Salix transect of Europe: additional leaf beetle (Chrysomelidae) records and insights from chrysomelid DNA barcoding

Roy Canty‡, Enrico Ruzzier§, Quentin C Cronk¶, Diana M Percy¶

‡ Natural History Museum, Cromwell Road, SW7 5BD, London, United Kingdom
§ University degli Studi di Padova, Legnaro (Padova), Italy
¶ Natural History Museum, London, United Kingdom
¶¶ University of British Columbia, Vancouver, Canada

Corresponding author: Diana M Percy (diana.percy@ubc.ca)

Abstract

Occurrence patterns of chrysomelid beetles (Coleoptera: Chrysomelidae), associated with willow (Salix spp.) at 42 sites across Europe, have previously been described. The sites form a transect from Greece (lat. 38.8 °N) to arctic Norway (lat. 69.7 °N). This paper reports additional records and the results of DNA sequencing in certain genera. Examination of further collections from the transect has added 13 species in the genera Aphthona, Chrysomela, Cryptocephalus, Epitrix, Galerucella (2 spp.), Goniocrena, Phyllotreta (2 spp.), Pachybrachis (3 spp.) and Syneta. We also report the sequencing of the DNA regions cytochrome oxidase 1 (CO1) and cytochrome B (cytB) for a number of samples in the genera Plagiodera, Chrysomela, Goniocrena, Phratora, Galerucella and Crepidodera. The cytB sequences are the first available for some of these taxa. The DNA barcoding largely confirmed previous identifications but allowed a small number of re-assignments between related species. Most notably, however, it was evident that the southernmost material (Greece and Bulgaria) of specimens, previously treated as Crepidodera aurata sens. lat., belonged to a distinctive molecular cluster. Morphological re-examination revealed these to be C. nigricoxis Allard, 1878. This is an example of how
morphotaxonomy and DNA barcoding can work iteratively to refine identification. Our sequences for *C. nigricoxis* appear to be the first available for this taxon. Finally, there is little geographic structure evident, even in widely dispersed species.

**Keywords**

Salicophagy, salicivorous insects, Salicaceae, Chrysomelidae, DNA barcoding, Europe, megatransect

**Introduction**

Since early pleas were made for the routine incorporation of a molecular component to taxonomy (“DNA barcoding”) (Hebert et al. 2003a, Hebert et al. 2003b, Tautz et al. 2003), a large amount of literature has accrued and a very large number of sequences backed by voucher specimens have been deposited in standard databases. It is now well established that, in many animal groups, sequencing mitochondrial cytochrome c oxidase subunit 1 (COI) provides a straightforward way of gaining taxonomic insight. Early concerns about molecular methods being somehow antagonistic to morphological taxonomy have given way to acceptance that molecular and morphological taxonomy are complementary, reciprocally illuminating and iterative processes.

As part of a study of lowland willow communities sampled from south to north across Europe, we have previously investigated the occurrence and abundance patterns of chrysomelid beetles (Coleoptera: Chrysomelidae) associated with *Salix* species (Canty et al. 2016). In this study, large numbers of individual beetles were processed and it was impossible with available resources to perform large numbers of genitalia dissections. For this reason, a broad morphospecies concept was used, identifying to species largely using external morphology. We have now been able to test some of these morphospecies assignments using DNA barcoding. This paper reports the new insights that this offers. We also take the opportunity to report additional chrysomelid records from the transect following examination of additional collections.

**Material and methods**

**Collecting methods**

Chrysomelid beetles were collected from willows (*Salix* spp.) by the authors ER and DP at all sites, as previously described (Canty et al. 2016). Details of the sites and the method of their selection have been given in previous papers (Cronk et al. 2015; Canty et al. 2016). The sample sites formed a megatransect from Greece to arctic Norway (Table 1). All collections are deposited in the Natural History Museum, London (BMNH).
| SITE# | Country     | Lat N  | Long E  | Alt (m) | Date of collection |
|------|-------------|--------|---------|---------|--------------------|
| 1    | Greece      | 38.80007 | 22.4629 | 37      | 21-iv-2015         |
| 2    | Greece      | 38.902  | 22.31015| 33      | 21-iv-2015         |
| 3    | Greece      | 39.306694 | 22.528323| 177    | 22-iv-2015         |
| 4    | Greece      | 40.032685 | 22.175437| 534    | 22-iv-2015         |
| 5    | Greece      | 41.113317 | 23.273893| 31      | 23-iv-2015         |
| 6    | Bulgaria    | 41.412468 | 23.318609| 90     | 23-iv-2015         |
| 7    | Bulgaria    | 42.165622 | 22.998141| 392    | 24-iv-2015         |
| 8    | Bulgaria    | 42.923989 | 23.810563| 339    | 24-iv-2015         |
| 9    | Bulgaria    | 43.739343 | 23.966755| 35     | 24-iv-2015         |
| 10   | Romania     | 44.260343 | 23.786781| 81     | 25-iv-2015         |
| 11   | Romania     | 44.961981 | 23.190337| 172    | 25-iv-2015         |
| 12   | Romania     | 45.510676 | 22.737225| 556    | 26-iv-2015         |
| 13   | Romania     | 46.518504 | 21.512839| 102    | 26-iv-2015         |
| 14   | Hungary     | 46.700744 | 21.31268 | 94     | 27-iv-2015         |
| 15   | Hungary     | 47.665648 | 21.261768| 91     | 27-iv-2015         |
| 16   | Hungary     | 48.374291 | 20.725264| 148    | 28-iv-2015         |
| 17   | Poland      | 49.463447 | 21.697255| 385    | 28-iv-2015         |
| 18   | Poland      | 50.470234 | 22.238372| 157    | 29-iv-2015         |
| 19   | Poland      | 50.673994 | 21.823391| 141    | 29-iv-2015         |
| 20   | Poland      | 51.775039 | 21.1971  | 101    | 30-iv-2015         |
| 20a  | Poland      | 51.775039 | 21.1971  | 101    | 11-vi-2015         |
| 21   | Poland      | 52.69398  | 21.8529  | 96     | 12-vi-2015         |
| 22   | Poland      | 53.55483  | 22.30299 | 128    | 12-vi-2015         |
| 23   | Poland      | 54.06943  | 23.11745 | 137    | 13-vi-2015         |
| 24   | Lithuania   | 54.92583  | 23.7742  | 28     | 13-vi-2015         |
| 25   | Lithuania   | 55.79557  | 24.56678 | 62     | 13-vi-2015         |
| 26   | Latvia      | 56.71141  | 24.25162 | 23     | 14-vi-2015         |
| 27   | Latvia      | 57.74963  | 24.4023  | 7      | 14-vi-2015         |
| 28   | Estonia     | 58.42257  | 24.44063 | 18     | 15-vi-2015         |
| 29   | Estonia     | 59.40289  | 24.93577 | 48     | 15-vi-2015         |
| 30   | Finland     | 60.27299  | 24.65843 | 33     | 16-vi-2015         |
| 31   | Finland     | 61.09965  | 25.6282  | 84     | 16-vi-2015         |
| 32   | Finland     | 62.04962  | 26.12369 | 174    | 17-vi-2015         |

Table 1.
Basic site details. See Cronk et al. (2015) for further details.
Morphological procedures followed those used in Canty et al. (2016). A selected subset of specimens was chosen for sequencing (Table 2). These included specimens deemed to be potentially problematic in the original identifications and samples from widespread and variable species. DNA was extracted from material preserved in 90% ethanol. Sequences of mitochondrial cytochrome oxidase subunit 1 (COI) and cytochrome B (cytB) were obtained following protocols for DNA extraction, polymerase chain reaction (PCR) and sequencing described in Percy et al. (2018) with additional primers used for COI (LCO1490 and HCO2198; Folmer et al. 1994). As numerous COI sequences are available on GenBank, we were able to align our own sequences with previously published ones (Table 3). Aligned sequences were analysed using neighbour-joining (NJ) with uncorrected (p) distances in PAUP* (Swofford 2003). Bootstrap support was obtained using 1000 replicates. Sequences generated as a result of this study are all deposited in GenBank (accession numbers MN629748 - MN629886) (Table 2).

Table 2.
Samples sequenced in this study, reassignments made, and sequences deposited in GenBank: COI (cytochrome oxidase 1), cytB (cytochrome B).

| Original species ID | Reassignment ID | Site | COI       | cytB       |
|---------------------|----------------|------|-----------|------------|
| Chrysomela vigintipunctata | correct     | 4    | MN629768  | MN629838  |
| Chrysomela vigintipunctata | correct     | 7    | MN629769  | MN629839  |
| Chrysomela vigintipunctata | correct     | 11   | MN629770  | MN629840  |
| Chrysomela vigintipunctata | correct     | 16   | MN629771  | MN6298341 |
| Chrysomela vigintipunctata | correct     | 21   | MN629772  | MN629842  |
| Crepidodera aurata     | Crepidodera nigricoxis | 3    | MN629760  | MN629830  |
| Crepidodera aurata     | Crepidodera nigricoxis | 4    | MN629762  | MN629832  |
| Crepidodera aurata     | Crepidodera nigricoxis | 4    | MN629763  | MN629833  |
| Original species ID       | Reassignment ID | Site | COI          | cytB          |
|--------------------------|----------------|------|--------------|---------------|
| Crepidodera aurata       | Crepidodera nigricoxis | 4    | MN629764     | MN629834      |
| Crepidodera aurata       | Crepidodera nigricoxis | 4    | MN629765     | MN629835      |
| Crepidodera aurata       | Crepidodera nigricoxis | 4    | MN629773     | MN629843      |
| Crepidodera aurata       | Crepidodera nigricoxis | 7    | MN629761     | MN629831      |
| Crepidodera aurata       | Crepidodera nigricoxis | 7    | MN629766     | MN629836      |
| Crepidodera aurata       | Crepidodera nigricoxis | 7    | MN629767     | MN629837      |
| Crepidodera aurata       | correct          | 7    | MN629759     | MN629829      |
| Crepidodera aurata       | correct          | 8    | MN629749     | MN629819      |
| Crepidodera aurata       | correct          | 8    | MN629750     | MN629820      |
| Crepidodera aurata       | correct          | 8    | MN629751     | MN629821      |
| Crepidodera aurata       | correct          | 8    | MN629752     | MN629822      |
| Crepidodera aurata       | correct          | 8    | MN629753     | MN629823      |
| Crepidodera aurata       | correct          | 8    | MN629754     | MN629824      |
| Crepidodera aurata       | correct          | 8    | MN629755     | MN629825      |
| Crepidodera aurata       | correct          | 8    | MN629756     | MN629826      |
| Crepidodera aurata       | correct          | 8    | MN629757     | MN629827      |
| Crepidodera aurata       | correct          | 8    | MN629758     | MN629828      |
| Crepidodera aurata       | correct          | 11   | MN629774     | MN629844      |
| Crepidodera aurata       | correct          | 18   | MN629775     | MN629845      |
| Crepidodera aurata       | correct          | 25   | MN629776     | MN629846      |
| Crepidodera aurata       | Crepidodera fulvicornis | 33   | /            | MN629847      |
| Crepidodera aurata       | Crepidodera fulvicornis | 39   | /            | MN629848      |
| Crepidodera fulvicornis  | correct          | 16   | MN629778     | /             |
| Crepidodera fulvicornis  (a) | correct      | 23   | MN629779     | /             |
| Crepidodera fulvicornis  (b) | correct      | 23   | MN629780     | MN629849      |
| Crepidodera fulvicornis  (c) | correct      | 23   | MN629781     | MN629850      |
| Crepidodera fulvicornis  | correct          | 27   | MN629782     | MN629851      |
| Crepidodera fulvicornis  | correct          | 31   | MN629783     | MN629852      |
| Crepidodera fulvicornis  | correct          | 35   | MN629784     | MN629853      |
| Crepidodera fulvicornis  | correct          | 39   | MN629785     | MN629854      |
| Crepidodera plutus       | correct          | 6    | MN629748     | MN629818      |
| Crepidodera plutus       | correct          | 9    | MN629786     | MN629855      |
| Crepidodera plutus       | correct          | 11   | MN629787     | MN629856      |
| Crepidodera plutus       | correct          | 13   | MN629788     | MN629857      |
| Crepidodera plutus       | correct          | 14   | MN629789     | MN629858      |
| Crepidodera plutus       | correct          | 19   | MN629790     | MN629859      |
| Original species ID       | Reassignment ID | Site | COI       | cytB       |
|--------------------------|----------------|------|-----------|------------|
| Crepidodera plutus      | correct        | 21   | MN629791  | MN629860   |
| Galerucella lineola     | correct        | 7    | MN629792  | MN629861   |
| Galerucella lineola     | correct        | 11   | MN629793  | MN629862   |
| Galerucella lineola     | correct        | 19   | MN629794  | MN629863   |
| Galerucella lineola     | correct        | 26   | MN629795  | MN629864   |
| Galerucella lineola     | correct        | 34   | MN629796  | MN629865   |
| Galerucella lineola     | correct        | 39   | MN629797  | MN629866   |
| Goniocynthia pallida   | correct        | 32   | MN629798  | MN629867   |
| Goniocynthia pallida   | correct        | 34   | MN629799  | MN629868   |
| Goniocynthia pallida   | correct        | 35   | MN629800  | MN629869   |
| Goniocynthia pallida   | correct        | 37   | MN629801  | MN629870   |
| Goniocynthia pallida   | correct        | 39   | MN629802  | MN629871   |
| Goniocynthia pallida   | correct        | 41   | MN629803  | MN629872   |
| Phratora vitellinae     | Phratora polaris | 7   | MN629804  | MN629873   |
| Phratora vitellinae     | Phratora vulgatissima | 15 | MN629805  | MN629874   |
| Phratora vitellinae     | Phratora polaris | 20  | MN629806  | MN629875   |
| Phratora vitellinae     | Phratora polaris | 26  | MN629807  | MN629876   |
| Phratora vitellinae     | correct        | 32   | MN629808  | MN629877   |
| Phratora vitellinae     | correct        | 41   | MN629809  | MN629878   |
| Plagiodera versicolora  | correct        | 6    | MN629810  | MN629879   |
| Plagiodera versicolora  | correct        | 12   | MN629811  | MN629880   |
| Plagiodera versicolora  | correct        | 16   | MN629812  | MN629881   |
| Plagiodera versicolora (a) | correct    | 20   | MN629813  | MN629882   |
| Plagiodera versicolora (b) | correct    | 20   | MN629814  | MN629883   |
| Plagiodera versicolora (c) | correct    | 20   | MN629815  | MN629884   |
| Plagiodera versicolora  | correct        | 33   | MN629816  | MN629885   |
| Plagiodera versicolora  | correct        | 39   | MN629817  | MN629886   |

Table 3.
GenBank sequences included in the phylogenetic analysis. The sample in **bold** under *Phratora polaris* was downloaded from GenBank as *P. tibialis*.

| Species (Chrysomelidae) | GenBank Accession numbers |
|-------------------------|---------------------------|
| Chrysomela vigintipunctata | AY027624, KM51318, KM443123, JN087422, KU188452, KM443640, KJ961764, KM443924 |
| Crepidodera aurata      | KJ966066, KJ962544, KF654801, KF656415, KF654798, KJ963892, KM450642, KM445873, KM444848, KM445803 |
Results

Taxonomic insights from molecular barcoding

We used DNA sequencing to test and, if necessary, refine our morphospecies assignments made previously (Canty et al. 2016). Generally, the barcoding results confirmed the morphospecies assignments and provide well-supported species clusters (Figs 1, 2). However, the Chrysomelidae barcoding analysis revealed that some specimens were incorrectly assigned in Canty et al. (2016) (Table 2; Fig. 2). These were all due to using broad morphospecies concepts for Phratora vitellinae (Linnaeus, 1758) and Crepidodera aurata Marsham, 1802. In Phratora, three specimens assigned to Phratora vitellinae clustered in the barcoding data with sequences identified on GenBank as P. polaris Schneider, 1886; and one specimen assigned to Phratora vitellinae clustered with
GenBank sequences of *P. vulgatissima* (Linnaeus, 1758). In *Crepidodera*, two specimens assigned to *Crepidodera aurata* clustered with GenBank sequences, plus our own sequences, for *C. fulvicornis* Fabricius, 1792.

In addition, we noted that certain specimens assigned to *Crepidodera aurata* formed a distinct molecular cluster, distinct from our own *C. aurata* sequences and from all others downloaded from GenBank. These specimens were the southernmost specimens of our *C. aurata* from sites 3 and 4 (Greece) and site 7 (Bulgaria). This prompted a morphological re-examination of these samples, including dissections of genitalia and these specimens were identified with *C. nigricoxis* Allard, 1878 (Fig. 3; Table 2). The two species are very similar in external morphology and variable (Fig. 3). Nevertheless, the molecular data clearly separates them (Figs 1, 2). Our sequences for *C. nigricoxis* appear to be the first to be made available for this taxon. Gavrilović and Ćurčić (2013) note that *C. nigricoxis* is found on *Salix alba* L. Although we did not distinguish willow species at the point of collection, *Salix alba* was present at all the sites where we recorded *C. nigricoxis* (Cronk et al. 2015).
Figure 2. DNA barcoding analysis using COI sequences generated in this study and from GenBank. Sequences from this study show the site number and those obtained from GenBank are indicated by a black circle (GenBank accessions given in Table 3). Node support shown only for nodes > 90% bootstrap support. Maximum intraspecific divergences are shown (for our transect samples only), estimated using uncorrected (p) distances (see methods).
Finally, our analysis indicates that a specimen from GenBank ([KM442534.1](https://www.ncbi.nlm.nih.gov/nuccore/KM442534.1): voucher GBOL_Col_FK_7108), identified as *Phratora tibialis* (Suffrian, 1851), may in fact be *P. polaris* (Table 3; Fig. 2).

**Phylogeographic patterns**

There is little phylogeographic structure evident from the sequence data, even for widely dispersed taxa along the transect. Fig. 2 (COI data) is suggestive of a split in *Crepidodera fulvicornis* between northern samples (Finland: 31, 35, 39) in one clade and southern samples (Hungary: 16, Poland: 23, Latvia: 27) in the other (e.g. a zoogeographic boundary around Estonia or the Gulf of Finland), but one sample from Finland (site 33) that only sequenced for cytB (Fig. 1) clusters with the southern clade. The absence of clear phylogeographic patterns in the chrysomelids is similar to our findings for curculionids (Canty et al. in review), but differs from those found in a hemipteran taxon (the nettle psyllid; Psylloidea, Hemiptera) sampled along the transect in which population structure suggests distinct regional clades (Wonglersak et al. 2017).

**Additional chrysomelid records from the transect**

Since the publication of Canty et al. (2016), examination of additional material from general collections by DP over the transect has brought to light some further records (all single individuals per site, unless otherwise stated). The additional records are: *Aphthona cf. lutescens* (Gyllenhal, 1808) (site 22); *Chrysomela lapponica* Linnaeus, 1758 (site 40 and also in supplementary site ii-I [site details in Cronk et al. 2015]); *Cryptocephalus ocellatus*...
Drapiez, 1819 (site 20a); *Epitrix* sp. (site 22 - two individuals); *Galerucella cf. nymphaeae* (Linnaeus, 1758) (site 37); *Galerucella cf. sagittariae* (Gyllenhal, 1813) (site 38); *Gonioctena cf. olivacea* (Forster, 1771) (site 39); *Phyllotreta cf. vittula* (Redtenbacher, 1849) (site 24); *Phyllotreta undulata* (Kutschera, 1860) (sites 27, 30); *Pachybrachis hieroglyphicus* Laicharting, 1781 (site 20a); *Pachybrachis* sp. (site 20); *Pachybrachis cf. salfii* Burlini, 1956 (site 31) ; and *Syneta* sp. (site 35). Some of these are not generally associated with willows and are probably accidental by-catch (e.g. *Galerucella nymphaeae* and *Galerucella sagittariae*). These additional records do not materially change the basic data or conclusions of Canty et al. (2016), but bring the total number of species to 47 (not 34).

**Discussion**

The barcoding, described here, provides a good example of the value of iterative molecular and morphological processes in taxonomy. In this case, a broad morphospecies concept allowed determination of those species that have the greatest geographic and morphological variation. These could then be targeted for barcoding to determine patterns of molecular variation. In the case of *Crepidodera aurata* sens. lat., this led to the distinguishing of two divergent molecular clusters. This in turn led to a re-appraisal of the morphology and to the refinement of the concept of *C. aurata* and the recognition of *C. nigricoxis* as its apparent replacement (at least in our sampling) in southern Europe (Greece and Balkans). This very small example thus serves to emphasise that morphological and molecular taxonomy, taken together and applied iteratively, are powerful adjuncts.

**Acknowledgements**

Funding for the fieldwork was partly provided by the Natural History Museum (London, UK) Life Sciences Departmental Investment Fund ([SDF13010](#)) to DMP. We thank Gavin Broad (NHM) for advice and help in the field.

**Author contributions**

RC identified and analysed the beetles, extracted DNA and contributed to the writing of the paper; ER collected the beetles and contributed to the writing of the paper; QC co-wrote the paper and contributed to the analysis and planning of the work; DP contributed to the collection of beetles, co-wrote the paper, analysed the molecular data, planned and directed the work and obtained funding for the study.

**Conflicts of interest**

None
References

• Canty R, Ruzzier E, Cronk Q, Percy D (2016) *Salix* transect of Europe: patterns in the most abundant chrysomelid beetle (Coleoptera: Chrysomelidae) herbivores of willow from Greece to Arctic Norway. Biodiversity Data Journal 4: e10194. https://doi.org/10.3897/BDJ.4.e10194

• Cronk Q, Ruzzier E, Belyaeva I, Percy D (2015) *Salix* transect of Europe: latitudinal patterns in willow diversity from Greece to arctic Norway. Biodiversity Data Journal 3: e6258. https://doi.org/10.3897/bdj.3.e6258

• Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3 (5): 294-299.

• Gavrilović BD, Ćurčić SB (2013) The diversity of the family Chrysomelidae (Insecta: Coleoptera) of the Obedska Bara Special Nature Reserve (Vojvodina Province, Serbia), with special reference to the host plants. Acta Zoologica Bulgarica 65 (1): 37-44.

• Hebert PN, Ratnasingham S, de Waard J (2003a) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London. Series B: Biological Sciences 270 https://doi.org/10.1098/rspb.2003.2218

• Hebert PN, Cywinska A, Ball S, deWaard J (2003b) Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences 270 (1512): 313-321. https://doi.org/10.1098/rspb.2002.2218

• Percy D, Crampton-Platt A, Sveinsson S, Lemmon A, Lemmon EM, Ouvrard D, Burckhardt D (2018) Resolving the psyllid tree of life: phylogenomic analyses of the superfamily Psylloidea (Hemiptera). Systematic Entomology 43 (4): 762-776. https://doi.org/10.1111/syen.12302

• Swofford DL (2003) PAUP*: phylogenetic analysis using parsimony (*and other methods). 4. Sinauer, Sunderland, MA..

• Tautz D, Arctander P, Minelli A, Thomas R, Vogler A (2003) A plea for DNA taxonomy. Trends in Ecology & Evolution 18 (2): 70-74. https://doi.org/10.1016/s0169-5347(02)00041-1

• Wonglersak R, Cronk Q, Percy D (2017) *Salix* transect of Europe: structured genetic variation and isolation-by-distance in the nettle psyllid, *Trioza urticae* (Psylloidea, Hemiptera), from Greece to Arctic Norway. Biodiversity Data Journal 5: e10824. https://doi.org/10.3897/bdj.5.e10824