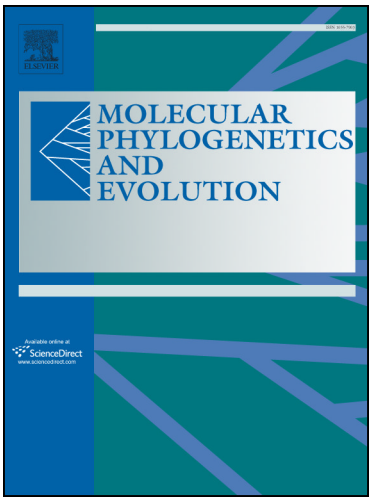

Species delimitation within the *Bothryorrhynchapion* weevils: multiple evidence from genetics, morphology and ecological associations

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Species delimitation within the *Bothryorrhynchapion* weevils: multiple evidence from genetics, morphology and ecological associations

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Abstract

Curculionidae is a hyperdiverse group of beetles, whose taxonomy and phylogenetics is still poorly understood, especially at the genus level. The latest work on the evolution of Apionini showed a noticeable "mess" in the subtribe Oxystomatina, where most of the morphology-based genera were found to be polyphyletic or paraphyletic. These discrepancies between classical taxonomy and molecular phylogenetics implied the need for further taxonomic revision of these groups. Here, we used sets of morphological, molecular and ecological characters to verify the taxonomic statuses and disentangle the phylogenetic relations among the *Bothryorrhynchapion* apionids, which are classified as a subgenus of *Cyanapion*. Morphological data including morphometrics, and multilocus molecular analyses confirmed the monophyly of the *Bothryorrhynchapion* and species statuses of five species. The morphological analyses showed that *Cyanapion* (*Bothryorrhynchapion*) *protractum* (Sharp, 1891) from the southeast Palaearctic is a synonym of *C.* (*B.*) *gyllenhalii* (Kirby). Moreover, ecological features (host plant use and presence/absence of the endosymbiotic bacteria *Wolbachia*) helped to unravel the relations among the examined weevils. The speciation of *Bothryorrhynchapion* apionids was probably affected by allopatric distribution, shifts in the preferred host plants (*Vicia* sp. or *Lathyrus* sp.) of sympatric taxa, and infection by different strains of *Wolbachia.* The paper presents the first comprehensive description of the species' morphology, biology and ecology, and includes a key to the species.

Key words: Curculionoidea**;** *Cyanapion*; *Wolbachia;* integrative taxonomy; phylogenetics; systematics

1. Introduction

Molecular data plays a central role in modern systematics for the delimitation of taxa and understanding relations among them (Avise and Ball, 1990; Sites and Marshall, 2003; Hebert and Gregory, 2005). This has been accelerated by the fast development of various techniques, methods and molecular databases, among which the most promising and reliable are based on DNA/RNA sequencing. Within hyperdiverse groups of insects, systematic studies are especially challenging in sibling taxa or groups of closely related species (e.g., Gómez-Zurita et al., 2012; Montagna et al., 2016). In such cases, various approaches can be used to shed light on the factors that have influenced the evolution of the taxa. When systematics are complex, an "integrative taxonomy" approach that integrates data on morphometrics, genetics and even ecological traits is often required to reliably delineate and identify species (Dayrat 2005; Schlick-Steiner et al., 2010). Such studies may consider the basic ecological traits of the examined taxa (e.g., habitat requirements and interactions with other organisms, such as the host plants of herbivores). Moreover, recent findings suggest that another ecological trait worth considering is the diversity of associated organisms (Valentini et al., 2009) – particularly that of bacteria (e.g., Steinert et al., 2000; Hosokawa et al., 2006). This latter factor could be especially important for arthropods, as some parasites or symbionts, particularly intracellular ones, can directly influence host fitness, development and diversity, which may in turn also have implications on host speciation (Hurst and Jiggins, 2000; Engelstädter and Hurst, 2009). Notable examples of such endosymbionts/parasites are the maternally inherited bacteria belonging to the genus *Wolbachia* (Bourtzis and O'Neill, 1998; Stouthamer et al., 1999; Kikuchi, 2009; White et al., 2011).

Although weevils have been the object of numerous taxonomic and phylogenetic studies based on morphology, ecology and/or genetics (e.g., Kuschel, 1995; Oberprieler, 2000; Wanat, 2001; Marvaldi et al., 2002;-McKenna et al., 2009), most of these studies have focused on family-level phylogenies or covered species of several major weevil groups. In contrast, comprehensive morphological and/or phylogenetic studies on Apioninae and especially on the most speciose tribe Apionini are still scarce (e.g., Kissinger, 1968; Alonso-Zarazaga, 1990; Wanat, 1995; Winter et al., 2017).

The latest work on the evolution of Apionini (Winter et al., 2017) presents the first molecular phylogenetic hypothesis considering a majority of the lineages within the tribe. It confirmed the monophyly of most of the subtribes erected earlier by Alonso-Zarazaga (1990), with the exception of Oxystomatina, Kalcapiina and Aspidapiina. "A noticeable mess" was

found in Oxystomatina where most of the morphology-based genera were found to be polyphyletic or paraphyletic. These discrepancies between classical taxonomy and molecular phylogenetics in Oxystomatina implied "(…) the need for further taxonomic revision of these groups." (Winter et al., 2017).

Here we focus on a small group of Oxystomatina that is currently classified in the genus *Cyanapion* Bokor 1923. Following basic re-classification of the Apionini by Alonso-Zarazaga (1990), this genus consists of two subgenera: *Cyanapion* s. str. and *Bothryorrhynchapion* Bokor, 1923. The subgenus *Bothryorrhynchapion*, as defined by Dieckmann (1976), contains five valid species. One of them, *C. (B.) offensum* Faust from Crimea, remains quite enigmatic and is still only known from a single holotype collected over a hundred years ago. Of the remaining four species, three are relatively common in the western Palaearctic and well recognized in many European identification keys (e.g. Dieckmann, 1977; Lohse, 1981; Ehret, 1990; Gønget, 1997). The fourth species, the little known *C. (B.) gnarum* Faust originally described from Abakan (Central Siberia), was long considered an Asiatic species, until Silfverberg (1992) recorded it in Russian Karelia (detailed data of the only available specimen was provided in 1997 by Gønget). There are no further records of this species from Scandinavia nor Northern Europe, but it has since been discovered in NE and SW Poland (Wanat, 1994, 2009). In the meantime, it has also been found in several regions of Russian Asia (Legalov, 2002): thus the species seems to represent a typical Eurosiberian zoogeographic element reaching Karelia and Poland, where it forms two disjunct metapopulations at the western extremes of its range.

Among the species of the *Bothryorrhynchapion* group, *C. (B.) gnarum* is morphologically very close to *C. (B.) platalea*. Morphological differences between *C. (B.) platalea* and *C. (B.) gnarum* are marginal and actually only concern the shape of their rostra, which are more subapically constricted in *C. (B.) platalea*. Considering the fact that their ranges overlap in Poland and Czechia (Wanat, 2005, 2009; Benedikt et al., 2010; Wanat et al., 2016), the question arises whether they are indeed distinct species. The question may also actually involve *C. (B.) afer*, since males of this species express much greater variability in the lengths and shapes of their rostra than the two allied species, and some specimens are difficult to distinguish on morphological grounds from males of *C. (B.) platalea* and *C. (B.) gnarum*. This encouraged us to employ genetic methods for testing the hypothetical taxonomic division of *Bothryorrhynchapion*, which has traditionally been based on morphology.

Except for Winter et al., (2017), there have been only two previously published hypotheses on the molecular phylogenetics of *Cyanapion* species. Ptaszyńska et al. (2012) included two species of *Cyanapion* (s. str.), namely *C. columbinum* and *C. spencii*, and Stüben et al. (2015) considered *C.* (*B.*) *gyllenhalii*. In both these studies phylogenetic analyses were based only on mitochondrial sequences. There are no studies on *Wolbachia* occurrence and diversity in Oxystomatina apionids, but also – the potential role of endosymbiotic bacteria in the speciation and systematics of Apionini, Apioninae and Brentidae has been completely neglected so far.

As the work of Winter et al. (2017) showed that the phylogenies within *Cyanapion* could be discordant with the classical taxonomies, in this study we focused on determining the species distinctiveness and relations of the *Cyanapion* subg. *Bothryorrhynchapion*. Using different sources of morphological, DNA-based and ecological data, we aimed to do the following: to i) disentangle the phylogenetic relationships among the species of *Bothryorrhynchapion* with respect to some selected *Cyanapion*, ii) verify species distinctiveness for pairs of sibling taxa, i.e. *C. (B.) gnarum* - *C. (B.) platalea, C. (B.) afer* - *C. (B.) offensum*, using combined genetic and morphological methods, iii) test the congruence between morphological and molecular species within *Cyanapion* and *Bothryorrhynchapion*, iv) verify if species relatedness is connected with an affinity to particular host plants, and v) test for the presence of *Wolbachia* in the selected species in order to verify its possible role in their speciation.

2. Material and methods

2.1. Depository abbreviations

The following institutional abbreviations were used for depositories of studied material: MIZW - Museum and Institute of Zoology, Polish Academy of Sciences, Warsaw; MNHW - Museum of Natural History, University of Wrocław; NHM - The Natural History Museum, London; SMTD - Senckenberg Natural History Collections, Dresden.

2.2. Sampling

All the 44 specimens of *Bothryorrhynchapion* species used for DNA sequencing were collected in 2005-2016 in Poland and Ukraine. Additionally, two species from the nominotypic subgenus (*C. (C.) columbinum* and *C. (C.) spencii*) (12 specimens of each) were collected in Poland (Table A.1). *Synapion ebeninum* (Kirby, 1808) was selected as a close outgroup, and the broad-nosed weevils ((Curculionidae: Entiminae): *Polydrusus inustus* Germar, 1824 and *Centricnemus leucogrammus* (Germar, 1824)) were selected as more

distant outgroups. In the case of *cox1* dataset, in order to avoid the possible long-branch attraction of ingroup taxa towards the root of the tree due to homoplasy in *cox1*, a further close outgroup was selected on the basis of the topology inferred by Winter et al. (2017), *viz*. *Ischnopterapion modestum* HQ165405 (Apionidae, Oxystomatini) and the two Entiminae species were excluded.

The morphological study was based on 633 specimens of the genus *Cyanapion* preserved with the junior author (M. Wanat) and at the historical museum collections at MNHW, MIZW and SMTD (the holotype of *Apion offensum* Faust). They originated from the various parts of the species' ranges, listed under the particular species descriptions in Appendix A.

2.3. Morphological measurements and analyses

The measurements used for counting the set of indices presented and explained in Table B.1 were carried out with a calibrated stereomicroscopic grid eyepiece from 70 specimens of five *Bothryorrhynchapion* species, which were selected in an attempt to cover the whole morphological variation. Body length was measured in side view from the base of the rostrum to the apex of elytra.

The morphological study resulted in the construction of concise differential diagnoses of the subgenus *Bothryorrhynchapion*, including all five of its species, supported with basic data on their distribution and biology and presented in full in Appendix A. Regarding terminology, terms for body parts including wing venation generally followed Oberprieler et al. (2014) and those for structures (e.g., elytral setae, aedeagal tegmen) specific for Apioninae followed Alonso-Zarazaga and Wanat (2014). The way, in which morphological micrographs were taken and prepared, is described in Appendix B.

Non-metric multi-dimensional scaling (NMDS, Kruskal, 1964) analyses were performed on the measurements of morphological features reported in Tables C.1-2 (males and females, respectively) to graphically ordinate the specimens and assess the differences among morphospecies. NMDS analyses were performed with the Bray-Curtis dissimilarity index using the *metaMDS* function of the R package vegan (Bray and Curtis, 1957). The correlation between the NMDS ordination scores and the morphospecies assignment was investigated with the *envfit* function in vegan. The permutation of the dissimilarity matrix allowed the significance of the fitted factor to be assessed and a squared correlation coefficient (r2) to be calculated.

2.4. Molecular analyses

2.4.1. Laboratory procedures

Three different weevil genes were amplified, sequenced and used for the following analyses: partial sequence of the mitochondrial cytochrome oxidase subunit I (*cox1*), the nuclear elongation factor 1-alfa (*ef-1α*) and the internal transcribed spacer 2 (*ITS2*) between *5.8S* rRNA and *LSU* rRNA genes. *Wolbachia* infection was genotyped in respect to all five genes included in Multilocus Sequence Typing system accepted for *Wolbachia* (Baldo et al., 2006). Applied laboratory protocols, as well as GenBank accession numbers of all specimens and gene fragments sequenced in this study can be found in Appendix C. After trimming of sequences, the final nucleotide alignments constituted of 760 bp for *cox1*, 680-810 bp for *ef-1α* and 591-659 bp for ITS2. Differences in the length of nuclear sequences were due to presence of indels, which were absent in mitochondrial dataset. Considering *Wolbachia* MLST genes, the nucleotide alignments resulted of a length of 415 bp for *gatB*, 438 bp for *coxA*, 457 bp for *hcpA*, 472 bp for *ftsZ* and 445 bp for *fbpA*.

2.4.2. Phylogenetic analyses and divergence time estimations for weevils

Saturation level of the used markers (*cox1*, *ef-1α* and ITS2) was assessed with the Xia test (Xia, 2009) implemented in DAMBE5 (Xia, 2013) and low level of saturation was recovered only for *cox1* ($Iss = 0.132 < Iss.c = 0.735$; $p < 0.0001$). The best partitioning scheme and models of nucleotide evolution fitting our sequence dataset were estimated by PartitionFinder2 (Lanfear et al. 2016) and are reported in Table D.1.

Phylogenetic analyses were performed adopting Bayesian inference (BI) and maximum likelihood (ML) approach on each single gene dataset, to obtain single-gene topology used in species delimitation analyses, as well as on the three markers using the selected partitioning scheme. BI was performed using MrBayes 3.2.2 (Ronquist et al., 2012) in two independent runs, with one cold and five heated Markov chains each, for $2 \times 10^{\wedge 7}$ generations that were sampled every 100 generations. Stationarity was considered to be reached when the average standard deviation of split frequencies was less than 0.01; the convergence of each run were also visually inspected using TRACER (Drummond et al., 2012). An appropriate number of sampled trees were discarded as burn-in and a majority-rule consensus tree was obtained. The ML analyses were performed using IQ-TREE v.1.4.0 (Nguyen et al., 2015), implementing the selected partitioning scheme and the model of nucleotide substitutions; branch support was obtained by the Approximate Likelihood-Ratio Test (aLRT) (Anisimova and Gascuel, 2006).

Dating analysis was inferred using the three datasets, *viz cox1*, *ef-1α* and ITS2. The datasets were tested for strict clock model against non-clock models using a Bayes factor comparison. The marginal likelihoods of the models were estimated using MrBayes 3.2.2 (Ronquist et al., 2012) by the stepping stone method, and then compared according to the

criterion reported in Kass and Raftery (1995). The strict clock model was preferred for all the tested datasets.

Ages and confidence intervals of branching events were estimated with BEAST v1.8.4 (Drummond et al., 2012). Considering the absence of fossils and biogeographic events useful for calibrating inner nodes, to tentatively provide a temporal framework for the evolution of *Cyanapion* species we adopted the estimated mean evolutionary rates of another family of the same coleopteran infraorder (Papadopoulou et al., 2010). The adopted evolutionary mean rates (mean number of substitutions per site divided by tree length ± standard deviation) were: 0.0177 ± 0.0019 for the partitioned *cox1* (corresponding to a divergence rate of 3.54% My^{-1}), 0.0017 ± 0.0003 for the partitioned *ef-1α* exons (divergence rate of 0.34% My^{-1}), and 0.0184 ± 0.0152 for ITS2 and *ef-1α* intron (divergence rate of 3.68% My^{-1}). We adopted a normal prior distribution for the clock rate with the previously reported values as the mean and standard deviation. Two independent runs were performed using the following parameters: Markov chain length of $3 \times 10^{\wedge 8}$ generations; sampling of trees and parameters every 5000 generations; models of nucleotide evolution as obtained by the model selection analysis; birth-death process as the tree prior (Gernhard, 2008); other priors were set to their default values. The convergence of the two runs was examined with TRACER (Drummond et al., 2012),the runs were then pooled after removal of the tree burn-in fraction. The majority-rule consensus tree was obtained using TreeAnnotator (Drummond et al., 2012).

2.4.3. Species delimitation

Molecular species delimitation analyses were performed with different independent methods with no *a priori* information about the morphospecies, as has been described in previous studies (Montagna et al., 2016a, b). In brief, tree-based methods represented by the generalized mixed Yule-coalescent model (GMYC; Pons et al., 2006), its Bayesian implementation,(bGMYC; Reid and Carstens, 2012) and the Poisson tree process model (PTP; Zhang et al., 2013) were adopted in this study to delimit the species. These methods have been used extensively to recognize and delimit species(e.g., Cranston and Krosh, 2015; Lecocq et al., 2015; Montagna et al., 2016b) as well as to help describe new insect taxa (e.g., Montagna et al., 2013). The Bayesian trees obtained by MrBayes analyses were used as input for the delimitation analyses, after ultrametric conversion with the penalized likelihood strategy implemented in r8s 1.7 (Sanderson, 2003)in the cases of GMYC and bGMYC, or without ultrametic conversion in the case of bPTP. Single-threshold GMYC and bGMYC

analyses were performed using the R packages SPLITS and bGMYC, respectively.*2.5. Wolbachia infection*

Allelic profiles of MLST genes were generated for each infected individual. For all MLST genotypes, as the best model were selected GTR + I (MLST genes from *Cyanapion* weevils) and GTR + G (MLST genes from *Cyanapion* plus the reference sequences). An approach similar to that of Montagna et al. (2014, 2015) was adopted to compare allelic profiles generated from *Cyanapion* beetles with some representative sequence types from other species that harboured bacteria belonging to different supergroups and from European beetles with a complete allelic profile (see caption of Figure 4). We then used the generated alignment of MLST genes for the construction of a phylogenetic tree and network. The phylogenetic tree was reconstructed for the whole dataset using ML approach implemented in PhyML 3.0 under the settings described above. The unrooted phylogenetic networks were prepared for each MLST gene separately in SplitsTree4 (Huson and Bryant, 2006) with the use of neighbour-net algorithm distance estimates. The PHI test implemented in SPLITSTREE v. 4 has been shown to identify the presence/absence of recombination within a range of sequence samples (both insect and bacterial markers) with a low false-positive rate (Bruen et al., 2006). The PHI test rejects hypotheses assuming recombination among MLST genes ($p = 0.986$ if consider all sequences, $p = 0.174$ if consider only unique strains). Additionally, the most similar hits to all MLST (gene) sequences generated from *Cyanapion* weevils were identified with the BLAST search tool (Altschul et al., 1990) against NCBI GenBank resources.

2.6. Host plant data

Information on host plant use of the examined *Cyanapion* weevils was primarily derived from literature searches for any information on their biologies and ecologies (e.g. Dieckmann, 1976, 1977; Ehret, 1983, 1990; Gønget, 1997), supplemented with unpublished field observations of M. Wanat.

3. Results

3.1. Morphological analyses

The morphological distinctness of all the studied species of the subgenus *Bothryorrhynchapion* was confirmed and is summarised in the identification key. The key and detailed morphological descriptions are provided in the supplementary files (Appendix A), which also contain diagnoses of the subgenera of *Cyanapion* and species of the subgenus

Bothryorrhynchapion, basic nomenclatural and distributional comments, and illustrations of relevant morphological characters.

The variation of metric characters is summarised in Table B.1 as a set of relative indices based on 11 measurements of rostra, heads, pronota and elytrae. The non-metric multi-dimensional scaling analyses, performed on morphometric tables, reached stable solutions at two dimensions, with stress values of 11% and 7.4% for male and female measurements, respectively. The NMDS ordination scores fitted significantly (p-value < 0.001) with the species-grouping factors, with r^2 correlation coefficients of 0.88 and 0.83 (for female and male specimens, respectively). This indicates that the selected morphometric variables efficiently discriminate between the selected *Cyanapion* species. The pattern is highlighted by the non-overlapping standard error ellipses representing the 95% confidence area around the mean of the grouping factor (Fig. 1). In female specimens, on the first NMDS axis a transition from *C. platalea*, *C. gnarum*, *C. afer* and *C. gyllenhalii* was observed (Fig. 1), while on the second axis a separation between the groups *C. gnarum – C. afer* and *C. platalea – C. gyllenhalii* was observed, with *C. offensum* clearly separated (Fig. 1). Male specimens also showed a similar pattern (Fig. 1).

3.2. Molecular analyses

3.2.1. Phylogenetics and dating

Phylogenetic trees inferred from each of the three markers separately (with one representative individual per species) as well as the tree inferred using the three markers with the selected partitioning scheme resulted in congruent topologies (Fig. 2). Firstly, the monophyly of *Cyanapion* weevils was confirmed (Fig. 2), with the monophyly of *Cyanapion* s. str. and *Bothryorrhynchapion* strongly supported in the phylogenetic trees inferred from the three markers together (Fig. 2D) and the nuclear *ef-1α*. ITS2 gave weak support for the monophyly of the two subgenera and *cox1* gave no support (Fig. 2A,C). All the obtained phylogenies strongly confirmed the close relationship between *C. (B.) gnarum* and *C. (B.) platalea*. These two sibling taxa constituted a sister clade to *C. (B.) afer* in all topologies except that based on *cox1* (Fig. 2A). The fourth examined species of this subgenus, *C. (B.) gyllenhalii*, appeared to be the most external with respect to the other *Bothryorrhynchapion.* This was based on topologies derived from the dataset with the three markers partitioned, from *ef-1α* and from ITS2, while based on the ITS2 topology this node had weak support (Fig. 2). Moreover, all phylogenetic trees (Fig. 2) consistently supported *C. (C.) columbinum* and *C. (C.) spencii* being sister taxa. The results of the BEAST analysis, even though they should be considered with caution due to the adopted evolutionary mean rates, supported the following timeframe

for *Cyanapion* weevil evolution: i) the last common ancestor of *Cyanapion* weevils ocurred approximately 30.2 Mya (CI 22.5 to 38.7 Mya); ii) that of *Bothryorrhynchapion* ocurred ~26 Mya (CI 19.6 to 34 Mya); iii) the split between *C. (B.) afer* and *C. (B.) gnarum-C. (B.) platalea* ~18 Mya (CI 13.5 to 23.9 Mya); iv) the divergence of *C. (B.) gnarum* and *C. (B.) platalea* ~ 5 Mya (CI 3.5 to 7 Mya) and that of *C. (C.) spencii* and *C. (C.) columbinum* approximately 15.6 Mya (CI 11 to 20.5 Mya).

3.2.1. Species delimitation

Species delimitation analyses performed with coalescent tree-based approaches gave almost identical results. These confirmed, based on the nucleotide sequences of three markers (i.e., the mitochondrial *cox1* and the nuclear *ef-1α* and ITS2), the species statuses of the analysed morphospecies. For the *cox1* marker (eight morphospecies were included in the dataset, of which two were outgroups), the GMYC model exhibited a significantly better likelihood than the null model (*p-value* < 0.001; $\log L_{GMYC} = 158.2$, $\log L_{NULL} = 144.2$) and nine maximum likelihood entities (95% CI [8,9]) were identified at the Yule-Coalescent threshold (Fig 3A). The identified entities corresponded to the eight morphospecies included in the dataset, with the exception of *Synapion ebeninum* where the two specimens were separated into two lineages due to their deep coalescence. At the lowest value of the confidence interval (i.e., eight entities), the two specimens of *S. ebeninum* clustered in a single entity. Similar results were obtained using bGMYC and bPTP (nine partitions recovered with Bayesian posterior probabilities of ≥ 0.95 and ≥ 0.9, respectively; Fig. 3A).

The analyses performed on the two nuclear markers confirmed the results obtained with *cox1*. GMYC models identified 9 maximum likelihood entities (p-value < 0.001; 95% CI) from both *ef-1α* and ITS2, which corresponded to the nine morphospecies. At the highest value of the confidence interval, *C. gnarum* was divided into two entities using *ef-1α,* while *Cyanapion columbinum* was split into two entities using ITS2 (Fig 3B,C). bGMYC and bPTP almost confirmed the GMYC results; the bGMYC method was an exception, *C. gnarum* was divided into two lineages using *ef-1α*, and *C. columbinum* was divided into two lineages using ITS2 (Fig. 3B,C).

Nucleotide divergence (presented in Table E.1) supported the abovementioned data on species distinctiveness. The two most closely related taxa (*C. (B.) gnarum* and *C. (B.) platalea*) had the shortest pairwise distances (approx. 5% for *cox1* and approx. 1% for nuclear markers), followed by *C. (C.) columbinum* and *C. (C.) spencii* (mtDNA approx. 9%, nucDNA 3-5%). Intraspecific distances were always shorter than interspecific distances, showing a clear barcoding gap between the previously defined taxa. All pairwise distances between *C.*

(B.) gnarum and *C. (B.) platalea* were intermediate between the other intra- and inter-specific pairwise distances calculated in this study (Fig. B.1).

3.3. Wolbachia diversity

Amplification of MLST genes confirmed the presence of *Wolbachia* in three out of the six examined *Cyanapion* species (i.e., *C. (B.) afer*, *C. (B.) gyllenhalii* and *C. (C.) spencii*). All examined specimens of the first two species were infected, while *C. (C.) spencii* from Mielnik (Csp-II) did not harbour *Wolbachia*. *Wolbachia* gene diversity differed between species and in some cases also within species (Table F.1). All populations of *C. (B.) afer* were infected by a single strain, except the Pińczów population (Baf-I), which was infected by two strains. *C. (B.) gyllenhalii* was infected by a single strain across its entire examined range. In *C. (C.) spencii* three strains were detected, but were restricted to particular locations − at each location only single strains were found. Interestingly, in some cases MLST (gene) sequences obtained from single individuals belonged to different *Wolbachia* supergroups (Table F.1), as was the case for *C. (B.) afer* where gatB, coxA and hcpA were from both supergroups A and B, while ftsZ and fbpA belonged only to supergroup A. A single strain from *C. (B.) gyllenhalii* had all its genes assigned to supergroup A, except *coxA*, which was closest to supergroup B. The most complicated patterns were found in *C. (C.) spencii*, as each strain constituted a mix of genes belonging to either A or B supergroup (Table F.1); in addition there were two variants of *coxA*, both from supergroup B (named here B1 and B2) (Table F.1).

Phylogenetic relations between the identified *Wolbachia* strains in the three infected species showed that Bgy-strain1, Baf-strain1 and Csp-strain3 were almost identical and both clearly belonged to supergroup A, whereas all other strains clustered between *Wolbachia* supergroups A and B (Fig. 4).

A comparison of MLST profiles obtained from the infected *Cyanapion* species with sequence types in the MLST database showed that there were no identical or even similar strains in the database: the same sequence types could only be found for one or two genes (Table G.1), e.g. ST 68 (which only fits for gatB and fbpA genes) from the cricket *Teleogryllus taiwanemma*, and ST 303 (only fits for gatB and hcpA genes) from the parasitic wasp *Leptopilina clavipes*. A similar procedure using GenBank (NCBI) (Table G.1) identified several insect taxa possessing at least some similar genes.

3.4. Host plant use

Field observations made by one of the authors (MW) have found that the examined *Cyanapion* species are oligophages or monophages of Fabaceae: namely they feed on *Vicia* spp. or *Lathyrus* spp. These observations are concordant with previous findings available in the literature and extended knowledge on host plants, particularly by confirming the monophagy of *C. (B.) gnarum* on *Lathyrus sylvestris* suggested by Wanat (1999) and adding *L. palustris* to the list of plants regularly visited by *C.* (*B.*) *platalea.* A full list of host plants identified for the examined *Cyanapion* weevils is presented in Table 1.

3.5. Taxonomic decisions

After the analysis of the original description and other available evidence, *Apion protractum* Sharp, 1891 is here newly synonymised with *C.* (*B.*) *gyllenhalii* Kirby, 1808. This case is discussed in more detail in Appendix A.

4. Discussion

The present study examines details of species delimitation and relations within a relatively small group of Oxystomatina – the subgenus *Bothryorrhynchapion*. According to Winter et al. (2017), the *Bothryorrhynchapion* may not be so closely related to other *Cyanapion* species as was assumed based on their general morphological characters (Alonso-Zarazaga 1990), and may even require a genus of their own.

First of all, our phylogenetic analyses support the monophyly of *Bothryorrhynchapion* apionids. The only exception being the confusing topology inferred from the mitochondrial DNA, where presumably the outgroup taxa − *Ischnopterapion modestum* and *Synapion ebeninum* (members of the sister subtribe Synapiina) − disrupted the monophyly of *Bothryorrhynchapion*. The most probable explanation for the observed pattern, which was also supported by the low Bayesian posterior probabilities and bootstrap values of the nodes, is the high homoplasy of *cox1,* which precludes the proper reconstruction of these relationships. This study did not attempt to verify the monophyly of the whole *Cyanapion* genus, as this problem had already been previously examined by Winter et al. (2017), who rejected the monophyly of these apionids, at least with respect to the used mitochondrial and nuclear genes. However, the phylogenetics of *Cyanapion* apionids based on the nuclear DNA data collected in this study is different than that based on mtDNA. Both *Cyanapion* and *Bothryorrhynchapion* consistently formed monophyletic clades on the *ef-1α* and ITS2 phylogenetic trees. This assumption needs to be re-examined with a much larger number of Oxystomatina species.

Within *Cyanapion* and particularly *Bothryorrhynchapion* exist pairs of sibling taxa, or even groups of hardly distinguishable species that had been previously described on the basis of their basic morphological characters. i) *C. (B.) gnarum* with *C. (B.) platalea*, (ii) *C. (B.) afer* with *C. (B.) offensum* and (iii) all these species together.

Morphologically, all these species can be unambiguously identified from their unique combinations of measured rostrum, head and postabdomen characters (Table B.1), which are illustrated in the descriptions and finally incorporated into a newly proposed identification key (Appendix A). Analysis of the morphological characters confirmed the close relationships of all species of the subgenus *Bothryorrhynchapion*. These were especially reflected in the uniform structure of male terminalia, which turned out to be practically useless for identifying all species except *C.* (*B.*) *gyllenhalii*, while served well for recognizing the subgenera *Bothryorrhynchapion* and *Cyanapion s. str*. *Cyanapion* (*B.*) *gyllenhalii* is by far the most morphologically distinct among the *Bothryorrhynchapion* both in rostrum and head characters and male terminalia, while the other species are much more similar to each other. Our thorough study discovered just a few new characters useful for identifying particular species, like the peculiar shape of the apex of the penal tectum in *C.* (*B.*) *gyllenhalii,* bursal sclerites in the female of *C.* (*B.*) *afer* or vanishing metaventral tubercle in the male of the same species. The latter character is especially helpful in distinguishing males of the triplet *afer-gnarum-platalea* because of the highly variable rostrum shape in *C.* (*B.*) *afer*. This character could be responsible for the low discrimination between *C. (B.) afer* and *C. (B.) gnarum* males in the multidimensional scaling plot.

With respect to molecular data, all these taxa form well defined evolutionary lineages and all delimitation methods used in this study consistently divided the examined individuals into four *Bothryorrhynchapion* morphospecies (unfortunately *C. (B.) offensum* could not be included in this step of the research as the species is known only from the holotype collected over a hundred years ago). This was also the case for the two *Cyanapion* s. str. species: *(C.) columbinum* and *C. (C.) spencii*, included as close outgroups in this study. Moreover, the nucleotide distances between members of the nominative subgenus *Cyanapion* and members of *Bothryorrhynchapion* species are relatively large (8-15% in *cox1,* 3-9% in *ef-1α* and 4-6% in ITS2), further indicating that all these taxa are undoubtedly separate species. The only exception is the pair *C. (B.) gnarum − C. (B.) platalea*, which are distant by approx. 5% (*coxI*) or approx. 1% (*ef-1α* and ITS2), so still within the general interspecific distance range observed in beetles (e.g., Langor and Sperling, 1997; Pons et al., 2006; Montagna et al., 2016). Species distinctiveness based on nucleotide distances was also supported by the

existence of a clear barcoding gap, and this refers not only to mtDNA but also to nuclear markers. This gap was only partially diffused by the lower interspecific distances observed between *C. (B.) gnarum* and *C. (B.) platalea* (especially with respect to mtDNA).

Regarding the timing of evolution, the *Cyanapion* genus probably originated 22-39 Mya in the Eocene/Miocene, whereas the subgenus *Bothryorrhynchapion* probably arose 20-34 Mya, between the end of the Eocene and beginning of the Miocene. The most similar group of species from this genus, *C. (B.) gnarum*, *C. (B.) platalea* and *C. (B.) afer* evolved 13-24 Mya at the end of the Oligocene or during the Miocene, and the "youngest" pair of species, *C. (B.) gnarum* and *C. (B.) platalea* probably split at the border between the Miocene and Pliocene (3.5-7 Mya). The presented divergence times for *Bothryorrhynchapion* apionids, even if estimated without calibration with fossils or biogeographic events, are consistent with estimates obtained in the recent work on Apionini (Winter et al., 2017). Speciation of *Cyanapion* apionids seems to be quite ancient, compared with, e.g., the divergence times estimated for similar groups of closely related leaf beetles from the *Cryptocephalus hypochaeridis / C. sericeus* and *Cryptocephalus flavipes* complexes, (Gómez-Zurita et al., 2012; Montagna et al., 2016) or weevils from the *Mecinus heydenii* species complex (Toševski et al., 2014), whose members speciated from the late Miocene until the late Pleistocene. On the other hand, there are also examples of species groups that show similar levels of interspecific divergence to *Cyanapion* (e.g., *Crioceris* leaf beetles; Kubisz et al., 2012). There probably exist great differences in the evolution of beetles belonging to different tribes and genera, which may be connected with the diversification of ancestral taxa onto different host plants (Bernays and Chapman, 1994; Farrell, 1998; Jolivet, 1998). This could apply to *Cyanapion* apionids, as these weevils are oligophages of Fabaceae and particular species feed on either *Vicia* spp. or *Lathyrus* spp. Interestingly, within both the *Cyanapion* and *Bothryorrhynchapion* subgenera there are species adapted to either vetches or vetchlings; this strongly suggests that in the evolution of these weevils, a switch between host plants must have occurred, which could have promoted sympatric speciation. The three most closely related species, *C. (B.) gnarum*, *C. (B.) platalea* and *C. (B.) afer*, develop on *Lathyrus* spp.; however, the first is a monophage of *L. sylvestris*, the second is an oligophage of pink-flowering vetchlings, and the last is apparently a monophage of the yellow-flowering *L. pratensis*. The only seemingly overlapping populations of *C. (B.) gnarum* and *C. (B.) platalea* are known locally in Poland and Czechia (Wanat, 2005, 2009; Benedikt et al., 2010; Wanat et al., 2016). Considering their total ranges, these two species most probably originated via

allopatric speciation in two distant refugia and then spread out and met in eastern and central Europe.

Wolbachia screening showed infection in three out of the six examined species − in *C. (B.) afer*, *C. (B.) gyllenhalii* and *C. (C.) spencii*. Interestingly, this bacterium was only found in half of the studied species, and generally was present in all individuals and populations of the infected species (with the minor exception of a single uninfected population of *C. (C.) spencii*). This pattern suggests that *Wolbachia* could have been responsible for divergence and speciation in *Cyanapion* and *Bothryorrhynchapion* apionids, possibly through cytoplasmatic incompability between infected and uninfected specimens in ancestral taxa (Dobson et al., 2002). Interestingly, in *C. (C.) spencii* the diversity of strains was quite high, whereas in *C. (B.) gyllenhalii* and generally in *C. (B.) afer*, only a single *Wolbachia* strain was present. This observation suggests that the transmission of bacteria strains between taxa is possible (Vavre et al., 1999; Yang et al., 2013). This was supported by a single population of *C. (B.) afer* from the Nida valley in Poland being double infected by the strain specific to this species, but also by a strain very similar to that found in *C. (C.) spencii*. The source of this similarity is uncertain, as despite sharing common habitat, the two species feed on different host plants. Moreover, this complex diversity of strains, with MLST genes belonging to different supergroups, suggests that transmission must have occurred in association with recombination between the different strainss. This is a known phenomenon in *Wolbachia* (Yang et al., 2013) and has been previously reported for some weevils (Lachowska et al., 2010). The speciation of *Cyanapion* and *Bothryorrhynchapion* could also have been connected with infection by intracellular bacteria that have known effects on host reproduction and speciation (O'Neill et al., 1992; Rousset 1992; Werren, 1997).

Conclusions

The integrative taxonomy approach used in this study, which included morphology, molecular phylogenetics, species delimitation and ecological features (host plants, endosymbionts), supported the monophyly of *Bothryorrhynchapion* apionids. Moreover, all of the examined species, including the sibling pairs of questioned taxa like *C. (B.) gnarum* with *C. (B.) platalea*, and *C. (B.) afer* with *C. (B.) offensum*, were found to be undoubtedly distinct species, with morphological, ecological and genetic features congruently dividing them. This subgenus originated between the end of the Eocene and beginning of the Miocene and the descendent species split later, from the Oligocene-Miocene transition to the Pliocene. Finally, the speciation of *Cyanapion* was probably caused by the use of different host plants by

sympatrically occurring species, geographical isolation of allopatric taxa, and could also have been facilitated by the infection of endosymbiotic bacteria like *Wolbachia*.

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References

Alonso-Zarazaga, M. A 1990. Revision of the supraspecific taxa in the Palaearctic Apionidae Schoenherr, 1823 (Coleoptera, Curculionoidea). 2. Subfamily Apioninae Schoenherr, 1823: introduction, keys and descriptions. Graellsia 46, 19-156.

Alonso-Zarazaga, M. A., 2011. Apionidae, pp. 148-176. - In I. Löbl & A. Smetana (ed.): Catalogue of Palaearctic Coleoptera, Vol. 7. Stenstrup, Apollo Books.

Alonso-Zarazaga, M.A., Wanat, M. 2014. Apionidae Schoenherr, 1823. In: Beutel, R.G., Leschen, R.A.B. (Eds), Handbook of Zoology, Coleoptera Vol. 3: Morphology and Systematics (Chrysomeloidea, Curculionoidea). Walter de Gruyter, Berlin, pp. 395-415.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Anisimova, M., Gascuel, O., 2006. Approximate likelihood ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55, 539-552.

Avise, J.C., Ball, R.M. Jr., 1990. Principles of genealogical concordance in species concepts and biological taxonomy. Oxf. Surv. Evol. Biol. 7, 45-67.

Bajtenov M. S., 1974. Zhuki-dolgonosiki (Coleoptera: Attelabidae, Curculionidae) Srednej Azii i Kazakhstana. Izdatelstvo "Nauka" Kazakhskoi SSR, Alma-Ata, 286 pp.

Baldo, L., Hotopp, J.C.D., Jolley, K.A., Bordenstein, S.R., Biber, S.A., Choudhury, R.R., Hayashi, C., Maiden, M.C.J., Tettelin, H., Werren, J.H., 2006. Multilocus Sequence Typing System for the Endosymbiont *Wolbachia pipientis*. Appl. Environ. Microbiol. 72, 7098-7110.

Benedikt S., Borovec R., Fremuth J., Krátký J., Schön K., Skuhrovec J., Trýzna M., 2011. Annotated checklist of weevils (Coleoptera: Curculionoidea excepting Scolytinae and Platypodinae) of the Czech Republic and Slovakia. Part 1. Systematics, faunistics, history of research on weevils in the Czech Republic and Slovakia, structure outline, checklist. Comments on Anthribidae, Rhynchitidae, Attelabidae, Nanophyidae, Brachyceridae, Dryophthoridae, Erirhinidae and Curculionidae: Curculioninae, Bagoinae, Baridinae, Ceutorhynchinae, Conoderinae, Hyperinae. Klapalekiana 46, 1-363.

Bernays, E.A., Chapman, R.F. 1994. Host-plant selection by phytophagous insects. Chapman and Hall, New York, New York.

Bokor, E., 1923. Dr Kaufmann Ernö gyüjteményének Apion-jai, Rovartani Lap. 26, 95-102.

Bouchard, P., Bousquet, Y., Davies, A.E., Alonso-Zarazaga, M.A., Lawrence, J.F., Lyal, C.H.C., Newton, A.F., Reid, C.A.M., Schmitt, M., Ślipiński, S.A., Smith, A.B.T., 2011. Family-group names in Coleoptera (Insecta). ZooKeys 88, 1-972.

Bourtzis, K., O'Neill, S.L., 1998. *Wolbachia* infections and arthropod reproduction. Bioscience 48, 287-293.

Bray, J. R., Curtis, J. T., 1957. An ordination of upland forest communities of southern Wisconsin. Ecol. Monogr. 27, 325-349.

Bruen, T.C., Philippe, H., Bryant, D, 2006. A simple and robust statistical test for detecting the presence of recombination. Genetics 172, 2665-2681.

Cranston, P.S., Krosh, M.N., 2015. DNA sequences and austral taxa indicate generic synonymy of *Paratrichocladius* Santos-Abreu with *Cricotopus* Wulp (Diptera: Chironomidae). Syst. Entomol. 40, 719-732.

Dayrat, B. 2005. Towards integrative taxonomy. Biol. J. Linn. Soc. 85, 407-415.

Dieckmann, L., 1976. Revision der *Apion platalea*-Gruppe (Coleoptera, Curculionidae), Entomologische Nachrichten 20, 117-128.

Dieckmann, L., 1977. Beiträge zur Insektenfauna der DDR: Coleoptera - Curculionidae (Apioninae), Beit. Entomol. 27, 7-143.

Dobson, S.L., Fox, C.W., Jiggins, F.M., 2002. The effect of *Wolbachia* induced cytoplasmic incompatibility on host population size in natural and manipulated systems. Proc. R. Soc. Lond. B 269, 437-445.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Phylogenet. Evol. 29, 1969-1973.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5, 113.

Ehret, J.-M., 1983. Les Apions de la Région Bourgogne. Fascicule IV. Bulletin trimestriel de la Société d'Histoire Naturelle et des Amis du Museum d'Autun, 108, 155-208.

Ehret, J.-M., 1990. Les Apions de France: clés d'identification commentées (Coleoptera Curculionidae Apioninae), Bulletin mensuel de la Société linnéenne de Lyon 59, 209-292.

Engelstädter, J., Hurst, G.D.D., 2009. The ecology and evolution of microbes that manipulate host reproduction. Annu. Rev. Ecol. Evol. Syst. 40, 127-149.

Farrell, B.D., 1998. "Inordinate fondness" explained: why are there so many beetles? Science, 281, 555-559.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C. 1994. Testing significance of incongruence. Cladistics 10, 315-319.

Gernhard, T. 2008. The conditioned reconstructed process. J. Theor. Biol. 253, 769-778.

Gómez-Zurita, J., Sassi, D., Cardoso, A. & Balke, M. 2012. Evolution of *Cryptocephalus* leaf beetles related to *C. sericeus* (Coleoptera: Chrysomelidae) and the role of hybridization in generating species mtDNA paraphyly. Zool. Scr. 41, 47-67.

Gønget, H., 1997. The Brentidae (Coleoptera) of Northern Europe. Fauna Entomologica Scandinavica 34, 1-289.

Gunter, N.L., Oberprieler R.G. & Cameron S. L. 2016. Molecular phylogenetics of Australian weevils (Coleoptera: Curculionoidea): exploring relationships in a hyperdiverse lineage through comparison of independent analyses. Austr. Ent., 55: 217-233.

Hall, T.A. 1999. "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT". Nucl. Acids. Symp. Ser. 41, 95-98.

Han, K., Park S., Park J., Hong K.-J., Bae S.-A. 2013. Insect Fauna of Korea, Vol. 12, No. 11. Arthropoda: Insecta: Coleoptera: Apionidae, Nanophyidae, Brentidae, Attelabidae. National Institute of Biological Resources, Ministry of Environment.

Hayashi, M., Morimoto, K., Kimoto, S. (eds.), 1984. The Coleoptera of Japan in Color Vol. IV. Hoikusha Publishing Co., Ltd., Osaka, i-vii + 407 pp.

Hebert, P. D. N., Gregory, T.R., 2005. The promise of DNA barcoding for taxonomy. Syst. Biol. 54, 852-859.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., Fukatsu, T. 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biology 4, e337.

Hughes, J., Vogler, A.P., 2004. The phylogeny of acorn weevils (genus *Curculio*) from mitochondrial and nuclear DNA sequences: the problem of incomplete data. Mol. Phylogenet. Evol. 32, 601-615.

Hurst, G.D.D., Jiggins, F.M., 2000 Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerging Infect. Dis. 6, 329-336

Huson, D.H., Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254-267.

Jolivet, P. 1998. Interrelationship between insects and plants. CRC Press, Boca Raton, FL.

Kass, R.E., Raftery, A.E., 1995. Bayes factors, J. Am. Stat. Ass. vol. 90 pp. 773-795.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772-780.

Kikuchi, Y., 2009. Endosymbiotic bacteria in insects: their diversity and culturability. Microbes Environ. 24: 195-204.

Kissinger, D.G., 1968. Curculionidae subfamily Apioninae of North and Central America, with reviews of the world genera of Apioninae and world subgenera of *Apion* Herbst (Coleoptera). Taxonomic Publications, South Lancaster, Massachusetts.

Kolasa, M., Montagna, M., Mereghetti, V., Kubisz, D., Mazur, M.A., Kajtoch, Ł,. 2017. Preliminary evidence of *Wolbachia* horizontal transmission between *Crioceris* leaf beetles and *Asparagus* host plants. European Journal of Entomology. 114: 446–454

Kruskal, J.B. 1964. Nonmetric multidimensional scaling: A numerical method. Psychometrika 29, 115-129.

Kubisz, D., Kajtoch, Ł., Mazur, M.A., Rizun, V., 2012. Molecular barcoding for central-eastern European *Crioceris* leaf-beetles (Coleoptera: Chrysomelidae). Centr. Eur. J. Biol. 7, 69-76.

Kuschel, G. 1995. A phylogenetic classification of Curculionoidea to families and subfamilies. – Memoirs of the Entomological Society of Washington 14, 5-33.

Lachowska, D., Kajtoch, Ł., Knutelski, S. 2010. Occurrence of *Wolbachia* in central European weevils: correlations with host systematics, ecology, and biology. Entomol. Exp. Appl. 135, 105-118.

Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T. and Calcott, B. 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Mol. Biol. Evol. 34, 772-773.

Langor, D.W., Sperling, F.A.H., 1997. Mitochondrial DNA sequence divergence in weevils of the *Pissodes strobi* species complex (Coleoptera, Curculionidae). Insect Mol. Biol. 6, 255–265.

Lecocq, T., Dellicour, S., Michez, D., Dehon, M., Dewulf, A., De Meulemeester, T., Brasero, N., Valterova, I., Rasplus, J. and Rasmont, P., 2015. Methods for species delimitation in bumblebees (Hymenoptera, Apidae, *Bombus*): towards an integrative approach. Zool. Scr. 44, 281-297.

Legalov, A.A., 2002. Spisok zhukov semejstv Nemonychidae, Urodontidae, Rhynchitidae, Attelabidae i Brentidae (Coleoptera, Curculionoidea) Aziatskoj Rossii. Zhivotnyj mir Dalnego Vostoka, 4: 105-116.

Lohse G. A., 1981. Apioninae. In: Freude, H., Harde, K. W., Lohse, G. A., Die Käfer Mitteleuropas: 127-183. Band 10, Goecke & Evers, Krefeld.

Marvaldi, A.E., Sequeira, A.S., O'Brien, C.W., Farrell, B.D. 2002. Molecular and morphological phylogenetics of weevils (Coleoptera, Curculionoidea): do niche shifts accompany diversification? Syst. Biol. 51, 761-785.

Mazur, M.A., Holecova, M., Lachowska-Cierlik, D., Lis, A., Kubisz, D., Kajtoch, Ł., 2016. Selective sweep of *Wolbachia* and parthenogenetic host genomes – the example of the weevil *Eusomus ovulum*. Ins. Mol. Biol. 25, 701-711

McKenna, D.D., Sequeira. A.S., Marvaldi, A.E., Farrell, B.D., 2009. Temporal lags and overlap in the diversification of weevils and flowering plants. Proc. Natl. Acad. Sci. U.S.A. 106, 7083-7088.

Montagna, M., Gómez-Zurita, J., Giorgi, A., Epis, S., Lozzia, G., Bandi, C., 2015. Metamicrobiomics in herbivore beetles of the genus *Cryptocephalus* (Chrysomelidae): toward the understanding of ecological determinants in insect symbiosis. Ins. Sci. 3, 340-352.

Montagna, M., Mereghetti, V., Lencioni, V., Rossaro, B., 2016b. Integrated taxonomy and DNA barcoding of Alpine midges (Diptera: Chironomidae). PLoS One 11: e0149673.

Montagna, M., Chouaia, B., Sacchi, L., Porretta, D., Martin, E., Giorgi, A., Lozzia, G.C., Epis, S., 2014. A new strain of *Wolbachia* in an alpine population of the viviparous *Oreina cacaliae* (Coleoptera: Chrysomelidae). Environm. Entomol. 43, 913-922.

Montagna, M., Kubisz, D., Mazur, M.A., Magoga, G., Ścibior, R., Kajtoch Ł., 2016a. Exploring species-level taxonomy in the *Cryptocephalus flavipes* species complex (Coleoptera: Chrysomelidae). Zool. J. Linn. Soc. doi: 10.1111/zoj.12445.

Montagna, M., Sassi, D., and Giorgi, A., 2013. *Pachybrachis holerorum* (Coleoptera: Chrysomelidae: Cryptocephalinae), a new species from the Apennines, Italy, identified by integration of morphological and molecular data. Zootaxa 3741, 243-253.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32: 268-274.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L., Robertson, H.M. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. U.S.A. 89, 209-216.

Oberprieler, R.G., Anderson, R.S., Marvaldi, A.E., 2014. Curculionoidea Latreille, 1802: Introduction, Phylogeny. In: Beutel, R.G., Leschen, R.A.B. (Eds), Handbook of Zoology, Coleoptera Volume 3: Morphology and Systematics (Chrysomeloidea, Curculionoidea). Walter de Gruyter, Berlin, pp. 255-300.

Oberprieler, R., 2000. The larvae of the weevil tribe Eurhynchini and the phylogeny of the Brentidae (Coleoptera: Curculionoidea). Invert. Taxon.14, 755-770.

Papadopoulou, A., Anastasiou, I., Vogler, A.P., 2010. Revisiting the insect mitochondrial molecular clock: the mid-Aegean trench calibration. Mol. Biol. Evol. 27, 1659-1672.

Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun, S., Sumlin, W.D, Vogler, A.P., 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol. 55, 595-609.

Ptaszyńska, A.A., Łętowski, J., Gnat, S., Małek, W., 2012. Application of COI sequences in studies of phylogenetic relationships among 40 Apionidae species. J. Ins. Sci. 12:16 available online: insectscience.org/12.16.

Reid, N.M., Carstens, B.C., 2012. Phylogenetic estimation error can decrease the accuracy species delimitation: A Bayesian implementation of the General Mixed Yule Coalescent model. BMC Evol. Biol. 12, 196.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61. 539-542.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., Solignac, M. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. B 250, 91-98.

Sanderson, M.J. 2003. r8s: inferring absolute rates of molecular evolution, divergence times in the absence of a molecular clock. Bioinformatics 19, 301-302.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E., Crozier, R.H., 2009. Integrative taxonomy: a multisource approach to exploring biodiversity, Ann. Rev. Entomol. 55, 421-443.

Sharp, D., 1891. The Rhynchophorous Coleoptera of Japan. Part II. Apionidae and Anthribidae. Trans. Entomol. Soc. Lond. 1891, 293-328.

Silfverberg, H., 1992. Enumeratio Coleopterorum Fennoscandiae, Daniae et Baltiae. Helsinki.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651-701.

Sites, J.W. Jr., Marshall, J.C., 2003. Delimiting species: A Renaissance issue in systematic biology, Trends Ecol. Evol. 18, 462-470.

Steinert, M., Hentschel, U., Hacker, J., 2000. Symbiosis and Pathogenesis: evolution of the microbe-host interaction. Naturwissenschaften 87, 1-11.

Stouthamer, R., Breeuwer, J.A., Hurst, G.D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Ann. Rev. Microbiol. 53,71-102.

Stüben, P.E., Schütte, A., Bayer, C., Astrin, J.J., 2015. The Molecular Weevil Identification Project (Coleoptera: Curculionoidea), Part II. Towards an Integrative Taxonomy.

Snudebiller: Studies on taxonomy, biology and ecology of Curculionoidea, 16, 237, 1-294.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Toševski, I., Caldara, R., Jović, J., Baviera, C., Hernández -Vera, G., Gassmann, A. & Emerson, B.C., 2014. Revision of *Mecinus heydenii* species complex (Curculionidae): integrative taxonomy reveals multiple species exhibiting host specialization. Zool. Scr. 43, 34-51.

Valentini, A., Pompanon, F., Taberlet, P. 2009. DNA barcoding for ecologists. TREE 24, 110-117.

Vavre F., Fleury F., Lepetit D., Fouillet P., Boulétreau M. (1999) Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Mol. Biol. Evol. 16, 1711-1723.

Wagner, H., 1932. Apioninae, pp. 1393-1401. In: Winkler A., Catalogus Coleopterorum Regionis Palaearcticae. Pars 12. A. Winkler, Wien, pp. 1393-1520.

Wanat, M., 1994. Ryjkowce (Coleoptera: Curculionoidea: Anthribidae, Rhinomaceridae, Rhynchitidae, Attelabidae, Apionidae, Curculionidae) Puszczy Białowieskiej. Pol. Pismo Entomol. 63, 37-112.

Wanat, M., 2005: Ryjkowce (Coleoptera: Curculionoidea bez Scolytinae) Biebrzańskiego Parku Narodowego i jego otuliny. In: Dyrcz A., Werpachowski C. (Eds.). Przyroda Biebrzańskiego Parku Narodowego. Biebrzański Park Narodowy, 301-324.

Wanat, M., 2009. Nowe dane o rozmieszczeniu kilkunastu rzadkich gatunków ryjkowców (Coleoptera: Curculionoidea) w Polsce. Wiad. Entomol. 28, 132-134.

Wanat, M., 1995. Systematics and phylogeny of the tribe Ceratapiini (Coleoptera: Curculionoidea: Apionidae). Genus (Suppl.), Wrocław, pp. 406.

Wanat, M., Mazur M.A., Celadyn R., Jałoszyński P., Ruta R., Kaźmierczak M., Mocarski Z., Szypuła J., Sienkiewicz P. 2016. Nowe dane o rozmieszczeniu 50 gatunków ryjkowców (Coleoptera: Curculionoidea) w Polsce. Acta Entomologica Silesiana, 24 (online 016): 1-20.

Wanat, M., 1999. Ryjkowce (*Coleoptera*: *Curculionoidea* bez *Scolytidae* i *Platypodidae*) Puszczy Białowieskiej - charakterystyka fauny. Parki Nar. Rez. Przyr. 18, 25-47.

Wanat, M., 2001. Genera of Australo-Pacific Rhadinocybinae and Myrmacicelinae, with biogeography of the Apionidae (Coleoptera: Curculionoidea) and phylogeny of the Brentidae (s. lato). Mantis, Olsztyn, pp. 432.

Werren, J.H. 1997. Biology of *Wolbachia*. Ann. Rev. Entomol. 42, 587-609.

White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., eds). Academic Press, New York, USA. pp. 315-322.

White, J.A., Kelly, S.E., Cockburn, S.N., Perlman, S.J. & Hunter, M.S., 2011. Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. Heredity 106, 585-591.

Wink, M., Mikes, Z., Rheinheimer, J., 1997. Phylogenetic Relationships in Weevils (Coleoptera: Curculionoidea) Inferred from Nucleotide Sequences of Mitochondrial 16 S rDNA. Naturwissenschaften, 84, 318-321.

Winter, S., Friedman, A.L.L., Astrin, J.J., Gottsberger, B., Letsch, H., 2017. Timing and host plant associations in the evolution of the weevil tribe Apionini (Apioninae, Brentidae, Curculionoidea, Coleoptera) indicate an ancient co-diversification pattern of beetles and flowering plants. Mol. Phylogen. Evol. 107, 179-190.

Xia, X. 2013. DAMBE5: A comprehensive software package for data analysis in molecular biology and evolution. Mol. Phylogenet. Evol. 30, 1720-1728.

Xia, X., Lemey, P., 2009. Assessing substitution saturation with DAMBE. Pp. 615-630 in Leme,y P., Salemi, M., Vandamme, A.M., eds. The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny. 2nd edition Cambridge University Press.

Yang, X.-H., Zhu, D.-H., Liu, Z., Zhao, L., Su, C.-Y. 2013. High levels of multiple infections, recombination and horizontal transmission of *Wolbachia* in the *Andricus mukaigawae* (Hymenoptera; Cynipidae) communities. PLoS ONE 8, e78970.

Zhang, J., Kapli, P., Pavlidis, P., Stamatakis, A., 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics 22, 2869-2876.

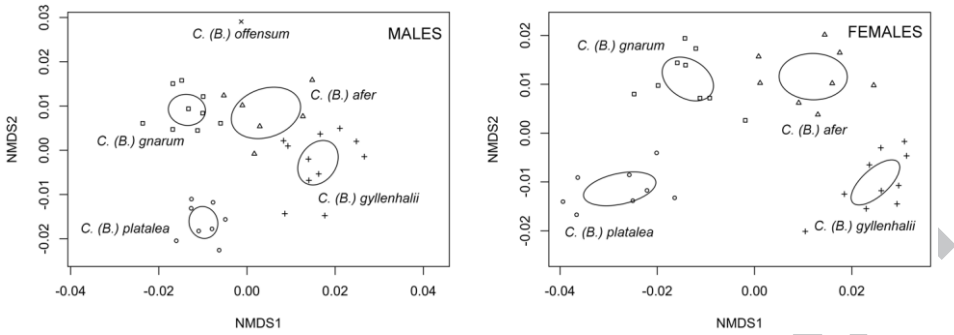
Figure captions

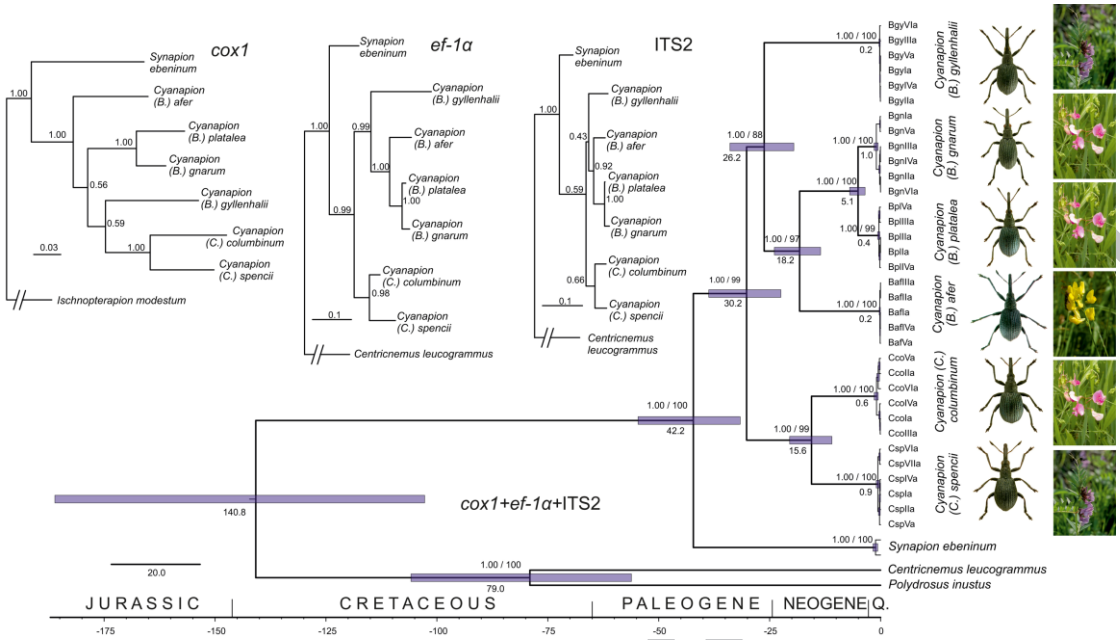
Figure 1. Non-metric multidimensional scaling plots showing relations among males and females of *Bothryorrhynchapion* species based on measurements of 11 morphometric parameters (see Tables C.1-2).

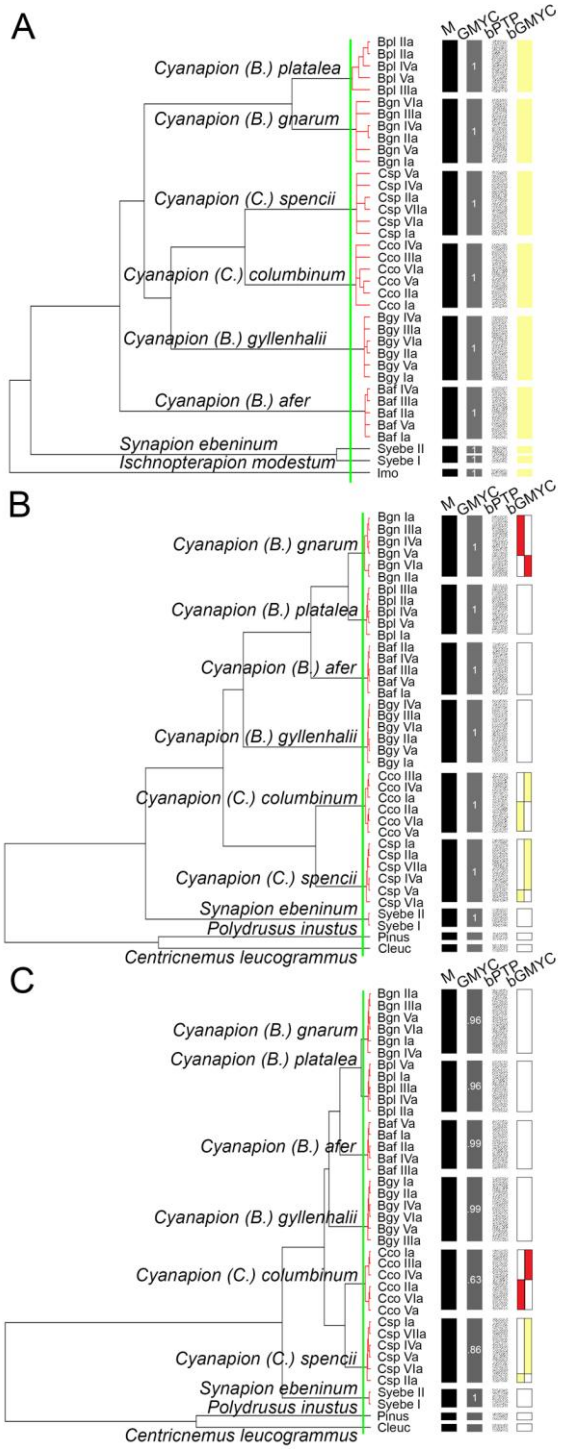
Figure 2. Phylogenetic relationships of the analysed *Cyanapion* species and divergence time estimation. Central graphic- Ultrametric tree inferred using BEAST on the three genetic markers: cytochrome oxidase I (*cox1*), nuclear elongation factor 1-alfa gene (*ef-1α*) and internal transcribed spacer 2 of nuclear ribosomal DNA (ITS2). Horizontal bars represent 95% age confidence intervals for each node. The scale bar indicates the distance in substitutions per site, and the geological time scale is recorded. Graphics in left upper part - Bayesian phylogenetic trees constructed for examined *Cyanapion* weevils on the basis of each single genetic marker. Values above branches indicate branch supports: Posterior Probabilities from Bayesian inference / Approximate Likelihood-Ratio Test supports from Maximum Likelihood approach (only Posterior Probabilities presented for single gene). Values below branches - the mean estimates of divergence dates. Pictures in right side – photographs of examined *Cyanapion* weevils with photographs of their host plants. Q – Quaternary.

Figure 3. Species delimitation analyses based on Bayesian ultrametric tree inferred from mitochondrial *cox1* (A), the nuclear *ef-1α* (B) and ITS2 (C). On each tree the vertical green line delimits clades corresponding to GMYC maximum likelihood clusters and entities (putative molecular species), also reported by dark grey vertical blocks; vertical black blocks indicating the identified morphospecies (M); putative molecular species identified by bPTP are represented by marbled vertical blocks, while those identified by bGMYC are represented by vertical solid colored boxes, colors indicate support values of Bayesian posterior probability (bpp) as following: 0.5 – 0.9 in red, 0.9 – 0.95 in ochre, 0.95 – 0.99 in yellow, 1 in white.

Figure 4. Median-joining network constructed on *Wolbachia* strains identified in infected *Cyanapion* weevils showed with several reference strains representing various *Wolbachia* supergroups known from the *Wolbachia* Multilocus Sequence Typing database. Allelic profiles generated from *Cyanapion* beetles are compared with some representative sequence types from other species that harboured bacteria belonging to different supergroups: A (ST-1 from *Drosophila melanogaster*), B (ST-15 from *Drosophila simulans*), D (ST-35 from nematode), F (ST-8 from *Cimex lectularius*), and H (ST-90 from *Zootermes angusticollis*), and from European beetles with a complete allelic profile: *Eusomus ovulum* weevil (Mazur et al., 2016), *Oreina cacaliae* (Montagna et al., 2014*)* and *Crioceris quinquepunctata* (Kolasa et al., 2017).







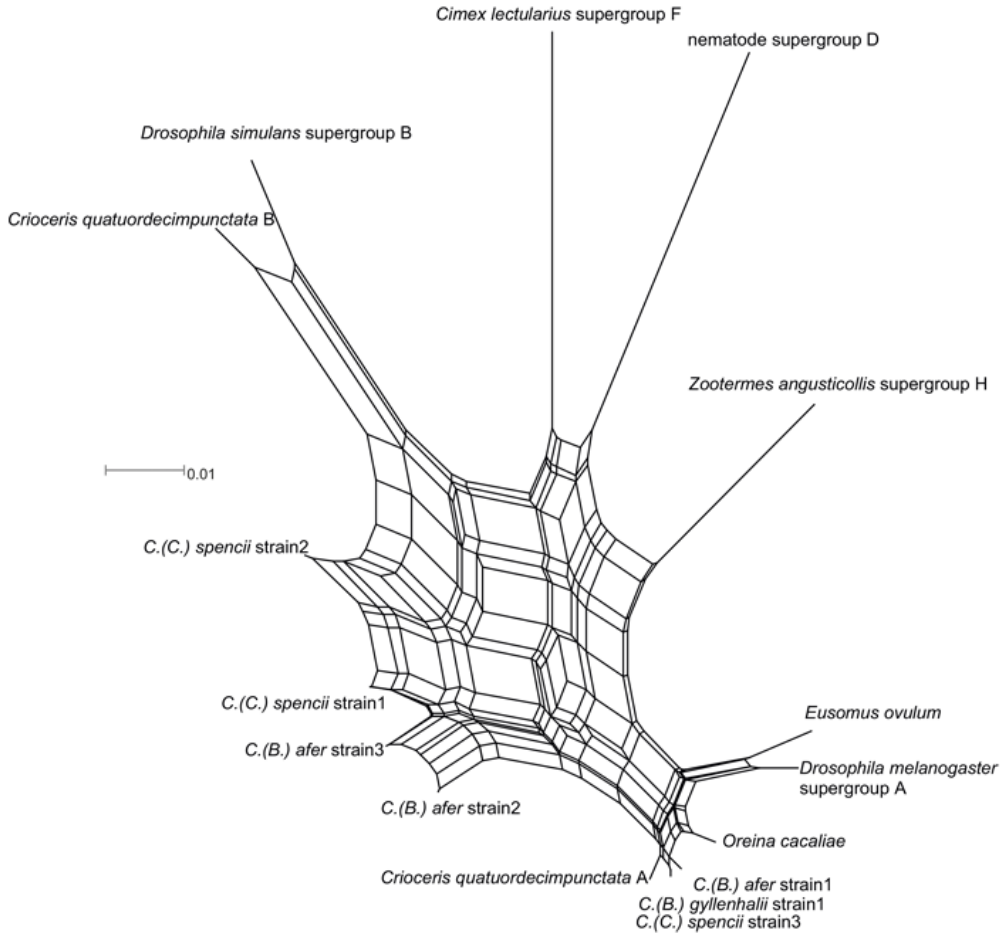
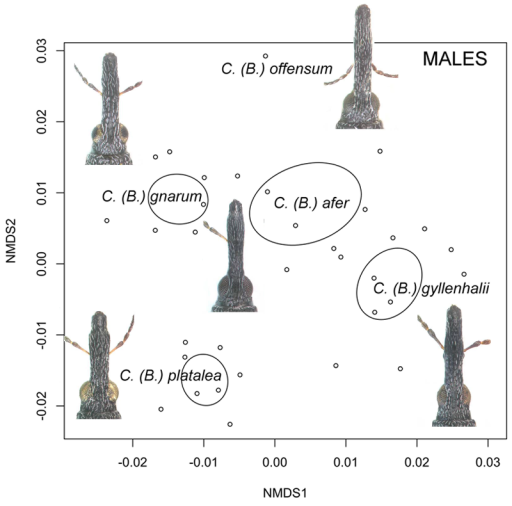
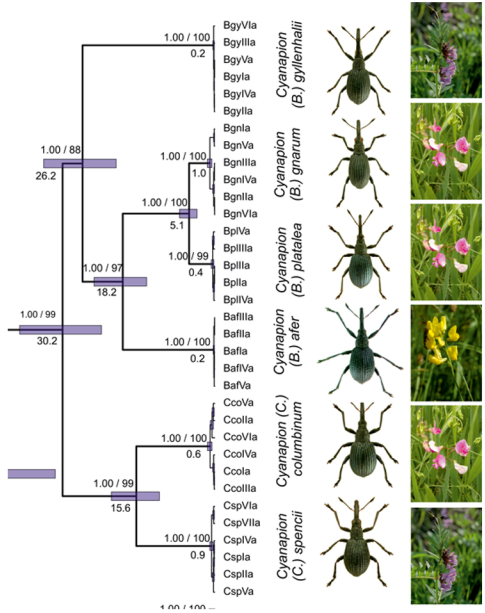


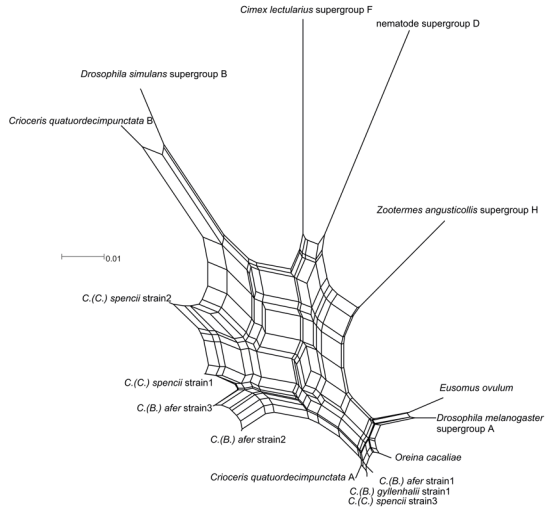
Table 1. A synopsis of host plant use by examined *Cyanapion* species.

Cyanapion species	Host plants	
	Lathyrus	*Vicia*
C. (C.) spencii	-	*V. faba, V. cracca, V. sepium, V. tenuifolia, V. villosa, V. dumetorum*
C. (C.) columbinum	*L. sylvestris, L. heterophyllus, L. latifolius, L. tuberosus, L. roseus*	-
C. (B.) gyllenhalii	-	*V. cracca, V. hirsuta, V. sepium, V. silvatica, V. tenuifolia, V. dumetorum, V. tetrasperma, V. sativa*
C. (B.) platalea	*L. tuberosus, L. sylvestris, L. latifolius, L. palustris*	-
C. (B.) gnarum	*L. sylvestris*	-
C. (B.) afer	*L. pratensis*	
C. (B.) offensum	?	?

MORPHOMETRICS PHYLOGENETICS HOST PLANTS *WOLBACHIA*







Highlights

- The monophyly of *Bothryorrhynchapion* weevils is supported,
- Morphology and molecular data generally congruently differentiated the examined species,
- Speciation of these apionids was associated with host plant shifts,
- *Wolbachia* infection could have facilitated speciation of sibling taxa.