# Taxonomy and DNA barcoding of *Stenostola ferrea* (Schrank, 1776) and *S. dubia* (Laicharting, 1784) (Coleoptera, Cerambycidae, Saperdini)

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The aim of this paper is to complete the studies of the genus *Stenostola* in Northern Europe. Together with previous published studies the characters presented form a better basis for separation of *S. ferrea* and *S. dubia* both as imagines and as larvae. The presented paper consists of three parts: Studies of the genitalia, morphological characters for identification of the larvae and DNA barcoding of both *Stenostola dubia* (Laicharting, 1784) and *S. ferrea* (Shrank, 1776). Although very similar, the male genitalia have good characters for species separation. The easiest characters to use are the parameres and tips of sclerite 2. The posterior ends of the elongated median sclerites of *S. ferrea* are irregularly curved or "zigzag" shaped, while the posterior ends of the median sclerites of *S. dubia* are straight. The genitalia characters presented are not previously published. No single morphological character alone is enough to identify the larvae to species. However, when the characters are used together the mature larvae are usually identifiable. The younger the larvae are, the more difficult they are to identify. Barcoding analysis of mitochondrial COI gene shows that *S. ferrea* and *S. dubia* are genetically different. The genetic distance between the species is 10.3 %. These results, in addition to previous findings, show that despite the morphological similarities, they are clearly two different species.

Key words: Cerambycidae, Saperdini, *Stenostola ferrea*, *Stenostola dubia*, DNA barcoding, larvae morphology, genital characters, taxonomy.

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## Introduction

The taxonomical and nomenclatorial confusion about *Stenostola ferrea* (Schrank, 1776) and *S. dubia* (Laicharting, 1784) was cleared up by Wallin et al. (2005). They did not study Norwegian specimens, thus leaving the question of the occurrence of *S. ferrea* in Norway unanswered. The status of the two species and the distribution in Norway was studied by Kvamme & Wallin (2011). They showed that all the old Norwegian specimens of *S. ferrea* were misidentified *S. dubia*. *S. ferrea* was documented occurring in the southernmost part of Norway based on newer specimens, and this species is considered to be a relict population in Norway. *S. dubia* is locally

common in the coastal areas of South Norway. A review of the biology showed that information on the species has been mixed up due to the taxonomical confusion. For general identification of imagines we refer to Wallin et al. (2005).

To our knowledge little has been published on the genus *Stenostola*. The aim of this paper is to study the genitalia in order to find new characters for identification of the species and to provide a basis for identification of larvae. Finally, we wanted to test the genetic differences of *S. dubia* and *S. ferrea* and to show the clustering of the two taxa using DNA barcoding sequences.

## Material and methods

### *Genitalia studies*

The following specimens of *S. ferrea* were included in this study: a total of 30 specimens (20 males and 10 females) from Czech Republic, Nizbor, 2002 (reared from *Tilia*), leg S. Snäll, 1 male from France, Hohnech, 1994-97, 1 male from Northern Italy, Castemonte, 1993-06-01, leg. P. Rapuzzi, 1 male from Denmark, Frejlev, Lolland, 1911-06-06, leg. E. Rosenberg. The following specimens of *S. dubia* were included: 1 male from Sweden, Vg., Mölndal, Gunnebo, 1962-06-14, leg. 1 female Vg., Göteborg, Kallebäck, 1955, leg. A. Törnvall, 20 specimens (15 males and 5 females) from Sweden, Tullgarn, 1995-04 (reared from *Tilia*), leg. H. Wallin, and approx. another 100 specimens of *S. dubia* from various Museum collections in Sweden also included in previous studies by Wallin et al. (2005) and Kvamme & Wallin (2011).

The sclerotized parts of the male genitalia (including the aedeagus, the sclerites inside the internal sac, the parameres and tergite VIII) were used to differentiate the two species of *Stenostola*. The spermatheca in *Stenostola* is well sclerotized, and was studied as a possible character. The method is described in detail by Wallin et al. (2009).

The spermathecae were embedded in 100% glycerol and photographed using a regular light microscope. The microscopic characters on the spermathecae are species-specific and have been used to identify new Palaearctic species of *Leiopus* (Sama 1985, 1994; Wallin et al. 2009), as well as other Lamiinae (Weigel & Skale 2009; Lin et al. 2009).

### *Identification of larvae*

The identification key to the *Stenostola* larvae (Table 1) is made by Petr Svacha (pers. com.) for this paper. Larvae of *Stenostola* used in the study are from central Europe. Classical comparative taxonomic methods were used. Additional information on larvae taxonomy and identification to genus level can be found in Svacha (2001).

### *DNA barcoding*

DNA was extracted from the following specimens:

***Stenostola ferrea.*** Norway, **VAY** Kristiansand: Nedre Timenes (EIS 2) (UTM 32V E447064 N6447010) 15.08.2008 (Leg. et det. F. Ødegaard) (Tissue sample in ethanol from a dried imago); Czech Republic, Bohemia Meridionalis, Hluboka upon Moldau (approx.10 km N of Ceske Budejovice) (49° 4'3.07"N, 14° 26'38.58"E), 17.05.2009 (Leg. Petr Svacha) (one larva found in a dead branch of *Tilia* and preserved in ethanol. A dead imago of *S. ferrea* was found in the same branch.).

***Stenostola dubia***. Norway, **VAY** Kristiansand: Nedre Timenes (EIS 2) (UTM 32V E447064 N6447010) 06.2011 (Leg. K. Berggren) (one imago preserved in ethanol); Norway, **VAY** Kristiansand; Nedre Timenes (EIS 2) (UTM 32V E447064 N6447010) 27.06.2011 (Leg. T. Kvamme) (one imago swept from a Tilia tree, preserved in ethanol. The locality is shown on Figure 1); Sweden, Bohuslän Province: Ärtan 1960 (Leg. G. Notini) (one pupa preserved in ethanol).

Tissue was preserved in absolute ethanol and extraction was performed on legs from imagines and tissue from the thorax of larvae and pupae. DNA extraction was carried out using the e.z.N.A tissue kit (Omega Biotek) according to the manufacturer's protocol. Tissue was dried at 50 ºC for approx. 30 minutes before

**FIGURE 1**. The forest edge with *Tilia cordata* at AAY Kristiansand: Nedre Timenes (EIS 2) (UTM 32V E447064 N6447010) where both *Stenostola dubia* (Laicharting, 1784) and *S. ferrea* (Schrank, 1776) have been collected (Photo Kai Berggren).

lysis, and the lysis reaction proceeded overnight. Amplification of a 657 base pair long cytochrome c oxidase I (COI) fragment from the COI 5` region was performed using the primers Lep-F1 (5′-ATTCAACCAATCATAAAGATAT-3′) and Lep-R1 (5′-′TAAACTTCTGGATGTCC-AAAAA-3′) (Hebert et al. 2004). The PCR profile used was as follows: 94 ºC for 2 min, 94 ºC for 30 sec, 45 ºC for 30 sec, 68 ºC for 45 sec, cycled 35 times and then held at 68 ºC for 10 min.

PCR reactions were performed in 10 µl reaction volumes. The final concentration of the various chemicals was: 1x buffer, 1.5mM $MgCl_2$, 0.8mM dNTPs, 0.5mM of forward and reverse primers, 3% DMSO, 1U/µl Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) and $dH_2O$ (Milli-Q) to make up the remaining reaction volume The samples were cleaned using ExoSAP-IT (United States Biochemical), diluted 10 times, and run with the following program: 37 ºC for 45 min and 80 ºC for 15 min. Cycle sequencing was performed in 10 µl reaction volume, by using BigDye v 3.1 cycle sequencing kit with 5x BigDye Terminator sequencing buffer (Applied Biosystems), and the program was run following the manufacturer's recommendations. Purification was performed using ABI's recommended ethanol/EDTA/sodium acetate precipitation. Electrophoresis and data analysis of samples were performed with an ABI 3130xl capillary electrophoresis instrument. The consensus sequences were aligned by ClustalW and manually edited in MEGA4 (Tamura et al. 2007).

Neighbour-joining analysis and genetic distances were calculated in MEGA4 (Tamura et al. 2007) with all sites included, the complete deletion option and Kimura 2-parameter (K2P) (Kimura 1980) was used as substitution model.

Bootstrap values were calculated using 10,000 iterations.

The GenBank accession numbers for the nucleotide sequences as provided by GenBank: *Stenostola dubia* Kristiansand-1 Accession nr: JQ907475; *Stenostola dubia* Kristiansand-2 Accession nr: JQ907474; *Stenostola ferrea* Czech Republic Accession nr: JQ907477; *Stenostola ferrea* Kristiansand Accession nr: JQ907476.

## Results

*Genitalia studies*

***Stenostola dubia***

*Aedeagus*: approx.1.6 mm long, slender, narrowed and weakly curved towards the relatively sharp, pointing apex, ventral ridge slightly wider than dorsal ridge at apex. The crescent-shaped sclerites at the proximal end of the basal segment consist of four separate parts (two rectangular and two crescent-shaped) (Figure 2), and three elongated median sclerites (approx. 1.8 mm long) inside the internal sac forming a slightly bent thread with the posterior end most narrowed and straight (Figure 3); surrounding intersegmental membrane with very fine, speckled microreticulation. *Tegmen*: approx. 1.6 mm long, parameres slender and flattened dorsoventrally, well separated medially along inner margin and towards apex forming a "U-shaped" cavity. Apex evenly rounded along entire posterior margin, with fringes of relatively long, yellowish hairs well concentrated at edge of apex. No microreticulation on parameres. Base of tegmen, at lower part of median lobe, extended and weakly curved dorsoventrally on middle. *Tergite VIII*: approx. 0.4 mm long, with fine, brownish pigmentation, rounded at the posterior margin, and covered with short, very fine yellowish hairs distally towards the posterior margin. Surface has weak microreticulation medially. *Spermatheca*: approx.0.12 mm wide, round and brownish (Figure 4).

***Stenostola ferrea***

*Aedeagus*: approx. 1.5 mm long, slender, narrowed and weakly curved towards the very sharp, pointing apex, dorsal ridge as wide as ventral ridge. The crescent-shaped sclerites at the proximal end of the basal segment consist of four separate parts (two rectangular and two crescent-shaped) (Figure 2), and three elongated median sclerites (approx. 1.8 mm long) inside the internal sac forming a slightly bent thread with the posterior end most narrow and not straight: forming an irregular curved or "zigzag" shape (Figure 3); surrounding intersegmental membrane with very fine, speckled microreticulation. *Tegmen*: approx.1.6 mm long, parameres slender and flattened dorsoventrally, less separated medially along inner margin and towards apex forming a "V-shaped" cavity. Apex evenly rounded along entire posterior margin, with fringes of relatively long, yellowish hairs well concentrated at edge of apex. No microreticulation on parameres. Base of tegmen, at lower part of median lobe, extended and weakly curved dorsoventrally on middle. *Tergite VIII*: approx. 0.5 mm long, with fine, brownish pigmentation, rounded at the posterior margin with a concave or notched part medially, and covered with short, very fine yellowish hairs distally towards the posterior margin. Surface has weak micro-reticulation medially. *Spermatheca*: approx. 0.12 mm wide, round and brownish (Figure 4).

## Identification of larvae

Table 1 and figures 5-11 makes it possible to identify larvae of *S. ferrea* and *S. dubia*.

## DNA results

Four out of five extracted samples were successfully DNA barcoded. Two attempts to extract DNA from different parts of the Swedish pupa of *S. dubia* failed, it is possible a different method is necessary for pupae. The results from the neighbour-joining analysis show that *S. ferre*a and *S. dubia* cluster into two distinct groups (Figure 12). The genetic distance between *S. ferrea* and *S. dubia* COI-haplotypes is 10.3 %.

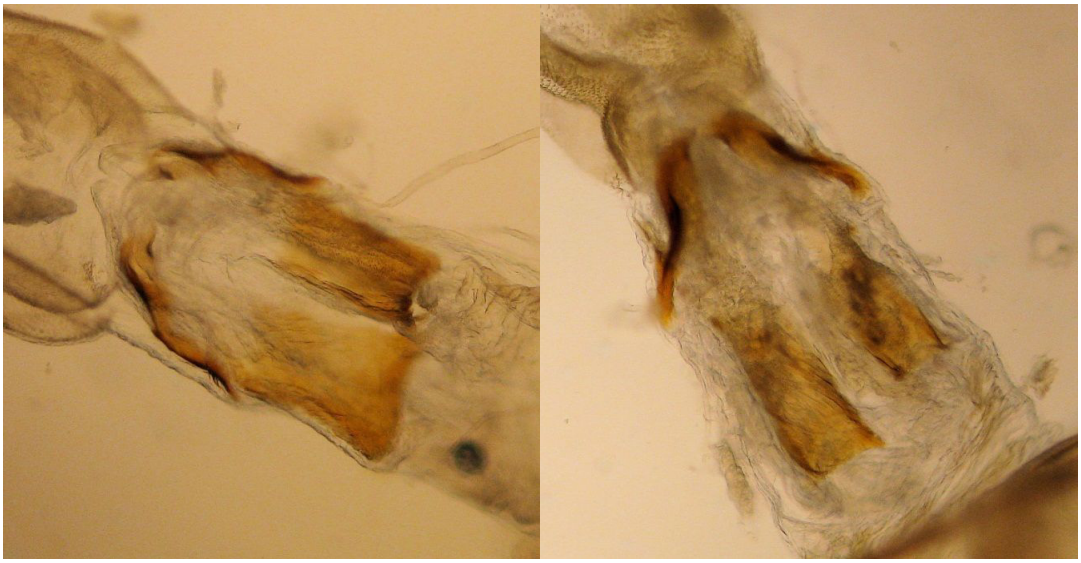


**FIGURE 2**. The crescent-shaped sclerites at the proximal end of the basal segment of *Stenostola dubia* (Laicharting, 1784) (left) and *S. ferrea* (Schrank, 1776) (right) (Photo H. Wallin).

**FIGURE 3**. The elongated median sclerites inside the internal sac of *Stenostola dubia* (Laicharting, 1784) (above) and *S. ferrea* (Schrank, 1776) (below) (Photo H. Wallin).

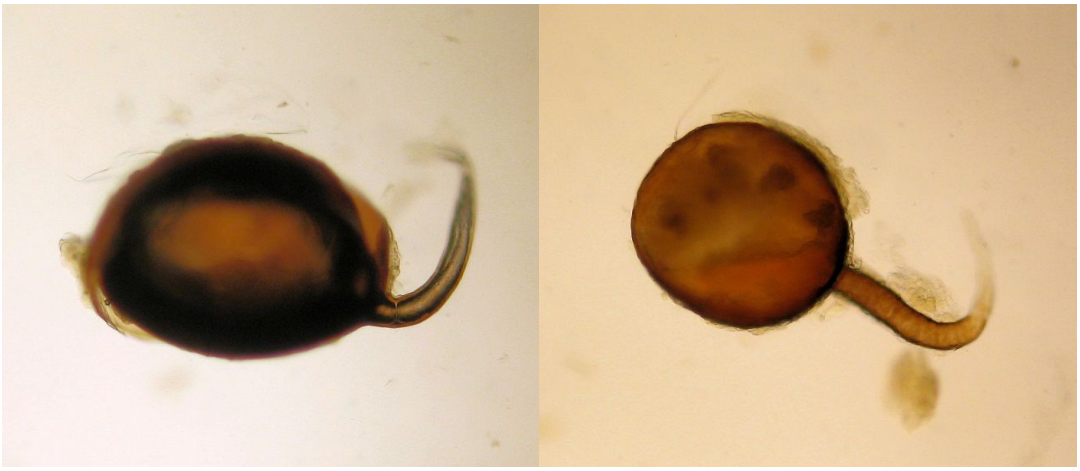


**FIGURE 4**. The crescent-shaped sclerites at the proximal end of the basal segment of *Stenostola dubia* (Laicharting, 1784) (left) and *S. ferrea* (Schrank, 1776) (right) (Photo H. Wallin).

**TABLE 1**. The morphological characters, which can be used to separate larvae of *Stenostola ferrea* (Schrank, 1776) and *Stenostola dubia* (Laicharting, 1784)

| ***Stenostola ferrea* (Schrank, 1776)** | ***Stenostola dubia* (Laicharting, 1784)** |
|---|---|
| *Morphological characters* | |
| Anterior sclerotized cranial margin on average paler, yellow-brown to ferruginous, less distinctly microsculptured and shinier. | Anterior cranium often strongly sclerotized, ferruginous to brown-black. Some areas (anterior half of ventral cranial sclerite (Figure 5) and adjacent parts of pleurostoma, anterior frontal margin) usually very distinctly microgranulate and usually matt. |
| Ventral sclerite almost always with at least one pair of distinct setae at lateral gular margin (Figure 6), often with additional setae. | Setae on ventral sclerite at gular margins usually absent (Figure 5) or very short (or present only on one side). Other setae are microscopic or absent. |
| Spines asperities on thoracic and abdominal terga and sterna are on average more numerous and finer (Figure 11); spines on prothoracic coxosternal fold as large as or even smaller than those on mesosternum (Figure 9), laterally usually arranged in more than one row, spines at middle not flattened; transverse furrows on ambulatory ampullae (Figure 7) often lined by two more or less distinct rows of spines from one or both sides (i.e. usually 6–8 rows in middle of dorsal ampullae (Figure 7) and 3–4 in middle of ventral ampullae). | Spines asperities on average sparser and coarser; spines on prothoracic coxosternal fold as large as or slightly larger than those on mesosternum (Figure 10), often arranged in a single (sometimes incomplete) row. Several medial asperities acquire a form of flattened transversely carinate sclerotized grains; transverse furrows of ambulatory ampullae (Figure 8) often lined by only one distinct row of spines from each side, and seldom by two (i.e. usually 4–6 transverse rows in middle of dorsal ampullae (Figure 8) and 2–3 in middle of ventral ampullae). |
| *Biology of the species* | |
| In dry branches of *Tilia* spp. Larvae are often numerous and many larvae can be found in one branch. | In dry branches of various trees, often in *Tilia* spp., but larvae can also be found on *Alnus*, *Ulmus*, *Quercus*, *Euonymus* and other species. The species is polyphagous. Larvae appear more sparsely in the substrate. |
| The host tree choice is discussed in detail by Kvamme & Wallin (2011). | |

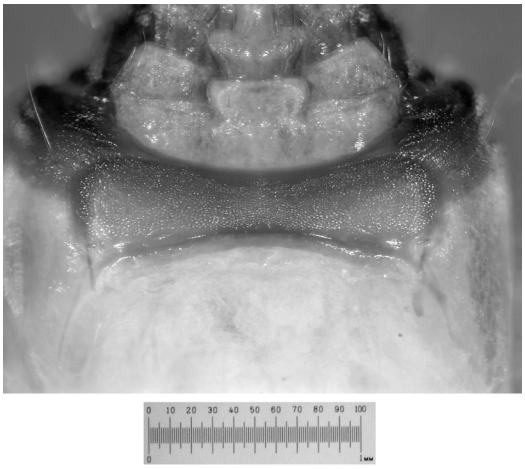


**FIGURE 5**. Ventral cranial sclerite of *Stenostola dubia* (Laicharting, 1784) (Photo © Petr Svacha).

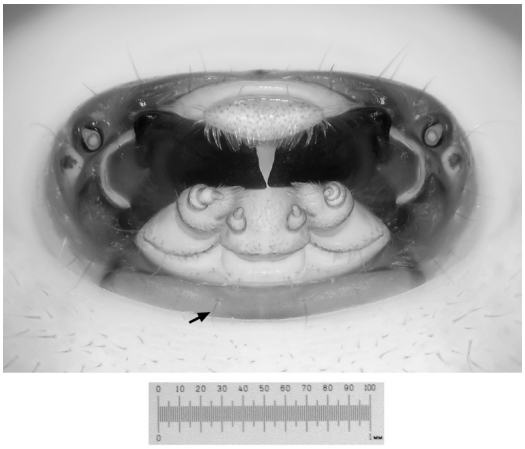
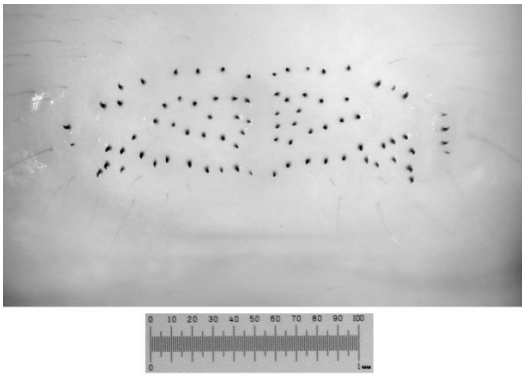


**FIGURE 6**. Head anteroventral of *Stenostola ferrea* (Schrank, 1776) (Photo © Petr Svacha). The arrow indicates the setae.



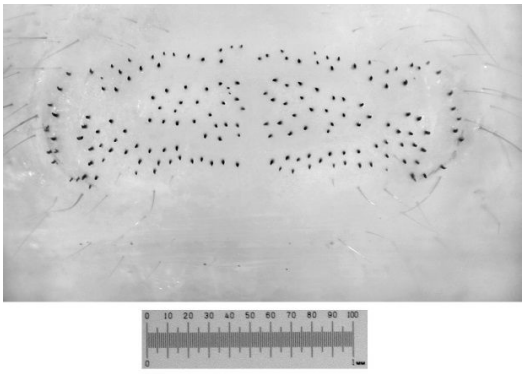


**FIGURE 7**. Dorsal ampulla 5 of *Stenostola ferrea* (Schrank, 1776) with spines minimum (left) and spines average (right) (Photo © Petr Svacha).

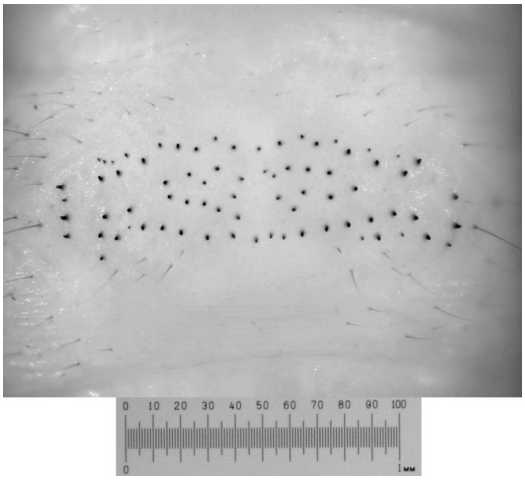


**FIGURE 8**. Dorsal ampulla 5 of *Stenostola dubia* (Laicharting, 1784) (Photo © Petr Svacha).

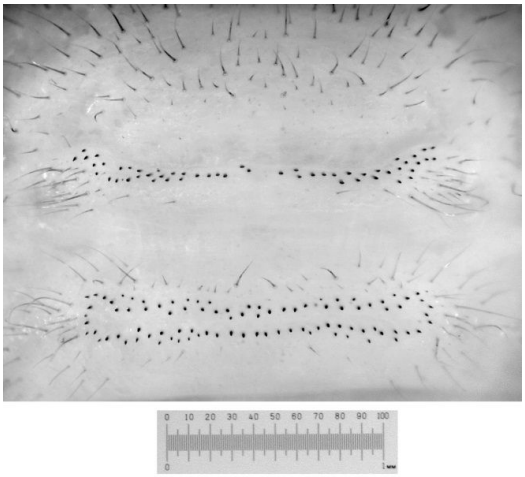


**FIGURE 9**. Pro- and mesosternum of *Stenostola ferrea* (Schrank, 1776) (Photo © Petr Svacha).

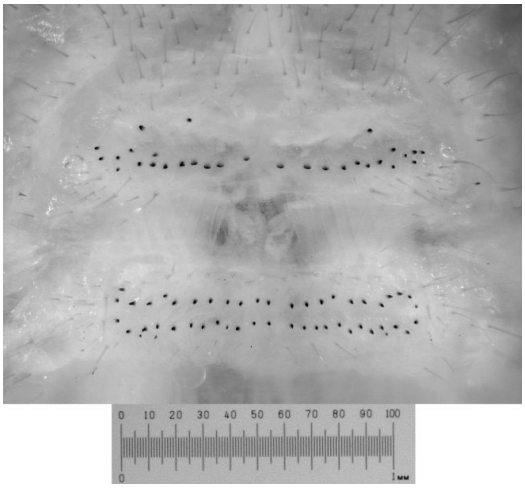


**FIGURE 10**. Pro- and mesosternum of *Stenostola dubia* (Laicharting, 1784) (Photo © Petr Svacha).

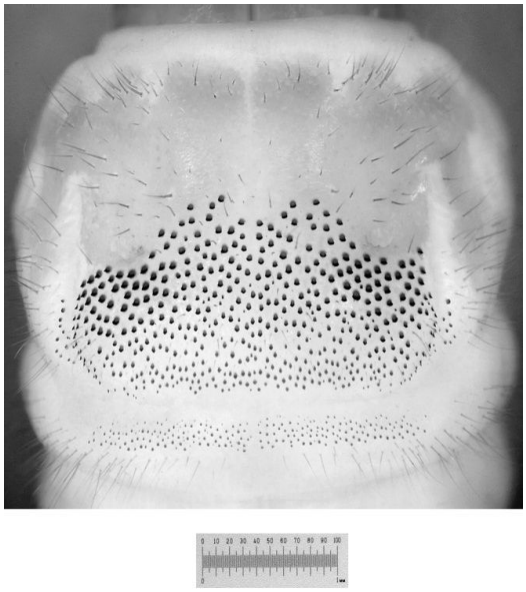


**FIGURE 11**. Pronotum of *Stenostola ferrea* (Schrank, 1776) (Photo © Petr Svacha).

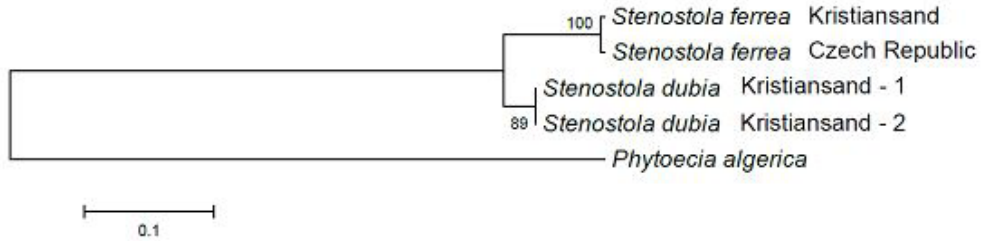



**FIGURE 12**. Neighbour-joining analysis of *Stenostola ferrea* (Schrank, 1776), *S. dubia* (Laicharting, 1784) and *Phytoecia algerica* Desbrochers des Loges, 1870, COI sequences. *S. ferrea* and *S. dubia* cluster into two distinct groups with 10. 3 % genetic distance. *P. algerica* (sequence downloaded from GenBank with accession number AM283245.1) is included as an outgroup. Bootstrap support is shown at each node.

## Discussion and conclusive remarks

The two species are easily separated by the differences in the shape of the inner margin of the parameres (cf. Wallin et al., 2005). In addition, tergite VIII in *S. ferrea* has a posterior margin with a concave or notched part medially which is absent in *S. dubia*, the apex of the aedeagus in *S. ferrea* is more pointed and sharp than in *S. dubia*, and the three elongated median sclerites exhibit some differences in the shape of the tip of the most narrow end.

Remaining genitalia characters, including the spermatheca, are similar and were not distinguished as species-specific characters. The distal and elongated sclerites inside the internal sac or "endophallus sclerites" of *S. dubia* have been illustrated in Sama (2008). These sclerites have a very close resemblance with the sclerites of e.g. *Saperda populnea* (Linnaeus, 1758). Sama

(2008) used this similarity as an argument that the genus *Stenostola* belongs to the subfamily Saperdini, not Phytoeciini. The distal sclerites of e.g. *Oberea* spp. and *Phytoecia* spp. are shaped very differently.

No single morphological character of the larvae shows reliable differences alone. Mature larvae can usually be identified by looking at a combination of characters. Younger larvae are more difficult to identify and might be impossible to assign to species.

The results from DNA barcoding of *S. ferrea* and *S. dubia* show that the two species are divided into two distinct groups with a relatively high genetic distance (10.3 %).These results are consistent with interspecific genetic distances found in previous studies performed on different insect groups (Hausmann et al., 2011; Park et al., 2011; Schilthuizen et al., 2011)..

The differences in morphology (Wallin et al. 2005), distribution and biology (Kvamme & Wallin 2011) and the results of the present study all support the conclusion that the species are truly separate despite the morphological similarities.

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