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Identification of the nymphal stages of two European seed bugs, *L. equestris* and *L. simulans* (Hemiptera: Heteroptera: Lygaeidae), using DNA barcodes

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The genus *Lygaeus* Fabricius, 1794 includes more than fifty species, mainly distributed in the Old World regions, with 17 species known from the Palearctic (Péricart 2001; Wachmann *et al.* 2007), and only two from Central Europe, i.e., *L. equestris* (Linnaeus, 1758) and *L. simulans* Deckert, 1985 (Péricart 2001).

The Harlequin bug, *L. equestris*, was for a long time the only known Central European species of the genus, until Deckert (1985) discovered that it consisted of two morphologically sibling species, i.e., *L. equestris* and *L. simulans*, the latter described by him as new. Their adults can easily be separated on the basis of morphological characters (see: Deckert 1985; J.A. Lis 1987; Melber *et al.* 1991; B. Lis 1998; Péricart 1998; Wachmann *et al.* 2007; Rabitsch and Deckert 2008). However, identification of nymphal stages of both species appears to be more complicated, and usually has been a source of problems.

The last two (4th and 5th) instars are, for both species, the only nymphal stages, whose identification is possible using the morphological characters for the adults (see, e.g., Melber *et al.* 1991; B. Lis 1998; Rabitsch and Deckert 2008); but ascribing the earlier (1st to 3rd) stages to the proper species was always problematic. The only suggestion regarding the differences in nymphal characters was the nymphal body color (Melber *et al.* 1991; B. Lis 1998; Péricart 1998; Wachmann *et al.* 2007; Rabitsch and Deckert 2008): i.e., when a ground body color of the nymph was entirely red, then the nymph was assigned to *L. equestris*; and, on the contrary, when the nymphal stage bears any creamy-white stripes and patches it was regarded as a nymph of *L. simulans*.

Because, both bugs are often considered as the model species in different types of biological studies (e.g., Gschwentner & Tadler 2000; Exnerová *et al.* 2008; Evans 2011), it seems very important to find a reliable way for the proper identification of nymphal stages of both species.

Because no morphological characters can be regarded as crucial for proper separation of their 1st to 3rd instars, we have decided to provide the DNA barcodes for both species, as well as to verify (based on the sequences of the cytochrome oxidase I - COI) whether the nymphal color characteristics used hitherto for species separation are reliable and should still be used.

Material and methods. Adults and nymphal stages of *Lygaeus equestris* and *L. simulans* were collected directly in pure ethanol (in August, 2010), from two localities where they co-occurred, i.e., "Kamienna Góra" Mt. reserve in Upper Silesia, Poland (B. Lis 1994, 1998), and the botanical garden in Štramberk, Czech Republic (B. Lis 2012) (see: Table). Adult specimens were identified according to morphological characters; 1st to 5th instars were assigned to species only on the basis of their body color, as follows: all nymphal specimens entirely red—to *L. equestris;* nymphs with at least a trace of creamy-white stripes or patches—to *L. simulans*.

DNA extraction, purification, and amplification were performed at the Centre for Biodiversity Studies (Department of Biosystematics, Opole University, Poland) with use of standard techniques (see: Lis J.A. *et al.* 2011). The remains of the studied specimens were lodged in a deep freezer with the Opole University sample numbers assigned for each specimen (see: Table). *Spilostethus hospes* (Fabricius, 1794), a species of the closely related genus, was used as the out-group (GenBank accession number: AY627338) (Li *et al.* 2005). Sequence alignments were made with Clustal X (using default parameters) in MEGA 4.0.2 software (Tamura *et al.* 2007; Kumar *et al.* 2008). Following two primer sequences were used for the PCR amplification, i.e., Jerry (COI-1) [C1-J-2183], and COI-2 [TL2-N-3014] (Simon *et al.* 1994, 2006; Damgaard *et al.* 2000). Sequencing was conducted at the Health Care Centre GENOMED (Warsaw, Poland).

All analyses were performed using MEGA 4.0.2 software (Tamura et al. 2007, Kumar *et al.* 2008); maximum parsimony (MP), minimum evolution (ME), neighbor-joining (NJ), and UPGMA (unweighted pairgroup method with

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