pellet in molecular grade deionized water, checked for quantity by electrophoresis using a MassRulerLow Range DNA Ladder (Thermo Scientific) as a standard and sequenced with an Abi Prism automatic DNA sequencer. Newly obtained sequences, submitted to Genbank under the accession numbers of KC422443 (*L. sticticalis*), KC422444 (*P. despicata*) and KC422445 (*P. sanguinalis*) were compared to those available in NCBI using the built-in BLAST utility (www.ncbi.nlm.nih.gov/Blast.cgi). The alignment, 615 bp long, of the newly obtained sequences with those belonging to other members of Crambidae family (see Results and Discussion) was done automatically using CLUSTAL W algorithm, edited manually and used for sequence identity calculation in BioEdit v7.0.8.0 [Hall, 1999]. For a phylogenetic study, an additional sequence of *Galleria mellonella* (Pyraloidea, Pyralidae) (Genbank accession number AF286298) was added to the alignment as an outgroup. Regions containing insertion/deletion sites (gaps) were removed, leaving an alignment of 614bp length. Phylogenetic reconstruction was carried out with Bayesian Inference (BI) in MrBayes v3.1.2 using the standard GTR+I+G model [Ronquist, Huelsenbeck, 2003]. MrBayes was run for 1000,000 generations and every 1000th generation was sampled. The first 25 % of samples were discarded as burn-in, parameter values were summarized, and a consensus tree was constructed. Standard deviation of split frequencies, which estimates the precision of the clade probabilities, reached 0.005 after 1000,000 generations. The tree was drawn and rooted using the outgroup in TreeViewv1.6.6 [Page, 1996].

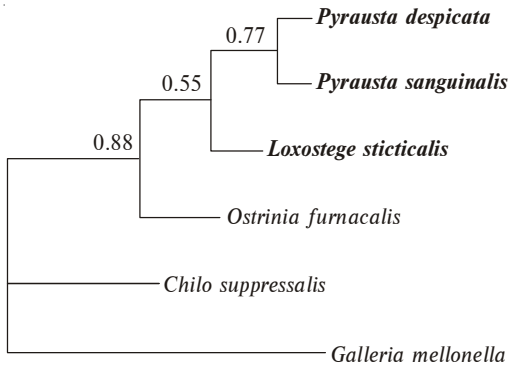

Fig. 2. Phylogram obtained using Bayesian Inference from an alignment of 18S ribosomal RNA gene sequences of the beet webworm *Loxostege sticticalis*, its closest relatives from the family Crambidae and *Galleria mellonella* used as an outgroup. Scale bar corresponds to 0.01 expected nucleotide substitution per site.

Рис. 2. Филограмма, полученная методом Байесовского заключения на основании элайнмента последовательностей гена 18S рибосомальной РНК лугового мотылька *Loxostege sticticalis*, его ближайших родственников из семейства Crambidae и *Galleria mellonella*, использованной в качестве внешней группы. Масштабная линейка соответствует 0,01 ожидаемой нуклеотидной замены на сайт.

Results and Discussion

Four specimens of each of the three species were assayed for molecular phylogenetic studies. The rRNA gene fragment was eagerly amplified and sequenced with rc18H:18L primers in all 16 samples tested. The obtained individual 18S rRNA gene sequences were identical within species, thus indicating absence of amplification and sequencing errors. BLAST analysis showed only two members of Crambidae family for which the homologous rRNA gene regions were accessible through Genbank, namely *Ostrinia furnacalis* (Accession number GU205787) and *Chilo suppressalis* (Accession number GQ265912). Sequence similarity between these taxa was comparatively high, as expected for members of a single family of Lepidoptera. *L. sticticalis* showed 99.6 % sequence identity to *P. despicata* and *P. sanguinalis*, 99.5 % to *O. furnacalis* and 99.1 % to *C. suppressalis*, *P. despicata* and *P. sanguinalis* were 100 % identical to each other at the aligned sequence region, with sequence similarity of 99.1 % to both *O. furnacalis* and *C. suppressalis*. The nucleotide differences between the examined sequences of *L. sticticalis* and *Pyrausta* sp. were restricted to the two transitions T/C at the positions 172 and 183 of the alignment. Two other transitions, T/C (pos. 194) and G/A (pos. 578), and one indel, -/A (pos. 193) were the source of sequence divergence between *L. sticticalis* and *O. furnacalis*. Further, *L. sticticalis* rRNA gene differentiation from that of *C. suppressalis* was contributed by as many as five nucleotide substitutions, including four transitions and one transversion. The observed dominance of transitions over transversions fairly corresponds to the observation that the former point mutations belong to the prevailing type of nucleotide substitutions in the evolution of genomes [Ebersberger et al., 2002; Xu et al., 2007; de Oliveira et al., 2008]. Notably, as many as 99 % of the first hundred 18S rRNA gene sequences found in Genbank using the megablast program as the most similar to those of *L. sticticalis* and *O. furnacalis* contained one to three insertion/deletion sites at positions 190 to 200 of the query subrange.

It is evident from the obtained results that the two species of the *Pyrausta* genus are the most closely related while genetic divergence of the beet webworm from both *Pyrausta* and *Ostrinia* is nearly the same. These relationships were displayed in the phylogram, as the two *Pyrausta* species formed a sister clade with adjoining *L. sticticalis* while *O. furnacalis* and *C. suppressalis* were in more basal positions, consequently

(Fig. 2). Genetic divergence of the beet webworm corresponds therefore to its taxonomic placing separate from both *Pyrausta* and *Ostrinia*, as suggested by the modern taxonomy. The 18S rRNA gene sequences in Lepidoptera are highly conservative yet suitable for taxa differentiation, at least at generic level, as highlighted by Wilson [2010] and exemplified in the present study.

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