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TAXONOMIC POSITION OF *ANASTRANGALIA REYI* AND *A. SEQUENSI* (COLEOPTERA, CERAMBYCIDAE) BASED ON MOLECULAR AND MORPHOLOGICAL DATA

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Taxonomic Position of *Anastrangalia reyi* and *A. sequensi* (Coleoptera, Cerambycidae) Based on Molecular and Morphological Data. Zamoroka, A. M., Semaniuk, D. V., Shparyk, V. Yu., Mykytyn, T. V., Skrypnyk, S. V. — *Anastrangalia reyi* (Heyden, 1889) and *Anastrangalia sequensi* (Reitter, 1898) are morphologically similar species described in late of XIX century. The recent barcoding revealed that *A. reyi* is almost identical to another species, *Anastrangalia dubia* (Scopoli, 1763), by the sequence of nucleotides in cytochrome C oxidase subunit I (COI). Consequently, the taxonomic position of these species is unclear. We have conducted a comprehensive meta-analysis of available data of COI sequences combined with a study of morphological characters of the male genitalia of *A. reyi*, *A. sequensi* and *A. dubia*. Based on 87 sequenced samples we built well-resolved phylogenetic maximum likelihood tree. We found the clades of *A. dubia*, *A. reyi* and *A. sequensi* to be closely related and arranged in the dense cluster. Despite this, numerous cases of introgressive hybridization of *A. reyi* and *A. dubia* were identified, indicating an inadequate reproductive barrier between them. The study of morphological features of male genitalia of *A. reyi*, *A. sequensi* and *A. dubia* shows minor differences between them. Based on these facts and the results of the phylogenetic analysis we propose to consider *A. reyi* and *A. sequensi* to be subspecies of *A. dubia*.

Key words: Cerambycidae, taxonomy, molecular phylogeny.

Introduction

Anastrangalia reyi (Heyden, 1889) and *Anastrangalia sequensi* (Reitter, 1898) are morphologically indistinguishable species, which initially were described as varieties of *Anastrangalia dubia* (Scopoli, 1763). *A. reyi* was formally described by Claudius Rey under the name *Leptura dubia* race *ochracea* as having black strip on the margin of the elytra (Rey, 1885). Heyden found this name to be preoccupied by *Leptura scutellata*

var. *ochracea* Faust, 1879 and proposed a new replacement name *Leptura reyi* for the junior homonym, "Ray's Leptura" (Heyden, 1889). However, he did not indicate any reason for erecting of a new species or providing its description. Decade later Edmund Reitter described *Leptura sequensi* as a species distinct from *L. dubia*. He also found three forms of *L. sequensi*: var. *rufopaca*, var. *pulchrina*, var. *tristina* from East Siberia. According to him, *L. sequensi* differs from *L. dubia* by smaller temples (Reitter, 1898). This feature is typical also for *A. reyi*. Both species have the pronotum subcylindrical with sparse erecting and dense decumbent pubescence. Despite the absence of morphological differences *A. reyi* and *A. sequensi* were recognized until now as distinct species based mainly on geographical separation.

During the last decade due to intensive barcoding of European Coleoptera it was revealed that *A. reyi* does not differ from *A. dubia* by the molecular signs of cytochrome c oxidase I (COI) (Rougerie et al., 2015; Hendrich et al., 2015; Wu et al., 2016). Moreover, Hendrich et al. (2015) noted that all sequenced specimens of both species were not grouped in separated clades on their phylogenetic trees; instead, they are completely mixed. Rougerie et al. (2015) assumed possibility of the past or current hybridization between *A. reyi* and *A. dubia*. They also supposed continuing speciation with low divergence of both species. Wu et al. (2016) in their study of the longhorn beetle larvae imported to the USA with solid wood packaging material found that *A. reyi* and *A. dubia* are indistinguishable by molecular methods. Thus, they indicated both species as *Anastrangalia* sp. in their study. The published data show some difficulties in molecular identification of *A. reyi* and *A. dubia* connected with low resolution of COI markers. Thus, the taxonomic position of *A. reyi* and *A. dubia* is unclear as well as *A. sequensi*, which is morphologically similar to *A. reyi*.

In the current study we have conducted the comprehensive a meta-analysis of available data of COI sequences, including analysis of *A. reyi*, *A. sequensi* and *A. dubia* morphological features of the male genitalia. We have found evidence that difference between all these species is considerably low and that they are conspecific. We proposed to consider *A. reyi* and *A. sequensi* to be subspecies of *A. dubia*.

Material and methods

The publicly available assembly data on DNA sequences of mitochondrial gene of COI of *Anastrangalia reyi* (20 samples), *Anastrangalia sequensi* (4 samples) and *Anastrangalia dubia* (23 samples) were obtained from GenBank as FASTA files. Additionally, we included 6 samples of *Anastrangalia* sp. that mentioned by Wu et al. (2016) as doubtful identification of *Anastrangalia dubia* /*reyi* to the phylogenetic analysis. For evaluating of the nesting of *A. reyi* and *A. sequensi* within *Anastrangalia* genera we added to phylogenetic analysis available sequences of *Anastrangalia sanguinea* (1 sample) and *Anastrangalia sanguinolenta* (33 samples). All imported nucleotide sequences were specifically labeled for their identifications on phylogenetic tree (see annex 1). In total 87 sequences of 5 species of *Anastrangalia* were included to phylogenetic analysis. Additionally, *Cerambyx cerdo* (KM285966) and *Cerambyx scopoli* (KU917190) COI sequences were used as outgroup. Both species belong to the type genus of family Cerambycidae.

Multiple alignments were generated using the Muscle software in the environment of SeaView 4 (Gouy et al., 2010). Alignments were provided with unlimited iterations, and were edited manually to correct regions containing missing data and to exclude unalignable positions. Phylogenetic trees were constructed using maximum-likelihood (ML) and Bayesian methods with PhyML (Guindon, Gascuel, 2003). Analyses were performed following a general time-reversible (GTR) model of sequence evolution using 4 categories of rates variation with a gamma correction. We performed an approximate likelihood-ratio test (aLRT) for branch support based on the log ratio between the likelihood value of the current tree and that of the best alternative (Anisimova, Gascuel, 2006; Guindon et al., 2010). The optimal trees structure was estimated using the nearest-neighbor interchange (NNI) algorithm for 5-branch trees. We also used neighbor-joining algorithm (BioNJ) optimaizing trees topology for estimation of branch distance from COI sequences (Gascuel, 1997).

We also dissected 16 males of *A. reyi*, *A. sequensi*, *A. dubia* and *A. sanguinolenta* for the comparative analysis of the genitalia morphology due to the classical practice in the taxonomical entomology (Simmons, 2014). Genitalia were mounted and studied under the stereomicroscope Nikon SMZ-1 at 40× zoom. Photos were taken and processed by USB camera DLT-Cam PRO 5 MP using DLTCamViewer x86, 3.7.7892 software package.

Measurements are given in the following format: min–max (M = mean). The following abbreviations are used in the diagnoses: AW/L = aedeagus width to length ratio, PW/L = paramere lobes width to length ratio.

Results

Haplotypes and haplogroups. Detail comparative analysis of COI sequences of *A. rei*, *A. sequensi* and *A. dubia* demonstrated presence of 20 nucleotide substitutions, which distinguishing these taxa. Since, COI sequences are 658 nucleotide lengths all of them ordered from 1st to 658th. Thus, nucleotide substitutions are nested in positions 22–206–247–250–271–316–331–370–379–451–463–496–523–548–550–565–619–625–631–658.

Annex 1. The list of *Anastrangalia* COI sequences used in the study

GenBank access code	Abbreviation	Country of origin	Coordinates
	Anastrangalia reyi		
KM449706	A_reyi_AU1	Austria	46.794 N 12.417 E
KM448004	A_reyi_AU2	Austria	46.708 N 12.587 E
KM446871	A_reyi_AU3	Austria	46.794 N 12.417 E
KM444576	A_reyi_AU4	Austria	46.796 N 12.409 E
KM444049	A_reyi_AU5	Austria	47.182 N 12.822 E
KM439252	A_reyi_AU6	Austria	46.796 N 12.409 E
KU918103	A_reyi_AU7	Austria	46.794 N 12.417 E
KU917280	A_reyi_AU8	Austria	46.708 N 12.587 E
KU916023	A_reyi_AU9	Austria	46.796 N 12.409 E
KU906588	A_reyi_AU10	Austria	46.796 N 12.409 E
KM451282	A_reyi_AU11	Austria	47.182 N 12.822 E
KM450755	A_reyi_AU12	Austria	46.794 N 12.417 E
KJ966266	A_reyi_FI1	Finland	60.238 N 25.14 E
KJ965629	A_reyi_FI2	Finland	60.675 N 27.005 E
KJ964792	A_reyi_FI3	Finland	66.288 N 29.646 E
KJ964213	A_reyi_FI4	Finland	65.116 N 25.829 E
KJ962569	A_reyi_FI5	Finland	65.116 N 25.829 E
KM286353	A_reyi_FR	France	44.774 N 6.955 E
KU918610	A_reyi_IT1	Italy	46.720 N 12.301 E
KU908627	A_reyi_IT2	Italy	46.720 N 12.301 E
KY683642	A_sequensi_RU1	Russia	43.17 N 132.79 E
AF332939	A_sequensi_KO	Korea	not available
NC_038090	A_sequensi_CN	China	not available
KY773687	A_sequensi_CN1	China	not available
KM447238	A_dubia_AU1	Austria	46.796 N 12.409 E
KM444513	A_dubia_AU2	Austria	46.804 N 12.453 E
KM451116	A_dubia_DE1	Germany	49.095 N 13.247 E
KM447620	A_dubia_DE2	Germany	47.491 N 11.095 E
KM444190	A_dubia_DE3	Germany	48.946 N 13.362 E
KM441763	A_dubia_DE4	Germany	49.095 N 13.247 E
KM439943	A_dubia_DE5	Germany	47.705 N 12.42 E
KM439442	A_dubia_DE6	Germany	47.705 N 12.420 E
KU910266	A_dubia_DE7	Germany	47.705 N 12.419 E
KU910193	A_dubia_DE8	Germany	47.705 N 12.420 E
KU908901	A_dubia_DE9	Germany	47.705 N 12.420 E
KM285974	A_dubia_FR1	France	42.739 N 2.200 E
KM286386	A_dubia_FR2	France	45.382 N 2.494 E
KM286142	A_dubia_FR3	France	46.332 N 6.063 E
KU918227	A_dubia_IT1	Italy	46.777 N 11.228 E
KM447713	A_dubia_IT2	Italy	46.778 N 11.242 E
KM444452	A_dubia_IT3	Italy	46.819 N 11.236 E
KM443724	A_dubia_IT4	Italy	46.778 N 11.242 E
KU913421	A_dubia_IT5	Italy	46.732 N 12.332 E
KM439224	A_dubia_IT6	Italy	46.733 N 12.332 E
KU906345	A_dubia_IT7	Italy	46.720 N 12.301 E

KM440821	A_dubia_SL	Slovenia	45.917 N 14.033 E
KY357754	A_dubia_USA	The United States	not available
	Anastrangalia sp.		
KY357756	Anastrangalia_sp_USA1	The United States	not available
KY357755	Anastrangalia_sp_USA2	The United States	not available
KY357753	Anastrangalia_sp_USA3	The United States	not available
KY357752	Anastrangalia_sp_USA4	The United States	not available
KY357751	Anastrangalia_sp_USA5	The United States	not available
KY357750	Anastrangalia_sp_USA6	The United States	not available
	Anastrangalia sanguinea		
KM849144	A_sanguinea_CA	Canada	51.126 N 115.726 W
	Anastrangalia sanguinolenta		
KU909168	A_sanguinolenta_AU1	Austria	46.708 N 12.587 E
KM445964	A_sanguinolenta_AU2	Austria	46.794 N 12.417 E
KM444617	A_sanguinolenta_AU3	Austria	47.013 N 11.306 E
KU919418	A_sanguinolenta_DE1	Germany	53.358 N 12.891 E
KU912934	A_sanguinolenta_DE2	Germany	50.778 N 10.921 E
KU912144	A_sanguinolenta_DE3	Germany	50.709 N 11.270 E
KU911644	A_sanguinolenta_DE4	Germany	50.908 N 11.611 E
KU909503	A_sanguinolenta_DE5	Germany	53.404 N 12.866 E
KU907670	A_sanguinolenta_DE6	Germany	53.404 N 12.866 E
KU907549	A_sanguinolenta_DE7	Germany	50.772 N 10.656 E
KU907417	A_sanguinolenta_DE8	Germany	53.4047 N 12.866 E
KM450185	A_sanguinolenta_DE9	Germany	49.276 N 8.0899 E
KM448291	A_sanguinolenta_DE10	Germany	47.675 N 12.014 E
KM444762	A_sanguinolenta_DE11	Germany	50.004 N 7.805 E
KM443756	A_sanguinolenta_DE12	Germany	49.070 N 13.131 E
KU915111	A_sanguinolenta_DE13	Germany	50.326 N 12.236 E
KU914828	A_sanguinolenta_DE14	Germany	53.404 N 12.866 E
KU914539	A_sanguinolenta_DE15	Germany	53.404 N 12.866 E
KU914346	A_sanguinolenta_DE16	Germany	53.404 N 12.866 E
KU913708	A_sanguinolenta_DE17	Germany	53.404 N 12.866 E
KU913364	A_sanguinolenta_DE18	Germany	47.705 N 12.419 E
KU915601	A_sanguinolenta_DE19	Germany	53.358 N 12.891 E
KJ964113	A_sanguinolenta_FI1	Finland	61.042 N 28.712 E
KJ963700	A_sanguinolenta_FI2	Finland	65.132 N 25.944 E
KJ963360	A_sanguinolenta_FI3	Finland	61.924 N 25.731 E
KJ963259	A_sanguinolenta_FI4	Finland	65.116 N 25.829 E
KJ962892	A_sanguinolenta_FI5	Finland	61.454 N 27.404 E
KJ962498	A_sanguinolenta_FI6	Finland	61.924 N 25.731 E
KM286319	A_sanguinolenta_FR	France	42.739 N 2.2 E
KU910516	A_sanguinolenta_IT1	Italy	46.732 N 12.332 E
KU908371	A_sanguinolenta_IT2	Italy	45.821 N 7.619 E
KU919382	A_sanguinolenta_IT3	Italy	46.628 N 12.229 E
KM452054	A_sanguinolenta_IT4	Italy	46.777 N 11.228 E

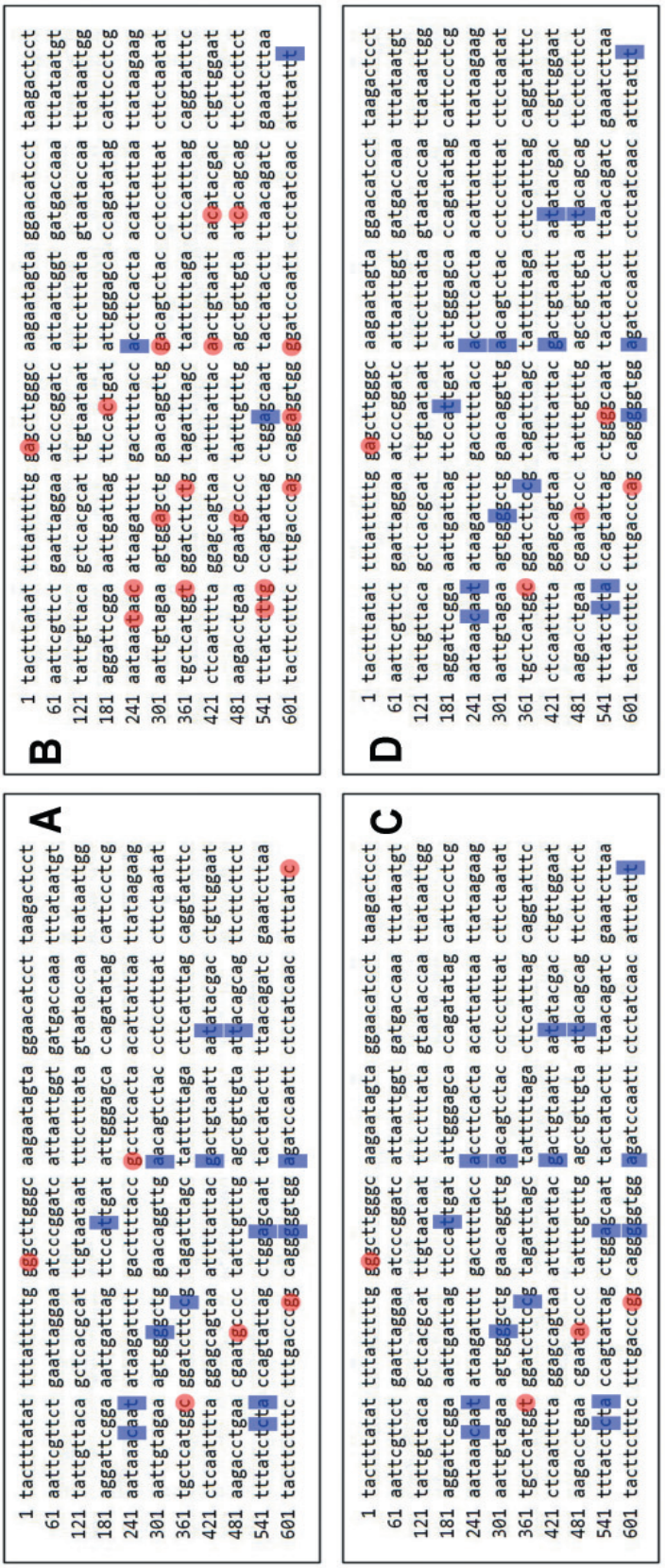

Fig. 1. Distribution of nucleotide substitutions within haplogroups: **A** — *A. reyi* (ArEu) (KJ964792); **B** — *A. sequensi* (AsFe) (KY683642); **C** — *A. dubia* (AdAl) (KM439943); **D** — *A. dubia* (AdPy) (KM285974). The unic nucleotide substitutions are red circled; the common substitutions are blue marked.

However, only 7 substitutions are crucial for distinguishing of *A. rei* and *A. dubia*. These found in positions 22–271–370–496–565–619–658. *A. sequensi* comprise 17 important substitutions in the following positions 22–206–247–250–304–316–331–379–451–463–496–523–548–550–619–625–631. The distribution of common and unic substitutions within COI haplogroups of *A. rei*, *A. sequensi* and *A. dubia* is shown on fig. 1.

We found a very low variatin of *A. reyi* COI sequences within all of Europe, which forming the homogenous haplogroup named us European (hereinafter: ArEu — *A. reyi* European). The haplogroup ArEu contains next substitutions in positions: 22 — a, 206 — c, 247 — c, 250 — t, 271 — g, 316 — g, 331 — a, 370 — c, 379 — c, 451 — g, 463 — t, 496 — g, 523 — t, 548 — c, 550 — a, 565 — a, 619 — g, 625 — g, 631 — a, 658 — c. The haplogroup ArEu consists two haplotypes (positions 22–271–370–496–565–619–658): ArEu-1 g–g–c–g–a–g–c occupying Europe from the Alps to Lapland; ArEu-2 (g–a–c–g–a–g–c) presented in Southern Finland.

All COI sequences of *A. sequensi* were origined from Pacific Coast of North Asia and belong to one haplogroup. This haplogroupe was named Far East (hereinafter: AsFe) and contains substitutions in positions: 22 — a, 206 — c, 247 — t, 250 — c, 271 — a, 316 — a, 331 — g, 370 — t, 379 — t, 451 — a, 463 — c, 496 — g, 523 — c, 548 — t, 550 — g, 565 — a, 619 — a, 625 — a, 631 — g, 658 — t. The haplogroup AsFe comprises two haplotypes (positions 22–206–247–250–304–316–331–379–451–463–496–523–548–550–619–625–631): AsFe-1 a–c–t–c–t–a–g–t–a–c–g–c–t–g–a–a–g distributed in North China and East Siberia (Russia); AsFe-2 a–c–t–c–c–a–g–t–a–c–g–c–t–g–a–a–g found in Korean Peninsula.

We identified two COI haplogroups for *A. dubia*, which we named "Alpine" (hereinafter: AdAl) and "Pyrenean" (hereinafter: AdPy). The AdAl haplogroup contains the next substitutions in positions: 22 — g, 206 — t, 247 — c, 250 — t, 271 — a, 316 — g, 331 — a, 370 — t, 379 — c, 451 — g, 463 — t, 496 — a, 523 — t, 548 — c, 550 — a, 565 — a, 619 — g, 625 — g, 631 — a, 658 — t. The AdPy haplogroup contains following substitutions in positions: 22 — a, 206 — t, 247 — c, 250 — t, 271 — a, 316 — g, 331— a, 370 — c, 379 — c, 451 — g, 463— t, 496 — a, 523 — t, 548 — c, 550— a, 565 — g, 619 — a, 625 — g, 631 — a, 658 — t.

The AdAl haplogroup comprises four haplotypes (positions 22–271–370–496–565–619–658): AdAl-1 g–a–t–a–a–g–t distributed in the Alps and the North-West Balkans; AdAl-2 g–g–t–a–a–g–t widespread among North foothills of Alps; AdAl-3 g–g–t–a–a–g–t with additional sunstituions in positions 625 — a and 646 — c occupied territory from the Northern Alps to the Ore Mountains and the Western Carpathians; AdAl-4 g–g–c–a–a–g–t, which is known by one sequence obtained from the wooden pacadge imported to US (Wu et al., 2016), unfortunately the territory of its origin is unknown.

The AdPy haplogroup includes the only haplotype (positions 22–271–370–496–565–619–658) AdPy-1 a–a–c–a–g–a–t occurring from the Pyrenees to the South-Western Alps.

Table 1. The percentage (%) variation of difference among haplotypes of *A. reyi*, *A. sequensi* and *A. dubia* (*A. sanguinolenta* is given for comparison)

Haplotype	ArEu-1	ArEu-2	AsFe-1	AsFe-2	AdAl-1	AdAl-2	AdAl-3	AdAl-4	AdPy-1
ArEu-1	0.00	0.15	2.74	2.43	0.61	0.46	0.76	0.46	0.91
ArEu-2		0.00	2.59	2.28	0.46	0.61	0.91	0.61	0.76
AsFe-1			0.00	0.15	2.43	2.58	2.59	2.89	2.43
AsFe-2				0.00	2.28	2.43	2.28	2.58	2.43
AdAl-1					0.00	0.15	0.46	0.46	0.61
AdAl-2						0.00	0.30	0.30	0.76
AdAl-3							0.00	0.61	1.10
AdAl-4								0.00	0.76
AdPy-1									0.00
A. sanguinolenta	12.16	12.01	12.46	12.61	12.01	12.16	12.16	12.31	12.01

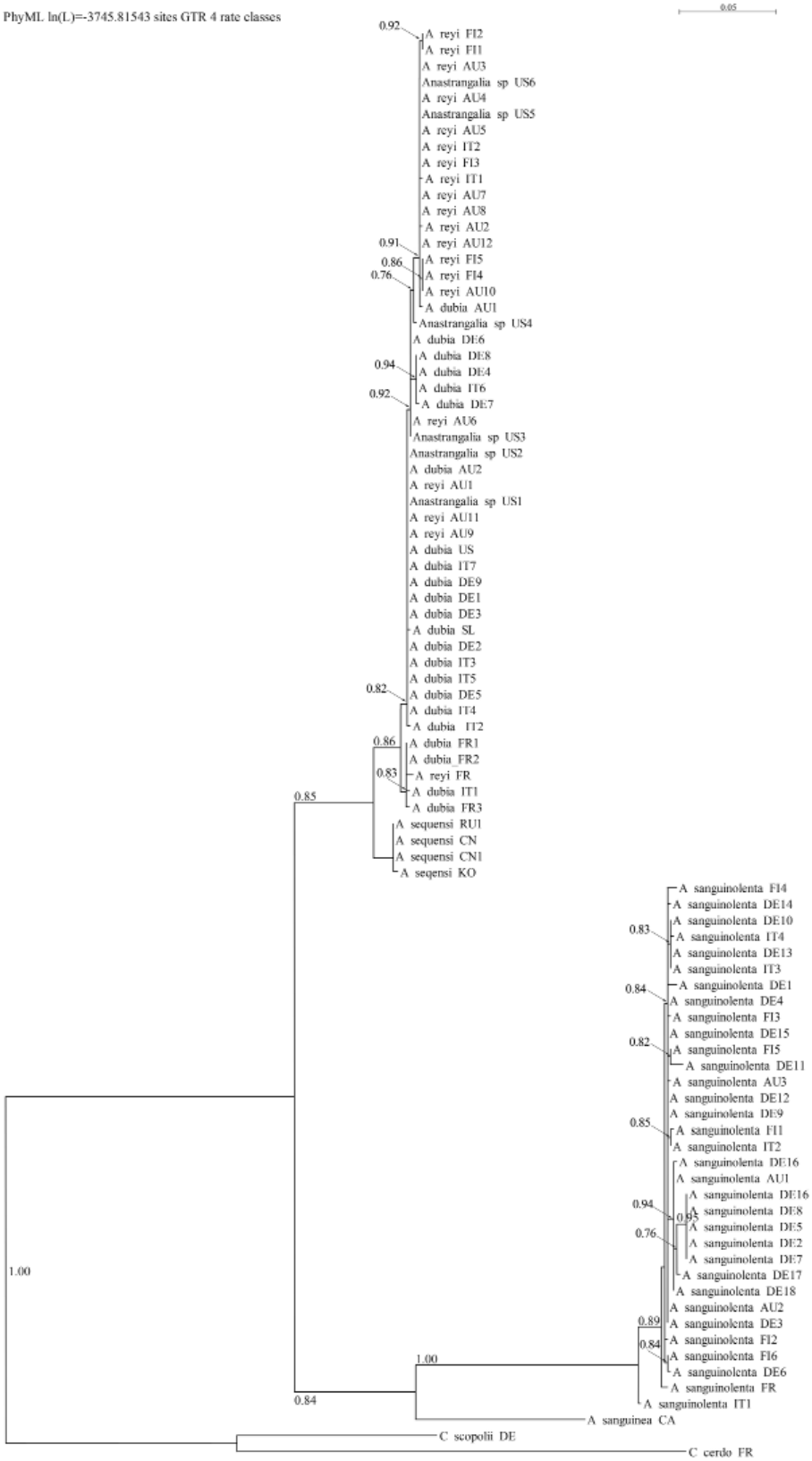

Fig. 2. Phylogenetic tree of the genus *Anastrangalia* (with *C. cerdo* and *C. scopolii* as outgrouped).

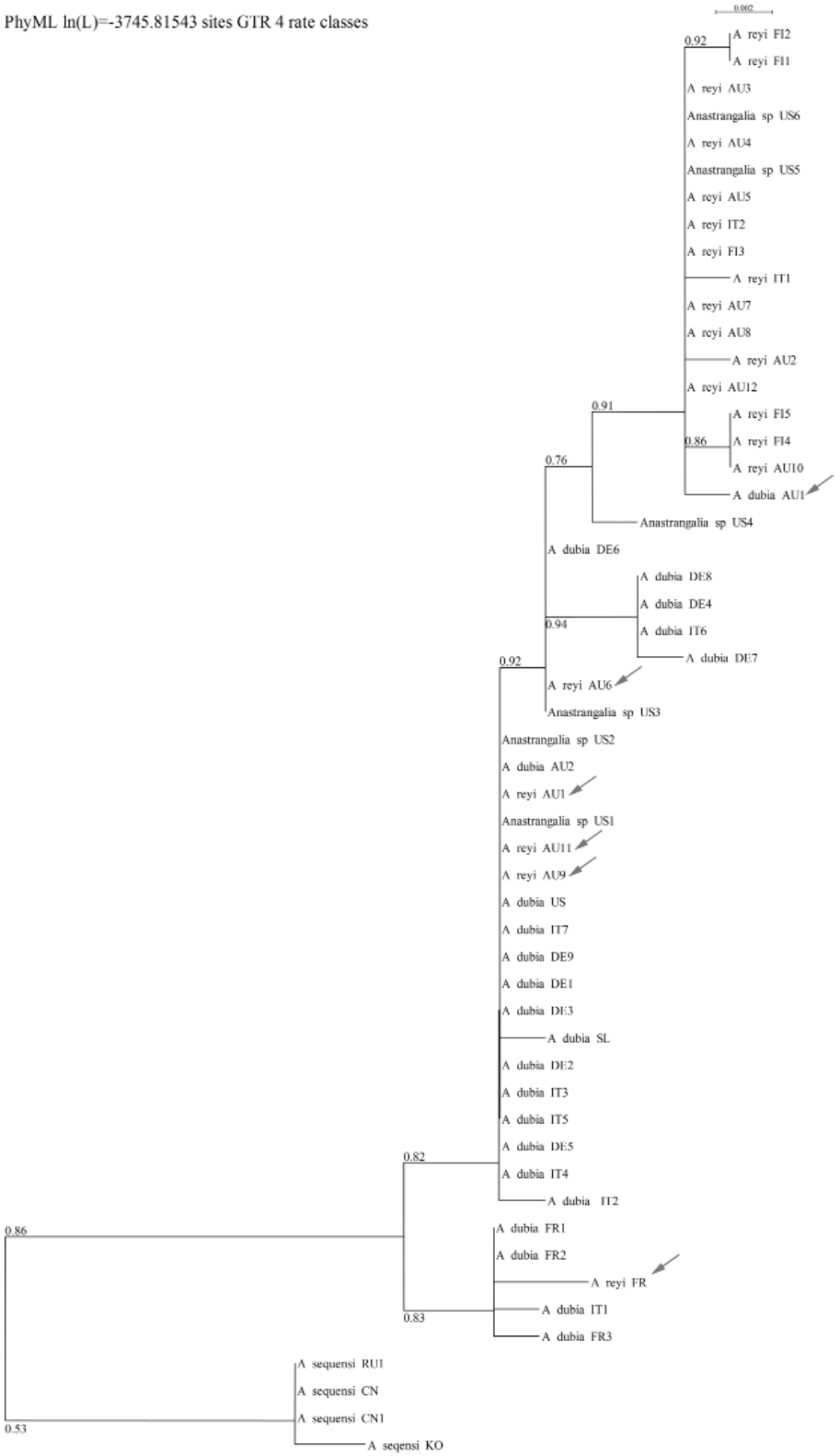

Fig. 3. Detailed phylogenetic subtree for the dubia group (hybrids of *A. reyi* and *A. dubia* are indicated by arrows).

The level of difference among COI haplotypes of *A. reyi*, *A. sequensi* and *A. dubia* is shown in table 2.

Surprisingly, the difference among *A. dubia* haplotypes is higher by an average of 0.2 % than between haplotypes of *A. dubia* and *A. reyi*. The COI sequences of *A. sequensi* differs from *A. reyi* by an average of 2.36 % and by 2.34 % from *A. dubia*. Comparison these species with *A. sanguinolenta* showed at least 12 % difference in the COI sequences. Thus, the divergence between of *A. reyi*, *A. sequensi* and *A. dubia* is a very weak comparing with the well separated species *A. sanguinolenta*.

Phylogenetic analysis. Based on 87 sequenced samples of the genus *Anastrangalia* and 2 sequences of outgroups we have built a well-resolved phylogenetic maximum likelihood tree (fig. 2). Nearly all branches are strongly supported based on an approximate likelihood-ratio test (aLRT). The *Anastrangalia* maximum likelihood tree consists of two strongly supported (1.00 aLRT) clades. The first clade includes the *dubia* group of species (e. g., *A. dubia*, *A. reyi*, *A. sequensi*). The *sanguinolenta* group (e. g., *A. sanguinolenta* and *A. sanguinea*) constitutes the second clade. We found that the *dubia*-group species grouped in a dense cluster, where the branches length do not exceed 0.01. This indicates that *A. dubia*, *A. reyi* and *A. sequensi* are poorly differentiated and their taxonomic status should be revised. In the subsequent analysis, we recognize *A. dubia*, *A. reyi* and *A. sequensi* as belonging to different evolutionary lineages (e. g., *dubia* lineage, *reyi* lineage and *sequensi* lineage) of the same species.

The detailed phylogenetic subtree of dubia-group is presented on the fig. 3. It consists of two weakly separated clades, which differ by 2.5 %. The lesser clade presents sequensi-lineage with two haplotypes AsFe-1 and AsFe-2. The bigger clade is an amount of the successive sister branches of the dubia-lineage and the reyi-lineage. We found that the AdPy haplogroup is well separated from the rest crown of the bigger clade. The AdPy branch includes a sample that is phenotypically *A. reyi*, however, the COI sequence indicates that it is *A. dubia*. The similarly we found several samples of phenotypically *A. reyi* in branches of AdAl haplogroup, which in fact contain *A. dubia* mitochondrion genome. The reyi-lineage (ArEu haplogrop) occupies top of the dubia-group successive tree and closely related to the AdAl haplogroup. The ArEu haplogroup contains a sample of phenotypically *A. dubia* with *A. reyi* COI sequence.

Discussion

Hybridization. The results of our phylogenetic analysis generally agree with the findings of other researchers that *A. reyi* and *A. dubia* are very similar in their COI sequences (Rougerie et al., 2015; Hendrich et al., 2015; Wu et al., 2016). Hendrich and colleagues (2015) noted that phenotypic *A. reyi* and *A. dubia* are mixed on the phylogenetic trees based on COI sequences. They indicate that molecular data are not consistent with morphological features. Rougerie et al. (2015) and Wu et al. (2016) have reached very similar conclusions on the impossibility of *A. reyi* and *A. dubia* discrimination based on the molecular data. On the contrary to them, we found crucial molecular markers for identification of *A. reyi* and *A. dubia*, which are described in the results (see above). This became possible after we added to our analysis COI sequences of *A. reyi* obtained by Pentinsaari et al. (2014) from Finland. Mentioned sequences are identical to the same from the Alps obtained by Hendrich et al. (2015). We revealed that *A. reyi* grouped in the dense monophyletic cluster (fig. 3), which are closely related to *A. dubia*. Despite that, we identified numerous cases of the introgressive hybridization of *A. reyi* and *A. dubia* in zone of overlapping of their areal in the Alps. This also has been noted by Rougerie et al. (2015). We found that some phenotypic *A. reyi* contains mitochondrial COI as in *A. dubia* and vice versa. These are the cases of current unimpeded hybridization between both species as the mitochondrial genome is non-recombinant and inherited only on the maternal line. The

Table 2. Distribution of haplotypes within samples of *A. dubia* and *A. reyi* and their hybrids

Haplotype	Positions of nucleotide substitutions in COI sequence (from 1 to 658): 22–271–370–496–565–619–658	Samples (see annex 1)
	Anastrangalia dubia	
AdPy-1	a–a–c–a–g–a–t	A_dubia_FR1, A_dubia_FR2, A_dubia_FR3, A_dubia_IT1
AdAl-1	g–a–t–a–a–g–t	A_dubia_DE1, A_dubia_DE2, A_dubia_AU2, A_dubia_DE3, A_dubia_DE5, A_dubia_DE9, A_dubia_IT2, A_dubia_IT3, A_dubia_IT4, A_dubia_IT5, A_dubia_IT7, A_dubia_SL, A_dubia_US
AdAl-2	g–g–t–a–a–g–t	A_dubia_AU1, A_dubia_DE6
AdAl-3	g–g–t–a–a–g–t (additional substitutions in positions 625 — a, 646 — c)	A_dubia_DE4, A_dubia_DE7, A_dubia_DE8, A_dubia_IT6
A. dubia (male) × *A. reyi* (female) ArEu-1	g–g–c–g–a–g–c	A_dubia_AU1
	Anastrangalia reyi	
ArEu-1	g–g–c–g–a–g–c	A_reyi_AU2, A_reyi_AU3, A_reyi_AU4, A_reyi_AU5, A_reyi_AU7, A_reyi_AU8, A_reyi_AU10, A_reyi_AU12, A_reyi_FI3, A_reyi_FI4, A_reyi_FI5, A_reyi_IT1, A_reyi_IT2,
ArEu-2	g–a–c–g–a–g–c	A_reyi_FI1, A_reyi_FI2,
A. reyi (male) × *A. dubia* (female) AdAl-1	g–a–t–a–a–g–t	A_reyi_AU1, A_reyi_AU9, A_reyi_AU11
A. reyi (male) × *A. dubia* (female) AdAl-2	g–g–t–a–a–g–t	A_reyi_AU6
A. reyi (male) × *A. dubia* (female) AdPy-1	a–a–c–a–g–a–t	A_reyi_FR
Anastrangalia sp.		
ArEu-1	g–g–c–g–a–g–c	Anastrangalia_sp_US5, Anastrangalia_sp_US6
AdAl-1	g–a–t–a–a–g–t	Anastrangalia_sp_US1, Anastrangalia_sp_US2
AdAl-2	g–g–t–a–a–g–t	Anastrangalia_sp_US3
AdAl-4	g–g–c–a–a–g–t	Anastrangalia_sp_US4

lack of reproductive barrier between *A. reyi* and *A. dubia* makes impossible to recognize them as separated species. The distribution of *A. reyi* and *A. dubia* haplotypes and their hybrids is presented in the table 3. By far, hybridization between *A. reyi* and *A, sequensi* is unknown, however their introgression is possible in the zone of their areal overlapping in the south of West Siberia.

Phylogeography. Generally, *A. dubia*, *A. reyi* and *A. sequensi* are vicariants, which replace each other from Atlantic coast to Pacific coast throughout all Eurasia. The altitudinal vicariance of *A. dubia* and *A. reyi* was observed by Brelih et al. (2006) and Hellrigl et al. (2012). While the differences on COI sequences between *A. reyi* and *A. dubia* is less than 1 % (table 1), the presence of numerous taxon-specific nucleotide substitutions evidences a period of their isolation. The extremely low interpopulation COI variation of A. *reyi* on the large territory from the Alps to Lapland indicates the relatively recent and a very rapid expanding of its areal. A very similar level of the interpopulation COI variation is typical for *A. sequensi*. On the contrary to them,

the COI sequences of A. *dubia* vary highly on the territory of Europe constituting at least 2 haplogroups and 5 haplotypes. Insufficient molecular data for *A. dubia* from the Carpathians, the Balkans, Asia Minor and the Caucasus makes its complete phylogeographic reconstruction impossible at the moment. Nevertheless, the high interpopulation variation of A. *dubia* COI points to several isolated centres of its formation. The time of the such centres existence refers to the Last Glacial Maximum (LGM) 26.5–18.5 ka, when rapid reducing of forests distribution was occurred (Terhurne-Berson, 2005; Svenning et al., 2008).

The current distribution of A. *reyi* coincides the spreading of *Picea abies* L. in Europe; *P. abies* is the host plant for *A. reyi* as well as *Pinus* L. in general. The areal of *A. reyi* (fig. 4) is disrupted into two parts: the eastern (Fennoscandia and the north of Eastern Europe) and western (the Alps). Populations of the eastern part occupy territories with dry and cold continental climate conditions reaching 65th parallel north (Danilevski, 2014). The western populations inhabit the wet and cold climate of the Alps and their spurs on the altitude over 1200 m a. s. l. (Brelih et al., 2006; Hellrigl et al., 2012). The presence of *A. reyi* in the Pyreneans is doubtful because it has not been mentioned in the detailed Cerambycidae Catalogue of the Iberian Peninsula (Gonzalez Pena et al., 2007), and only generally marked "SP" [Spain] in the Catalogue of Palearctic Coleoptera without any additional data (Löbl, Smetana, 2010). Since the published papers that prove its presence in the Pyreneans are unknown for us, we do not consider this area to the phylogeographyc analysis. As *A. reyi* lives in a cold climate, we consider that it has evolved in the periglacial refugia of *P. abies*. There are three main LGM refugia of P. *abies* known in Europe: The Massif Central (France), the Pannonian Plain (Hungary) and the Dnister Valley (Moldova) (Terhurne-Berson, 2005). However, all these refugia were located far in the south, where introgressive hybridization of *A. reyi* and *A. dubia* is highly probable. It should be noted that hybrids of *A. reyi* and *A. dubia* are known only from the Alps and have not been found in Fennoscandia. Thus, the LGM refugia of *A. reyi* are to be located in Eastern Europe. The most possible periglacial refugia of *A. reyi* were isolated forests of *P. abies* in the Don Valley (deposits age 30 ka) or surroundings of Plesheevo and Nero Lakes (deposits age 18–15 ka) in Russia (Terhurne-Berson, 2005). The isolated microsites of *P. abies* forests were scattered in the periglacial zone during LGM (Svenning et al., 2008). The current extremely low variability of COI sequences in populations of *A. reyi* indicates that it had to be emerged from a very small population restricted in the such microsite and the founder effect had a place. The expanding of *A. reyi* areal is believed to occur during rapid warming in Bølling-Allerød interstadial (11.7–10.7 ka). During this period there was a rapid expansion of *P. abies* forests from the eastern refugia to the west and north (Simakova & Puzachenko, 2005) and from the southern refugia to the north and east (Terhurne-Berson, 2005). These well agree with the molecular phylogeography of *P. abies* (Sperisen et al., 2001). At that time *A. reyi* was widespread in Eastern and Central Europe, reached the Carpathians and the Alps but not Britain. The areal had to be disrupted during the last cooling period in Younger Drias (10.7–9.7 ka). The following warm and dry conditions in Boreal (9.7–7.5 ka) and especially in Atlantic time (7.5–5 ka) caused extinction of *A. reyi* in the Carpathians and its isolation on the Alps highlands.

Anastrangalia sequensi occupies the same areal (fig. 4) as its host plants the East Siberian *Picea obovata* Ledeb. and the Far East *Picea jezoensis* (Siebold and Zuccarini) Carriere. Along with *Picea*, *Anastrangalia sequensi* also infests *Pinus*, *Abies*, and *Larix*, however their areals partly overlap. The lack of molecular data for *A. sequensi* from the broad territories of Siberia does not allow us to reconstruct its phylogeography completely and determinate of its LGM refugia. Nevertheless, the little data of available COI sequences points to extremely high homogeneity of *A. sequensi* populations at

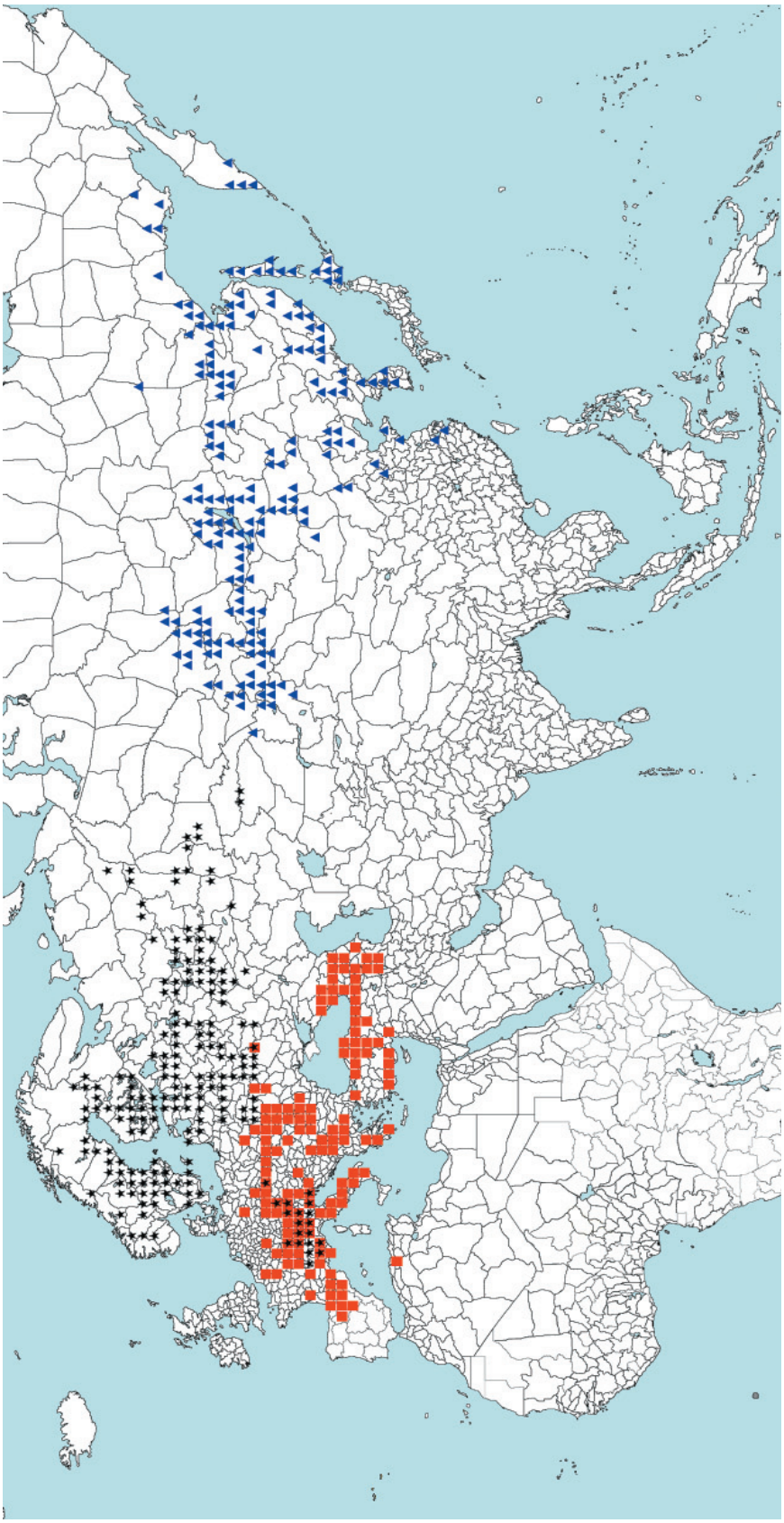

Fig. 4. Current distribution of *A. reyi* (stars), *A. sequensi* (triangles) and *A. dubia* (squares) in Eurasia.

least from the Far East. Alike *A. reyi*, *A. sequensi* had to be spread from a restricted LGM refugium to occupy current areal. Therefore, we indirectly identified possible LGM refugium and ways of post-glacial migration of *A. sequensi* using the data on the current and glacial vegetation cover of East Siberia and the Far East. At least three LGM refugia of *P. obovata* are known in South Ural, Altai and Baykalia (Blyakharchuk, 2010). During the Bølling-Allerød interstadial, and later during Holocene *P. obovata* had widespread within Siberia (Bezrukova, 2000; Blyakharchuk, 2010; Tollefsrud et al., 2015). *Picea obovata* migrated from South Ural to West Siberia and the north of Eastern Europe, from Altai it spread to the basin of Ob River and from Baikalia to the basins of Yenisei River and Lena River and further to the Pacific coast of Asia (Tollefsrud et al., 2015). We consider LGM refugia of *A. sequensi* to be located in isolated *P. obovata* forests in the south of East Siberia. The most probable LGM refugium for surviving of *A. sequensi* had to be Baykalia where *P. obovata* existed at the time (Bezrukova, 2000). We rejected the possibility of South Ural and Altai as *A. sequensi* LGM refugia for two reasons: firstly, *A. sequensi* is unknown from Ural and West Siberia where *A. reyi* is widespread; secondly, Altai is the westernmost limit of *A. sequensi* range (Danilevsky, 2014; Semaniuk, Zamoroka, 2018). We suggest that spreading of *A. sequensi* to Altai is a Late Holocene event, occurred probably during the Subboreal Time (5.7–2.5 ka). The assumption that there were also North Chinese or Korean LGM refugia connected with *P. jezoensis* is unlikely. First of all, *P. jezoensis* is widespread in the Far East including most of the Japanese Islands. *Picea jezoensis* is known to have spread to Kyushu and Honshu Islands via the land bridge from Korean Peninsula during LGM (Aizawa et al., 2007). However, *A. sequensi*, despite continental Asia including Korean Peninsula, distributed also on the Northern Japanese Islands (e. g. Sakhalin, Hokkaido, Kurile Islands) but it absents in the rest Japan (Semaniuk, Zamoroka, 2018). Thus, spreading of A. *sequensi* in the Far East happened much later after *P. jezoensis* migration. Nevertheless, the complete phylogeography of A. *sequensi* will be explained only after wider molecular studies across all of its areal.

The current areal of *Anastrangalia dubia* (fig. 4) coincides the spreading of *Abies* Mill. species in Europe (*Abies alba* Mill., *A. cephalonica* Loudon and their hybrids), Asia Minor and the Caucasus (*Abies nordmanniana* (Steven) and its hybrids, *Abies cilicica* (Antoine & Kotschy), North Africa (*Abies numidica* de Lannoy ex Carriere). *Abies* is the main food plant for *A. dubia*, but the larva also develops in decaying wood of *Picea*, *Pinus* and other conifers. We consider *A. dubia* to survive during last glaciation in *A. alba* LGM refugia in Europe. This explains the presence of *A. dubia* in the Pyreneans, the Apennines, the Balkans, Asia Minor and the Caucasus, where *P. abies* is completely absent at the present and during the last glaciation. The main LGM refugia of *A. alba* located in the Southern Pyreneans, the South-Western Alps, the Apennines and the Balkans (Terhurne-Berson, 2005). Locations of the LGM refugia of *Abies* in North Africa, Asia Minor and the Caucasus are still unclear. The post-glacial *A. alba* recolonization in Europe was started from the Pyreneans *c*. 10 ka ago invading South France and the Western Alps (Terhurne-Berson, 2005). The AdPy haplogroup of *A. dubia* spread from this LGM refugium. The different haplotypes of AdAl haplogroup, apparently have colonized Central Europe independently. The most probable LGM refugium of AdAl-2 and AdAl-3 situated in the Western Alps, where *A. alba* forests were known at that time (Terhurne-Berson, 2005). Their migrations occurred along the Northern Alps to the Ore Mountains and further to the Eastern Carpathians (Terhurne-Berson, 2005). Migration has finished with *A. alba* in the Eastern Carpathians at Subboreal Time (5.7–2.5 ka) (Kalinovych, 2003). The AdAl-1 haplotype is believed to migrate to the Alps from the Apennines LGM refugium. The Southern Carpathian *A. dubia* had to migrate with *A. alba* forests from the Balkans, the most probably from the Dinaric Mountains.

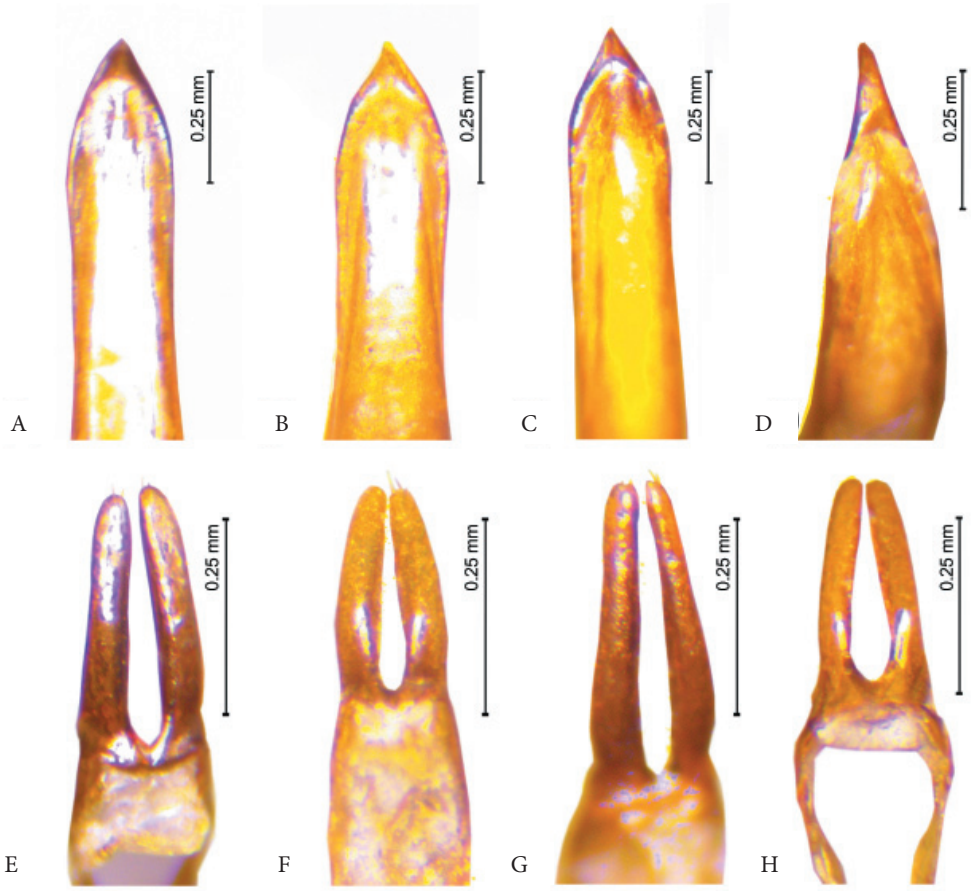

Fig 5. Male aedeagi (A–D) and parameres (E–H) of *A. reyi* (A, E), *A. sequensi* (B, F), *A. dubia* (C, G) and *A. sanguinolenta* (D, H).

Discrepancy between morphological and molecular data. We found a number of the morphological and molecular incompatibilities between *A. reyi*, *A. sequensi* and *A. dubia*. First of all, *A. reyi* and *A. sequensi* are completely identical in their morphology. Second, the COI sequences of *A. reyi* and *A. dubia* are nearly identical. Third, the morphology of *A. dubia* differs from both *A. reyi* and *A. sequensi*. Finally, the COI sequences of *A. sequensi* are different from both *A. reyi* and *A. dubia*. Thus, we conducted study of the male genitalia of *A. reyi*, *A. sequensi* and *A. dubia*, which is a classical entomological practice for the identification of sibling species (Simmons, 2014). The aedeagus morphology of *A. reyi*, *A. sequensi* and *A. dubia* (fig. 5, A, B, C) is nearly identical: the apical part of their aedeagus is slightly expanding and then narrowing, sharply terminating by a small sclerotized tip. The main morphological difference is the aedeagus width to length ratio: 0.179–0.185 (M = 0.182) in *A. reyi*, 0.207–0.227 (M = 0.217) in *A. sequensi*, and 0.160–0.173 (M = 0.167) in *A. dubia*. Morphology of the male parameres is generally very similar for *A. reyi*, *A. sequensi*, and *A. dubia* (fig. 5, E, F, G) differing by the lobes width to length ratio: 0.190–0.194 (M = 0.192) in *A. reyi*, 0.212–0.232 (M = 0.222) in *A. sequensi*, and 0.126–0.148 (M = 0.137) in *A. dubia*. Consequently, the aedeagus and paramere lobes of *A. dubia* are the longest and narrowest; *A. sequensi* — the shortest and widest; *A. reyi* —

intermediate between them. We also studied male genitalia of *A. sanguinolenta* (fig. 5, D, H) and found them to have crucial differences from *A. reyi*, *A. sequensi*, and *A. dubia*. This emphasizes close affinity of the specimens assigned to *A. reyi*, *A. sequensi* and *A. dubia*.

The minor differences of the male genitalia of *A. reyi*, *A. sequensi*, and *A. dubia* as well as numerous introgressive hybrids of *A. reyi* and *A. dubia* indicate insufficiency of the reproductive barrier between them. While morphological and molecular features of *A. sequensi* and *A. dubia* are the more or less differentiated, the positon of *A. reyi* is unclear. Based on the facts of the intermediated morphology of the male genitalia, the similarity of COI sequences and the results of the phylogenetic analysis shows *A. reyi* is to be considered a hybridogenic lineage originated from hybridization of *A. sequensi* and *A. dubia* in the past. We assume that *A. sequensi* was widespread from East Europe to Siberia during the Riss-Würm interstadial (130–115 ka), where it introgressed and hybridized with *A. dubia*. The climate changes during the Last Glaciation (115–9 ka) induced disappearing of the forest ecosystems in North Eurasia and extinction of *A. sequensi* in Europe, South Ural and West Siberia. However, the surviving of the hybrid population of *A. sequensi* and *A. dubia* restricted in the periglacial *P. abies* microsites gave rise of Holocene lineage of *A. reyi*. This is the outstanding case of the founder effect.

Taxonomic summary. Consequently, the morphological and molecular differences between specimens assigned to *A. reyi*, *A. sequensi* and *A. dubia* are sufficient for their recognition as conspecific rather than belonging to separate species, and as subspecies of the same species. We therefore consider *A. reyi* syn. n. and *A. sequensi* syn. n. to be junior synonyms of *A. dubia* and establish a subspecies rank for both of them: *Anastrangalia dubia reyi* **new rank** and *Anastrangalia dubia sequensi* **new rank**. The position of the nominal subspecies *Anastrangalia dubia melanota* (Faldennann, 1837) and *Anastrangalia dubia moreana* (Pic, 1906) is unclear. Both of them possibly belong to *Anastrangalia dubia dubia* (Scopoli, 1763).

Anastrangalia dubia (Scopoli, 1763)

Leptura dubia Scopoli, 1763: 47[1], *Leptura limbata* Laicharting, 1784: 166*; *Leptura notata* Olivier, 1795: 11*; *Leptura cincta* Fabricius, 1801: 356*; *Leptura chamomillae* Fabricius, 1801: 359*; *Marthaleptura dubia*, K. Ohbayashi, 1963a: 9*; *Anastrangalia dubia*, Villiers, 1978: 177*; *Anoplodera dubia*, Silfverberg, 2004*; *Corymbia dubia*, Zeegers & Heijerman, 2008: 78*; *Anastrangalia reyi* **syn. n.**; *Anastrangalia sequensi* **syn. n**.

Diagnosis: Body completely black. Elytra coloration vary. Male elytra fulvous with black edging, apex and suture, rarely completely black. Female elytra red with black edging and apex, frequently with big black stain on the disc or completely black. Pronotum longer than wider (for males 1.6 times, for female 1.3 times) with median furrow, covered by the dense decumbent hairs and the sparse standing hairs. The head shape varies due to temple size (e. g. sharply angled or smoothed). Male aedeagus curved, subapical slightly expanded then narrows sharply terminating by small sclerotized tip. Parameres long, narrow, touch each other by the apex where covered by bunch of long hair.

Distribution: Europe (except Britain Island, Iceland), North Africa (North Algeria), Asia Minor, the Caucasus, Siberia (except the most of the West Siberia), the Far East.

[1] Hereafter, if not otherwise stated, for references with asterisk (*) see Löbl, Smetana (2010) and Danilevsky (2014).

Anastrangalia dubia dubia (Scopoli, 1763)

Leptura dubia Scopoli, 1763: 47*; *Leptura limbata* Laicharting, 1784: 166*; *Leptura notata* Olivier, 1795: 11*; *Leptura cincta* Fabricius, 1801: 356*; *Leptura chamomillae* Fabricius, 1801: 359*; *Anastrangalia dubia dubia*, Slama et Slamova, 1996: 132*.

Type locality: "Carniolia" (= Krajina, Slovenia).

Diagnosis: Head: temple sharply angled. Aedeagus long and narrow, 0.175>AW/L [0.160–0.173 (M = 0.167))]. Paramere long and narrow, 0.165 > PW/L [0.126–0.148 (M = 0.137)]. Typical nucleotide substitutions of COI sequence in positions 22–271–370–496–565–619–658: 1) a–a–c–a–g–a–t; 2) g–a–t–a–a–g–t; 3) g–g–t–a–a–g–t; 4) g–g–c–a–a–g–t.

Distribution: North Africa (North Algeria), Mediterranean Basin, Central Europe, Asia Minor, the Caucasus.

Anastrangalia dubia reyi **new rank**

Leptura reyi Heyden, 1889: 203; *Leptura dubia* race *ochracea* Rey, 1885: 277; *Leptura inexspectata* Jansson & Sjöberg, 1928: 209, 212*; *Marthaleptura inexpectata*, K. Ohbayashi, 1963a: 9*; *Anoplodera reyi*, Lundberg, 1986: 114*; *Anastrangalia reyi*, Villiers, 1978: 180*; Danilevsky et Smetana, 2010: 97*.

Type locality: Germany.

Diagnosis: Head: temple smoothed. Aedeagus relatively long and narrow, 0.175 < AW/L < 0.2 [0.179–0.185 (M = 0.182)]. Paramere relatively long and narrow, 0.165 < PW/L < 0.207 [0.190–0.194 (M = 0.192)]. Typical nucleotide substitutions of COI sequence in positions 22–271–370–496–565–619–658: 1) g–g–c–g–a–g–c; 2) g–a–c–g–a–g–c.

Distribution: The Alps, Fennoscandia, Eastern Europe, the Southern Ural, Northern Kazakhstan.

Coment: it is believed to be a hybridogenic lineage originated from *A. sequensi* and *A. dubia*.

Anastrangalia dubia sequensi **new rank**

Leptura sequensi Reitter, 1898: 194, *Leptura sequensi* v. *rufopaca* Reitter, 1898: 194; *Leptura sequensi* v. *pulchrina* Reitter, 1898: 194; *Leptura sequensi* v. *tristina* Reitter, 1898: 194; *Leptura* (*Leptura*) *sequensi* var. *baikalensis* Pic, 1907: 6*; *Leptura sequensi* v. *baicalica* Pic, 1911: 4*; *Leptura* (s. str.) *sachalinensis* Matsushita, 1933: 104*; *Anoplodera* (s. str.) *sequensi,* Gressitt, 1951 a: 89*; *Marthaleptura sequensi*: Ohbayashi. 1963 a: 9*; *Anastrangalia sequensi*: Lobanov et al., 1981: 801; Sama, Lobl, 2010: 97*.

Type locality: "Ost-Sibirien: Quellgebiet des Irkut, Amur-Länder, Lena-Gebiet".

Diagnosis: Head: temple smoothed. Aedeagus relatively short and wide, AW/L > 0.2 [0.207–0.227 (M = 0.217)]. Paramere relatively short and wide; PW/L > 0.2 [0.212–0.232 (M = 0.222)]. Typical nucleotide substitutions of COI sequence in positions 22–206–247–250–304–316–331–379–451–463–496–523–548–550–619–625–631: 1) a–c–t–c–t–a–g–t–a–c–g–c–t–g–a–a–g; 2) a–c–t–c–c–a–g–t–a–c–g–c–t–g–a–a–g.

Distribution: Altai, Eastern Siberia, the Far East (except central and southern Japanese Islands).

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