CHEMOECOLOGY

AGRIOTES PROXIMUS AND A. LINEATUS (COLEOPTERA: ELATERIDAE): A COMPARATIVE STUDY ON THE PHEROMONE COMPOSITION AND CYTOCHROME C OXIDASE SUBUNIT I GENE SEQUENCE

--Manuscript Draft--

Manuscript Number:	CHEM346R2
Full Title:	AGRIOTES PROXIMUS AND A. LINEATUS (COLEOPTERA: ELATERIDAE): A COMPARATIVE STUDY ON THE PHEROMONE COMPOSITION AND CYTOCHROME C OXIDASE SUBUNIT I GENE SEQUENCE
Article Type:	Original Research
Keywords:	Pheromone extraction; chemical communication; Mitochondrial cytochrome c oxidase subunit I; Agriotes spp.; Geranyl butanoate; Geranyl octanoate
Corresponding Author:	Jozsef Vuts Harpenden, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Jozsef Vuts
First Author Secondary Information:	
All Authors:	Jozsef Vuts
	Till Tolasch
	Lorenzo Furlan
	Éva Bálintné Csonka
	Tamás Felföldi
	Károly Márialigeti
	Teodora B Toshova
	Mitko Subchev
	Amália Xavier
	Miklós Tóth
All Authors Secondary Information:	
Abstract:	The presence of geranyl octanoate, previously found in pheromone gland extracts of Agriotes lineatus females, was also demonstrated in gland extracts of A. proximus females. Similarly to A. lineatus, geranyl butanoate was present only in trace amounts in A. proximus female gland extracts. In air entrainment samples of female A. lineatus and A. proximus beetles, the relative ratio of geranyl butanoate and geranyl octanoate was higher than that in gland extracts. In addition, comparison of a segment of the mitochondrial cytochrome c oxidase subunit I gene of feral specimens of A. lineatus and A. proximus showed >99% similarity. Both pheromone profile and nucleotide sequence analysis delineate close relationship between the investigated taxa and postulate taxonomic revision. Further studies on sympatric populations of A. lineatus and A. proximus are underway to investigate and better understand possible processes of species diversification.
Response to Reviewers:	Description of the morphological characters used to separate the two species is incorporated (page 2, lines 51-55).

AGRIOTES PROXIMUS AND A. LINEATUS (COLEOPTERA: ELATERIDAE): A COMPARATIVE STUDY ON THE PHEROMONE COMPOSITION AND CYTOCHROME C OXIDASE SUBUNIT I GENE SEQUENCE

József Vuts · Till Tolasch · Lorenzo Furlan · Éva Bálintné Csonka · Tamás Felföldi · Károly Márialigeti · Teodora B. Toshova · Mitko Subchev · Amália Xavier · Miklós Tóth

J. Vuts · É. Bálintné Csonka · M. Tóth
Plant Protection Institute HAS, Department of Zoology, Herman O. u. 15. Budapest, 1022 Hungary
e-mail: joci0617@gmail.com

T. Tolasch
University of Hohenheim, Department of Zoology, Garbenstraße 30. Stuttgart, 70599 Germany

L. Furlan
Veneto Agricoltura, Viale dell´Università, 14 – Agripolis Legnaro (Pd), 35020 Italy

T. Felföldi · K. Márialigeti
Eötvös Loránd University, Department of Microbiology, Pázmány Péter stny. 1/c, Budapest, 1117 Hungary

T. B. Toshova · M. Subchev
Institute of Biodiversity and Ecosystem Research, ul. Gagarin 2., Sofia, 1113 Bulgaria

A. Xavier
DRAEDM, Rua da Resauração 336, Porto, P-4050-501 Portugal

Abstract The presence of geranyl octanoate, previously found in pheromone gland extracts of *Agriotes lineatus* females, was also demonstrated in gland extracts of *A. proximus* females. Similarly to *A. lineatus*, geranyl butanoate was present only in trace amounts in *A. proximus* female gland extracts. In air entrainment samples of female *A. lineatus* and *A. proximus* beetles, the relative ratio of geranyl butanoate and geranyl octanoate was higher than that in gland extracts. In addition, comparison of a segment of the mitochondrial cytochrome c oxidase subunit I gene of feral specimens of *A. lineatus* and *A. proximus* showed >99% similarity. Both pheromone profile and nucleotide sequence analysis delineate close relationship between the investigated taxa and postulate taxonomic revision. Further studies on sympatric populations of *A. lineatus* and *A. proximus* are underway to investigate and better understand possible processes of species diversification.

Keywords Pheromone extraction · Chemical communication · Mitochondrial cytochrome c oxidase subunit I · *Agriotes* spp. · Geranyl butanoate · Geranyl octanoate

Introduction

Agriotes lineatus (Linnaeus, 1767) (Coleoptera: Elateridae) is widely distributed throughout Europe, West and East Asia, and was introduced into Canada, Brasil and New Zealand as well (Scsegolev 1951; Tóth 1990; Furlan et al. 2000; Laibner 2000; Vernon and Tóth 2007). The closely related *Agriotes proximus* (Schwarz, 1891) is a ponto-mediterranean species which can be found in Portugal, in Bulgaria, in Turkey, and also in Russia, with a more scarce occurrence towards the northern and central parts of Europe (Yatsynin et al. 1996a; Laibner 2000; Subchev et al. 2005, 2006; Mertlik and Platia 2008). The two species are morphologically very similar and can be identified based on slight differences in the width-length ratio of the pronotum (1:1 in *A. lineatus* and 1.2:1 in *A. proximus*), shape of the metasternum (half of metasternum width equals its height in *A. lineatus*, while metasternum width is greater than its height in *A. proximus*), and subtle differences in genital morphology (shape of paramere apex of aedeagus) (Lohse 1979; Laibner 2000).

Pheromone gland extracts of *A. lineatus* contain large amounts of geranyl octanoate (Borg-Karlson et al. 1988; Kudryavtsev et al. 1993; Siirde et al. 1993) and only trace amounts of the related geranyl butanoate (Yatsynin et al. 1996a; Tóth et al. 2003). A blend of 10:1 geranyl octanoate/geranyl butanoate was found to be effective in field trapping of *A. lineatus* in many European countries (Tóth et al. 2003). However, in Portugal and Bulgaria, traps with this bait also caught specimens of the closely related *A. proximus* (Subchev et al. 2005, 2006; Tóth and Furlan 2005). Catches of *A. proximus* were unexpected because two very different compounds, *(E,E)*-farnesyl acetate and neryl isovalerate, had previously been identified as pheromone components of a Russian population of *A. proximus* (Yatsynin et al. 1980, 1996b). Subsequent field experiments, testing different ratios of geranyl butanoate and geranyl octanoate revealed that the largest catches for both species were observed with a 1:1 blend of geranyl butanoate and geranyl octanoate (Tóth et al. 2008). In electroantennographic (EAG) studies, testing synthetic click beetle pheromone components, antennal responses of male *A. lineatus* and *A. proximus* showed a very similar pattern. In dose-response tests, antennae of males of both species gave greater responses to geranyl butanoate than to geranyl octanoate, even at low doses (Tóth et al. 2008).

Considering the strong similarity between *A. lineatus* and *A. proximus* in morphology and pheromone communication, the aims of the present study were: 1) to demonstrate the presence of geranyl octanoate and geranyl butanoate in gland extracts of *A. proximus* females; 2) to prove that both geranyl octanoate and geranyl butanoate are emitted by *A. lineatus* and *A. proximus* females by collecting air entrainment samples; 3) to carry out preliminary molecular studies using the mitochondrial cytochrome c oxidase subunit I gene for the purpose of investigating the similarities and differences between the two species also at the genetic level.

Materials and methods

Gland extracts

For gland extraction, *A. proximus* females were collected in May 2007 in Porto, Portugal, using ca. 0.5 x 1 m plastic sheets placed on the ground of a potato field with no vegetation. Freshly collected plant material (preferably Gramineae) was put on the sheets serving as shelter for the nocturnal *A. proximus* during the day (method by L. Furlan, unpublished). Thirty sheets arranged in 5 lines were used with 10m between lines and 5m between sheets within the lines. Beetles were collected by manual inspection of the plant material twice a day.

The abdominal tips of 5 *A. proximus* females containing the pheromone glands (Borg-Karlson et al. 1988; Merivee and Erm 1993) were removed and extracted in pentane for 15 mins. Two extracts were prepared, the final volume of each was 25 µl. Extracts were analyzed by gas chromatography linked to mass spectrometry (GC-MS).

Collection of volatiles

For air entrainment studies, feral beetles were collected by the method described above. *A. lineatus* females were collected in June 2008 in Trento, Italy, while *A. proximus* females in April 2007 in Porto, Portugal,

and in May 2007 in Belchin, Bulgaria. Beetles were placed into the glass tube of a closed-loop stripping apparatus (CLSA) (Boland et al. 1984), equipped with a DC12/16NK vacuum pump (Erich Fürgut GmbH, Tannheim, Germany) (5.0 l/min) and a collection filter containing activated charcoal (1.5 mg) (Brechbühler AG, Schlieren, Switzerland).

Collections were run for 23-72 hours as the daily rhythm of pheromone emission is unknown in click beetles. Trapped volatiles were eluted from the charcoal filter with dichloromethane (25 µl; Merck KGaA, Darmstadt, Germany).

Gas chromatography

For analysis of air entrainment samples collected from *A. lineatus* and *A. proximus* females, a HP 5890 GC (Hewlett Packard Company, USA) equipped with a SP 2340 column (30m x 0.32mm x 0.20µm film thickness; Supelco Inc., USA) was used. Injections were made in the splitless mode (220°C). The oven temperature was held at 60°C for 5 min, then programmed at 10°C/min to 120°C, then at 5°C/min to 220°C and held for 20 min. The carrier gas was helium with a flow rate of 1.5 ml/min.

For authentic standards, synthetic geranyl butanoate and geranyl octanoate (Bedoukian Inc., USA) were used. Both compounds were > 95% pure as stated by the supplier.

Extracts showing peaks at the retention time of geranyl butanoate and geranyl octanoate were further analyzed by GC-MS for confirmation of the structure.

Coupled gas chromatography–mass spectrometry (GC–MS)

Analyses of extracts were carried out using a 6890 GC/5973N MSD quadrupole instrument (Agilent Technologies, Santa Clara, CA, USA) in electron impact mode at 70 eV and 230°C. Compounds were separated with a 30 m × 0.25 mm ID, 0.25-µm film thickness HP5-MS fused silica capillary column (Agilent), starting at 60°C for 3 min, then programmed to 300°C at a rate of 10°C /min. The carrier gas was helium with a flow rate of 1.2 ml/min.

Molecular analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene of *A. proximus* and *A. lineatus*

For molecular analyses of the COI gene, feral *A. lineatus* females were collected in June 2008 in Trento, Italy, by the same method as described above. *A. proximus* females were collected in May 2007 in Belchin, Bulgaria, by pheromone traps (codenamed YATLORf, RO-SA Micromecanica, San Dona di Piave, Venice, Veneto, Italy; Furlan et al. 2004). Genomic DNA was extracted with the DNeasy® Tissue Kit (Qiagen, Hilden, Germany) from wing muscles of one selected specimen from both species conserved in 70% ethanol.

Amplification of the COI gene was performed with a final volume of 50 µL using 3 µL of purified genomic DNA, 0.2mM of each deoxynucleotide, 2mM $MgCl_2$, 1 U LC *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1X PCR buffer (Fermentas), 0.3 µM of CW-3031 [5' - TTT GC(A/C) CT(A/T) ATC TGC C(A/C)T ATT, Heinze et al. 2005] and C1-J-2195 (5' - TTG ATT TTT TGG TCA TCC AGA AGT, Simon et al. 1994) primers and the following temperature profile: initial denaturation at 98°C for 5 min, followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 48°C (annealing), 2 min at 72°C (extension), and final extension at 72°C for 5 min. PCR amplicons were examined by electrophoresis in an ethidium-bromide-stained 1% (w/v) agarose gel under UV light. PCR products were purified with the PCR-M™ Clean Up System (Viogene, Sijhih, Taiwan) according to the manufacturer's instructions.

Sequence analysis was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The chromatograms were corrected manually with the Chromas software, version 1.45 (Technelysium Pty Ltd, Australia). The generated sequences were compared to the GenBank nucleotide database using the Blast program (Altschul et al. 1997). The neighbor-joining phylogenetic tree was constructed with the MEGA4 software (Tamura et al. 2007) using ClustalW alignment (Thompson et al. 1994). The determined sequences were deposited in GenBank under the following accession numbers: HQ330979 *(A. lineatus)* and HQ330980 *(A. proximus)*.

Results

Gland extract analysis of *A. proximus* females

The presence of geranyl octanoate was demonstrated in gland extracts of *A. proximus* females in high amounts (Fig. 1). Geranyl butanoate was detected only in traces. Beside geranyl octanoate and geranyl butanoate, small amounts of geranial were also detected.

Analysis of air entrainment samples from *A. proximus* and *A. lineatus* females

Both GC-MS data and injection of authentic standards confirmed the presence of geranyl butanoate and geranyl octanoate in the headspace collections of both species (Fig. 2). The relative ratio of geranyl butanoate and geranyl octanoate in headspace extracts was higher than that in gland extracts: an average ratio of 1:10 of geranyl butanoate:geranyl octanoate in both species was observed, in contrast to gland extracts where geranyl butanoate was present in traces only.

Apart from the above two compounds, GC-MS revealed the presence of geranyl hexanoate, neral, geranial, and geraniol in both species, and nerol only in *A. proximus*.

Molecular analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene

The COI gene is a widely used phylogenetic marker of beetles (e.g. Hunt et al. 2007) and with the analysis of this region species-level assignments can be obtained (Hebert et al. 2003). Furthermore, there are publicly available COI sequences from some *Agriotes* species for comparison.

Comparison of the sequences of feral *A. proximus* and *A. lineatus* with each other and with other click beetle sequences available in the GenBank database belonging to the same genus indicated high pairwise similarity values (99.5-99.8%) between *A. proximus* and *A. lineatus* (Fig. 3). These sequences separated well from the other two *Agriotes* species, *A. sputator* and *A. acuminatus* (sharing 85.4-85.5% and 80.3-81.1% pairwise similarities to the *A. proximus/A. lineatus* group, respectively).

Discussion

In previous field trials that tested different ratios of geranyl butanoate and geranyl octanoate, the greatest catches of *A. lineatus* were obtained with a blend containing relatively large amounts of geranyl butanoate (ratio of 1:1) (Tóth et al. 2008). These results were unexpected, since, in chemical studies, this compound was detected only in trace amounts in female pheromone glands (Siirde et al. 1993; Yatsynin et al. 1996b; Tóth et al. 2003). Similarly, *A. proximus* males showed the strongest attraction to the 1:1 blend in the field (Tóth et al. 2008). In contrast in this present study, geranyl octanoate was detected in high amounts, and geranyl butanoate was present only in traces (less than 0.1% of geranyl octanoate) in gland extracts of the females. One possible explanation can be that ratio of compounds extracted by direct solvent extraction of pheromone glands does not reflect the ratio of components emitted into the air by a calling female (Millar and Sims 1998). In this study, analyses of samples collected by air entrainment from intact insects showed a higher (1:10) geranyl butanoate:geranyl octanoate ratio in both species than those prepared by direct solvent extraction.

In preliminary tests, the ratio of geranyl butanoate:geranyl octanoate, released by the 1:1 synthetic blend under laboratory conditions at 20ºC, has been determined as 1:0.1 (J. Vuts et al. unpublished). This indicates that, due to considerable differences in volatility, the actual ratio of the two compounds released differs from the ratio of the mixture that had been administered onto the dispenser. Direct comparison of the ratio of geranyl butanoate:geranyl octanoate in air entrainment samples collected from female beetles, and that of the optimal 1:1 synthetic bait, is therefore problematic.

(E,E)-Farnesyl acetate and neryl isovalerate were identified as pheromone components of a Russian population of *A. proximus* (Yatsynin et al. 1980, 1996b). In this present study, these compounds were not found either in gland extracts or in headspace samples of *A. proximus* females. This corresponds well with our previous field trapping data, as no catches of *A. proximus* were observed to these compounds (Tóth et al. 2008). A possible explanation of this controversial phenomenon could be the different pheromonal composition of the European versus Russian populations of *A. proximus*, similar to inter-

populational differences observed in *A. lineatus* (Tóth et al. 2008). To clarify this, specimens used by Yatsynin et al. (1980, 1996b) need to be compared with specimens in the present study at the morphological and genetic level. Unfortunately, at present, such voucher specimens are unavailable.

The slight morphological differences, similarities in the composition of pheromones from glands resp. air entrainments, and data on their electrophysiological and behavioural activities (Tóth et al. 2008), as well as the preliminary molecular comparison of the two species in the present study may suggest the taxa to which the two described morphological forms belong, represent a stage of species diversification, or may just be geographical populations or races of the same species. This may be supported by Staudacher et al. (2011) who also found high similarity between the two species based on the analysis of a region of the COI gene different from that investigated in this study. To test this hypothesis, additional studies concerning the similarities/differences in other regions of the genom (e.g. mitochondrial 16S rRNA gene) of sympatric populations of *A. lineatus/A. proximus*, as well as comparison of pheromone composition of females and antennal responses of males to synthetic click beetle pheromone components and to natural extracts from these populations are required, since it cannot be excluded that the two morphological forms described as *A. lineatus* and *A. proximus* differentiate in their pheromone biology only in case of sympatry, but not in allopatry. Ontogenetic changes in the pheromone bouquets of the beetles were not considered in this study, because to the best of our knowledge changes in pheromone composition in click beetles have never been observed.

Examples of differing pheromone composition within one species are not exceptional, although not very frequent. In Lepidoptera, the European *Zeiraphera diniana* Guenée (Lepidoptera: Tortricidae) has two host races: one feeds on larch, the other one on pine; females of each race utilize a pheromone compound unique and characteristic to the given race (larch population: *(E)*-11-tetradecenyl acetate, pine population: *(E)*-9-dodecenyl acetate), and males respond only to the pheromone produced by females from the same race. The two races occur both in sympatry and allopatry (Baltensweiler and Priesner 1988). On the other hand, in *Ostrinia nubilalis* Hbn. (Lepidoptera: Pyralidae), two races utilize blends of *(E)*-11-tetradecenyl acetate and *(Z)*-11-tetradecenyl acetate in different ratios. The E race produces a blend of *E/Z* in 99:1, the Z race in 3:97. In places where the areas of the two races overlap, hybrids are produced, and females of the hybrid populations emit an intermediate blend (Roelofs et al. 1985).

Consequently, future work needs to focus on characterizing the pheromone composition, male pheromone perception and genetic background of populations from overlapping areas of *A. proximus* and *A. lineatus*. A better understanding of the factors playing a role in the regulation of chemical communication can contribute to a more precise decision making in the control of the two species within Integrated Pest Management.

Acknowledgement This work was partially supported by grant DO02-244/2008 of the Bulgarian National Scientific Fund and by OTKA grant K 81494 of HAS. Comments by anonymous referees and the editors greatly improved the relevance of the work presented.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402. doi:10.1093/nar/25.17.3389

Baltensweiler VW, Priesner E (1988) A study of pheromone polymorphism in *Zeiraphera diniana* Gn. (Lep., Tortricidae). 3. Specificity of attraction to synthetic pheromone sources by different male response types from two host races. J Appl Entomol 106:217–231. doi: 10.1111/j.1439-0418.1988.tb00587.x

Boland W, Ney P, Jaenickea L, Gassmann G (1984) A ”closed-loop-stripping” technique as a versatile tool for metabolic studies of volatiles. In: Schreier P (ed) Analysis of volatiles. Walter de Gruyter, Berlin, pp 371-380

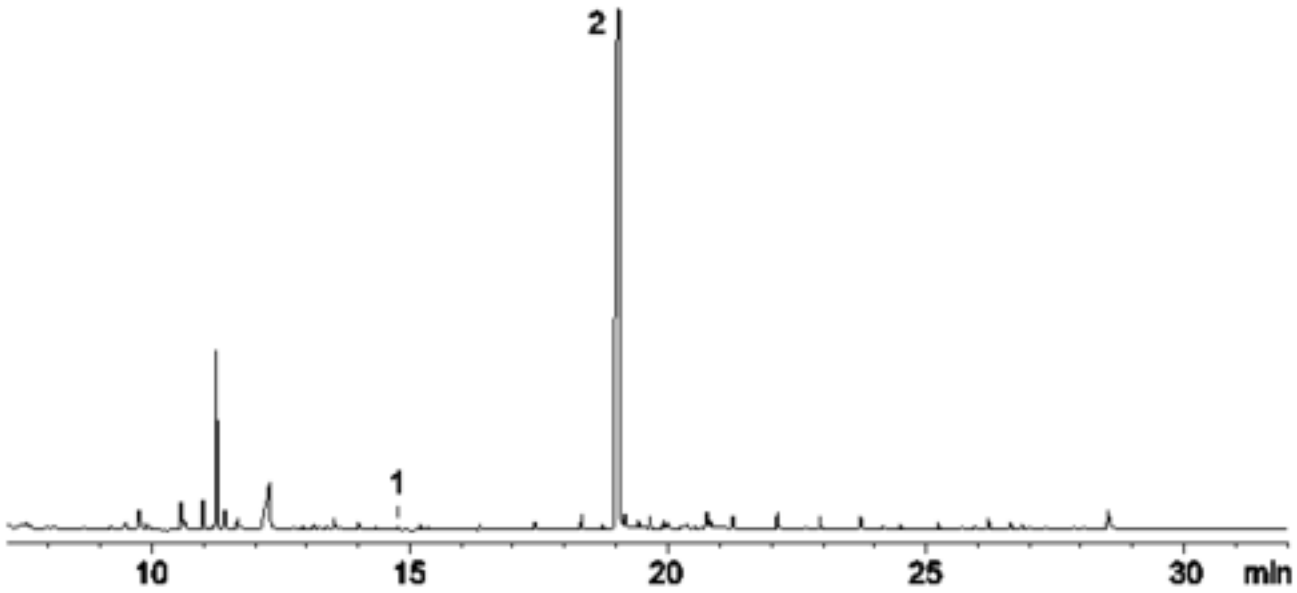
262 Borg-Karlson AK, Agren L, Dobson HB, Bergström BG (1988) Identification and electroantennographic
263 activity of sex-specific geranyl esters in an abdominal gland of female *Agriotes obscurus* (L.) and
264 *A. lineatus* (L.) (Coleoptera: Elateridae). Experientia 44:531–534. doi: 10.1007/BF01958940
265 Furlan L, Curto G, Ferrari R, Boriani L, Bourlot G, Turchi A (2000) Wireworm species damaging crops in
266 Po Valley. Informatore Fitopatologico 5:53-59
267 Furlan L, Garofalo N, Tóth M (2004) Biologia comparata di *Agriotes sordidus* Illiger nel Nord e Centro-
268 Sud dę Italia. Informatore Fitopatologico 10:49-54.

269 Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes.
270 Proc R Soc B 270:313–321. doi: 10.1098/rspb.2002.2218
271 Heinze J, Trindl A, Seifert B, Yamauchi K (2005) Evolution of male morphology in the ant genus
272 *Cardiocondyla*. Mol Phylogenet Evol 37: 278-288. doi: 10.1016/j.ympev.2005.04.005
273 Hunt T, Bergsten J, Levkanicova Z, Papadopoulou A, John OS, Wild R, Hammond PM, Ahrens D, Balke
274 M, Caterino MS, Gómez-Zurita J, Ribera I, Barraclough TG, Bocakova M, Bocak L, Vogler AP
275 (2007) A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation.
276 Science 318:1913-1916. doi:10.1126/science.1146954
277 Kudryavtsev I, Siirde K, Lääts K, Ismailov V, Pristavko V (1993) Determination of distribution of harmful
278 click beetle species (Coleoptera, Elateridae) by synthetic sex pheromones. J Chem Ecol 19:1607–
279 1611. doi: 10.1007/BF00982295
280 Laibner S (2000) Elateridae of the Czech and Slovak Republics. Kabourek, Zlin
281 Lohse GA (1979) 68. Familie: Elateridae. In: Freude H, Harde KW, Lohse GA (eds) Die Käfer
282 Mitteleuropas. Band 6., Diversicornia. Goecke & Evers, Krefeld, pp 103-186
283 Merivee E, Erm A (1993) Studies on sex pheromone gland morphology and pheromone components, in
284 female elaterid beetles *Agriotes obscurus* L. and *Agriotes lineatus* L. (Coleoptera, Elateridae).
285 Eesti Teaduste Akadeemia Toimetised Bioloogia 42:108-117.
286 Mertlik J, Platia G (2008) Catalogue of the family Cebrionidae, Elateridae, Lissomidae, Melasidae and
287 Throscidae (Coleoptera) from Turkey. Elateridarium 2:1-40.
288 Millar JG, Sims JJ (1998) Preparation, cleanup, and preliminary fractionation of extracts. In: Millar JG,
289 Haynes KF (eds.) Methods in chemical ecology I: Chemical methods Kluwer Academic
290 Publishers, Boston/Dordrecht/London, pp 1-37
291 Roelofs WL, Du JW, Tang XH, Robbins PS, Eckenrode CJ (1985) Three European corn borer populations
292 in New York based on sex pheromones and voltinism. J Chem Ecol 11:829–836. doi:
293 10.1007/BF01012071
294 Scsegolev VN (1951) Mezőgazdasági rovartan. Akadémiai Kiadó, Budapest, pp 695
295 Siirde K, Lääts K, Erm A, Kogerman A, Kudryavtsev I, Ismailov V, Pristavko V (1993) Structure-activity
296 relationships of synthetic pheromone components in sex communication of click beetles
297 (Coleoptera, Elateridae). J Chem Ecol 19:1597–1606. doi: 10.1007/BF00982294
298 Simon C, Frati F, Beckenback A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic
299 utility of mithocondrial gene sequences and a compilation of conserved PCR primers. Annals
300 Entomol Soc Am 87:651-701. doi: 0013-8746/94/0651-0701$02.00/0
301 Staudacher K, Pitterl P, Furlan L, Cate PC, Traugott M (2011) PCR-based species identification of Agriotes
302 larvae. Bull Entomol Res doi: 10.1017/S0007485310000337
303 Subchev M, Toshova T, Furlan L, Tóth M (2005) Click beetles (Coleoptera: Elateridae) and their seasonal
304 swarming as established by pheromone traps in different plant habitats in Bulgaria: 2. Maize. Acta
305 Zool Bulg 57:321–332.
306 Subchev M, Toshova T, Furlan L, Tóth M (2006) Click beetles (Coleoptera: Elateridae) and their seasonal
307 swarming as established by pheromone traps in different plant habitats in Bulgaria: 3. Potato. Acta
308 Zool Bulg 58:361–370.

309 Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis
310 (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599. doi:10.1093/molbev/msm092
311 Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple
312 sequence alignment through sequence weighting, position-specific gap penalties and weight matrix
313 choice. Nucleic Acids Res 22:4673-4680.
314 Tóth Z (1990) Family: Elateridae. In: Jermy T, Balázs K (eds.) A növényvédelmi állattan kézikönyve 3/A,
315 Akadémiai Kiadó, Budapest, pp 30-70
316 Tóth M, Furlan L, Yatsynin VG, Ujváry I, Szarukán I, Imrei Z, Tolasch T, Francke W, Jossi W (2003)
317 Identification of pheromones and optimization of bait composition for click beetle pests in Central
318 and Western Europe (Coleoptera: Elateridae). Pest Manag Sci 59:417-425. doi: 10.1002/ps.629
319 Tóth M, Furlan L (2005) Pheromone composition of European click beetle pests (Coleoptera, Elateridae):
320 common components—selective lures. IOBC/WPRS Bull 28:133–142
321 Tóth M, Furlan L, Xavier A, Vuts J, Toshova T, Subchev M, Szarukán I, Yatsynin V (2008) New sex
322 attractant composition for the click beetle *Agriotes proximus*: similarity to the pheromone of *A.*
323 *lineatus*. J Chem Ecol 34:107-111. doi: 10.1007/s10886-007-9398-7
324 Vernon RS, Tóth M (2007) Evaluation of pheromones and a new trap for monitoring *Agriotes lineatus* and
325 *Agriotes obscurus* in the Fraser Valley of British Columbia. J Chem Ecol 33:345–351. doi:
326 10.1007/s10886-006-9217-6
327 Yatsynin VG, Oleschenko IN, Rubanova EV, Ismailov VY (1980) Identification of active components of
328 the sex pheromones of click beetles *Agriotes gurgistanus, A. litigiosus* and *A. lineatus*. Khim
329 Sel'sk Khoz Edition Moskva, Khimiya, pp 33–35
330 Yatsynin VG, Rubanova EV, Okhrimenko NV (1996a) Identification of female-produced sex pheromones
331 and their geographical differences in pheromone gland extract composition from click beetles
332 (Col., Elateridae). J Appl Entomol 120:463-466. doi: 10.1111/j.1439-0418.1996.tb01636.x
333 Yatsynin VG, Karpenko NN, Orlov VN (1996b) Sex pheromone of the click beetle *Agriotes sputator* L.
334 (Coleoptera: Elateridae). Khim Komm Zhivot, Edition Moskva, Nauka pp 53–57.
335
336
337 Figure legends
338
339 **Fig. 1** GC trace of a gland extract of *A. proximus* females. (1) geranyl butanoate, (2) geranyl octanoate. For
340 a GC-trace of *A. lineatus* female gland extracts, see Tóth et al. 2003
341
342 **Fig. 2** GC traces of air entrainment samples of *A. proximus* and *A. lineatus*. (1) geranyl butanoate, (2)
343 geranyl octanoate
344
345 **Fig. 3** Neighbor-joining phylogenetic tree of COI sequences retrieved from various *Agriotes* species (based
346 on 639 unambiguously aligned nucleotide positions; evolutionary distances were computed using the
347 Maximum Composite Likelihood method)

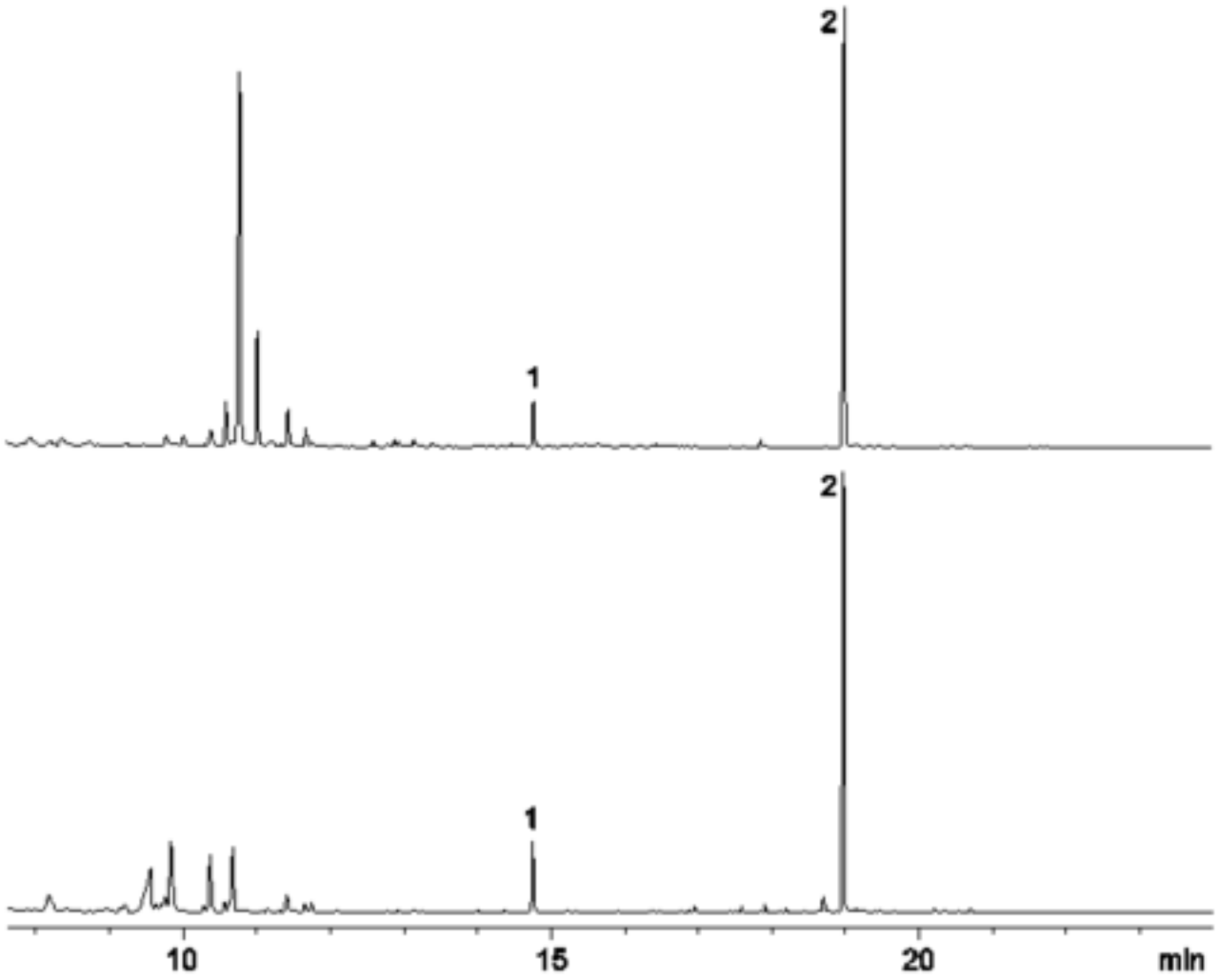
line figure

Click here to download high resolution image



line figure

Click here to download high resolution image



line figure

Click here to download high resolution image