SHILAP Revista de lepidopterología, 51 (201) marzo 2023: 37-50 eISSN: 2340-4078 ISSN: 0300-5267
https://doi.org/10.57065/shilap.433

The taxonomic separateness of the species *Aporophyla lueneburgensis* (Freyer, 1848) and *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775) occurring in Poland (Lepidoptera: Noctuidae)

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Abstract

Morphological and genetic comparisons were carried out of specimens of two sister species *Aporophyla lueneburgensis* (Freyer, 1848) and *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775), which are distributed allopatrically in Poland. The specimens from different populations of these two species hardly differ morphologically but do differ genetically. The mean genetic distance between the groups of *A. lueneburgensis* and *A. lutulenta* examined here indicates that their geographic populations can be treated as belonging to separate species. Hence, *A. lueneburgensis* and *A. lutulenta* occurring in Poland can be regarded as two separate but sister species.
Keywords: Lepidoptera, Noctuidae, *Aporophyla lueneburgensis*, *Aporophyla lutulenta*, distribution, Poland.

La separación taxonómica de las especies *Aporophyla lueneburgensis* (Freyer, 1848) y *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775) presentes en Polonia (Lepidoptera: Noctuidae)

Resumen

Se han realizado comparaciones morfológicas y genéticas de ejemplares de dos especies hermanas, *Aporophyla lueneburgensis* (Freyer, 1848) y *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775), que se distribuyen alopátricamente en Polonia. Los ejemplares de las distintas poblaciones de estas dos especies apenas difieren morfológicamente, pero sí genéticamente. La distancia genética media entre los grupos examinados aquí de *A. lueneburgensis* y *A. lutulenta* indica que sus poblaciones geográficas pueden tratarse como pertenecientes a especies distintas. Por lo tanto, *A. lueneburgensis* y *A. lutulenta* que se encuentran en Polonia pueden considerarse como dos especies separadas pero hermanas.
Palabras clave: Lepidoptera, Noctuidae, *Aporophyla lueneburgensis*, *Aporophyla lutulenta*, distribución, Polonia.

Introduction

The Western Palearctic genus *Aporophyla* Guenée, 1841, with a Ponto-Mediterranean distribution, occurs from the Atlantic coasts in the west, to eastern Turkey, Iraq and western Iran in the east. Six species of this genus are known from Europe, three of which - *Aporophyla lueneburgensis* (Freyer, 1848), *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775) and *Aporophyla nigra* (Haworth, 1809) - occur in central Europe (Ronkay et al. 2001).

The taxonomic separateness of *A. lueneburgensis* as a species has been debated by lepidopterologists since the late 19th century. Some authors were inclined to accept the specific separateness of this taxon. Ultimately, however, it came to be regarded as a synonym of *A. lutulenta* (Buszko & Nowacki, 2000; Nowacki, 1998; Nowacki & Fibiger, 1996). This view prevailed because of the great external similarity of the two species, despite the considerable individual variation within each group, especially as the type localities in Europe, given in the original descriptions of both taxa, are not so far apart: the Vienna region for *A. lutulenta* and Lüneburg (Lower Saxony) in Germany in the case of *A. lueneburgensis* (Orhant, 2012). However, meticulous examination of the structure and functioning of the genitalia of both species were crucial for restoring the specific status of *A. lueneburgensis* (Ronkay et al. 2001). At the same time, the ranges of the two species in Europe are generally allopatric. *A. lueneburgensis* is an Atlantic-Mediterranean species distributed from Spain and Portugal, through northern Italy, Switzerland, France, Great Britain, the Netherlands, Belgium, Denmark, Germany and southern Sweden, reaching its eastern boundary in Austria, the Czech Republic, Poland, Lithuania, Latvia, and southern Finland. In contrast, its sister species *A. lutulenta* has a Pontic distribution in south-eastern Europe, from the Caspian Sea across the steppes of southern Russia, Ukraine, all the countries on the Balkan Peninsula and Italy as far as central Europe, where it has been recorded in Hungary, the Czech Republic, Slovakia, Austria, south-eastern Germany and southern Poland (Aarvik et al. 2017; Buszko & Nowacki, 2017; Rákosy, 1996; Ronkay et al. 2001; Varga et al. 2005). It must be emphasized that the distributions of *A. lutulenta* and *A. lueneburgensis* to the north of the Carpathians and Sudetes are distinctly allopatric, and their range boundaries meet in Poland. Hitherto, *A. lutulenta* was thought to inhabit south-eastern Poland; its localities in southern Podlasie and Polesie clearly lie on its north-western range boundary. In contrast, *A. lueneburgensis* was earlier reported solely from western and central Poland, at numerous localities from Pomerania, through central Wielkopolska, as far as Lower Silesia (Buszko & Nowacki, 2017). In the light of recent records of *A. lueneburgensis* from Lithuania, Latvia and southern Finland (Aarvik et al. 2017), it appears crucial to re-examine the specific status of specimens regarded as *A. lutulenta*, reported earlier from eastern Poland (Nowacki, 2002).

Using genetic techniques, Orhant (2012) attempted to resolve the doubts surrounding the specific separateness of *A. lueneburgensis* and *A. lutulenta* from western Europe. However, his erroneous interpretation of the results led him to infer that only *A. lutulenta* was present in Europe, and that *A. lueneburgensis* was its junior synonym. In contrast, Andrillo (2019) expressed a different opinion. Based on the genetic code of the remains of *A. lueneburgensis* discovered in bat guano, he was able to document the presence of that species in the Geneva region of Switzerland, at the same time accepting that *A. lutulenta* was an eastern European species.

In view of the above, we set up the hypothesis that the two sister species *A. lueneburgensis* and *A. lutulenta*, occurring allopatrically in Poland, though exhibiting only slight morphological differences, are genetically different. This may be due to the historical breakup of the contiguous distribution of a precursor species in Europe and the subsequent long-term isolation of its geographical populations.

For these reasons, the aim of this research was to perform a morphological analysis and also an analysis of genetic separateness based on molecular studies of specimens from isolated populations from western Poland regarded as *A. lueneburgensis* and from eastern Polish populations regarded as *A. lutulenta*.

Materials and methods

The research material, in the form of imagines of both species, was accumulated from 2005 until 2019 during fieldwork at the following localities: for *A. lueneburgensis* in western Poland at Biedrusko, Bielinek, Borne Sulinowo, Marianka ad Brody, Poznań and Strzeszyn, and for *A. lutulenta* in eastern Poland at localities in five regions: Podlasie - Mielnik; Polesie - Macoszyn, Kosyń, Wola Uhruska; Bieszczady Mts. - Krzywe; Beskid Mts. - Umieszcz; and Pieniny Mts. - Sromowce Niżne. The moths

were caught at night, having been attracted to a white screen illuminated by a 250 W mercury vapour lamp. Each specimen intended for the molecular studies was preserved in ethanol.

- *A. lueneburgensis*: western POLAND: Bielinek 23-IX-2016, 1 ex., leg. R. Wąsala, Borne Sulinowo 26-V-2016 ex-larva, 1 ex., leg. A. Berezowski, Marianka ad Brody 18-IX-2012, 1 ex., leg. R. Wąsala, Poznań 2-X-2016, 2 exx., leg. R. Wąsala, Strzeszyn 25-IX-2010, 1 ex., leg. Ł. Matuszewski.

- *A. lutulenta*: eastern POLAND: Mielnik 17-IX-2015, 1 ex., leg. D. Wasiluk, Macoszyn 16-IX-2015, 1 ex., leg. M. Hołowiński, Kosyń 7-IX-2019, 1 ex., leg. M. Hołowiński, Wola Uhruska 30-IX-2016, 2 exx., leg. Ł. Dawidowicz, southern Poland: Krzywe 10-IX-2013, 2 exx., leg. Ł. Matuszewski, Umieszcz 15-IX-2014, 1 ex., leg. K. Mazur, and Sromowce Niżne 31-VIII-2005, 1 ex., leg. R. Wąsala.

The individuals intended for morphological analysis were killed with ethyl acetate, then mounted in the usual way for lepidopterans. Preparations of male and female genitalia were also made.

Morphological analysis

The span, structure and coloration of both pairs of wings were compared in 10 individuals of each species. The genitalia of males and females were also compared: 5 preparations each of males and females of both species were made in accordance with the technique given in Nowacki (1995).

Molecular analysis

For the molecular analysis two legs were taken from 10 individuals of *A. lutulenta* and from 10 of *A. lueneburgensis*. DNA was extracted using Macherey-Nagel's Nucleospin Tissue extraction kit, following the manufacturer's protocol. Amplification of mitochondrial gene COI was performed in a volume of 20 µl, in one or two parts, depending on the sample condition. The PCR protocol followed Wahlberg and Wheat (2008) with the following primer pairs (Simón et al. 1994; Folmer et al. 1994): LCO-1940 / HCO-2198 for COI sequenced in one piece; LCO-1940 / K699 and C1-J-1751(alias Ron) / C1-N-2191(alias Nancy) for COI sequenced in two pieces. The PCR products were sent for purification and sequencing to Macrogen Europe (Amsterdam, Netherlands); all the other analyses were conducted in the molecular laboratory of the Nature Education Centre of the Jagiellonian University, Kraków.

An additional 24 sequences of four species of *Aporophyla* were imported from the GenBank and BOLD Systems databases: *A. lutulenta*, *A. lueneburgensis*, *A. nigra* and *A. australis* (Boisduval, 1829). The final matrix involved 41 nucleotide sequences with a total of 612 positions in the final dataset. All sequences were aligned manually in Bioedit, version 7.0.9.0. (Hall, 1999). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). Pairwise distances were calculated using the Maximum Composite Likelihood model (Tamura et al. 2004) and the pairwise deletion option. A Maximum Likelihood tree was inferred using the Tamura 3-parameter (Tamura, 1992) model and the partial deletion option. The branch support for internal nodes was measured using 1000 rapid bootstrap replicates. The final tree was edited in Corel DRAW 2018 to enhance picture quality. The analyses were performed in the Molecular Laboratory of the Nature Education Centre of the Jagiellonian University.

Estimation of divergence times

Divergence times were estimated using the COI gene in Beast V.2.6.3 (Bouckaert et al, 2019) using the StarBEAST2 package (Ogilvie et al. 2017). The GTR nucleotide substitution model with four gamma categories and estimated base frequencies was implemented, and the three codon

positions were unlinked in order to be estimated independently. For this study, the constrained root age was obtained by using the results of Wang et al. (2014) as a secondary calibration, so that three secondary points were used: 1st: *Egira acronyctoides* Wileman, 1914, *Pseudopanolis heterogyna* Bang-Haas 1927, *Panolis flammea* ([Denis & Schiffermüller], 1775), *Panolis pinicortex* Draudt, 1950 (mean: 8.17), 2nd: *P. flammea*, *P. heterogyna* and *P. pinicortex* (mean: 7.31, 95% HPD: 4.99-9.87), and 3rd: *P. flammea* and *P. pinicortex* (mean: 6.54, 95% HPD: 4.52-8.87). Using the strict molecular clock model, the conventional mutation rate was fixed for the arthropod mitochondrial COI gene from the literature: 2.3% (0.0115 substitutions/site/million years) (Browner 1994; Wang et al. 2014; Nowacki et al. 2019). A Yule speciation process model was selected and two independent MCMC analyses were run for 30 000 000 generations, with Markov chains sampled every 5 000 generations. The results of the two runs were combined in LogCombiner 2.6.3 with the initial 10% of the trees discarded as burn-in. Tracer V. 1.7 (Rambaut et al. 2018) was used to determine convergence, measure the effective sample size of each parameter, and calculate the mean and 95% highest posterior density (HPD) intervals for the divergence times.

Results

The analyses confirmed the occurrence in Poland of both sister species *A. lueneburgensis* and *A. lutulenta.* Importantly, however, genetic analysis provided incontrovertible evidence that all the moths from Polesie and Podlasie in eastern Poland belonged to *A. lueneburgensis.* This will therefore require a revision of the earlier information given by Nowacki (2002) regarding the distribution of *A. lutulenta* in this region of Poland. By contrast, the occurrence of *A. lutulenta* in southern Poland was confirmed, i. e. in the Bieszczady, Beskid and Pieniny mountain ranges of the Carpathians.

Morphology

Morphological analysis of the two species revealed only slight differences between them. *A. lueneburgensis* from Poland has slightly narrower, clearly pointed forewings. The coloration is variable, from ash grey through brown to brownish-black, and the inner, outer and wavy cross-lines (bands) are clearly visible. The kidney-mark and oval are also fairly distinct (Figure 1). The wingspan is 33-38 mm. *A. lutulenta* from southern Poland has slightly broader forewings with slightly more rounded tips. In coloration it is more uniformly tawny to brown, the cross-lines are faint and the kidney-mark and oval are almost invisible (Figure 2). The wingspan is 35-40 mm.

Analysis of the genitalia in several individuals of both species did not reveal any substantial differences in their overall structure, either in males or in females. There are, however, some conspicuous differences in the organs of both sexes. In males the shape of the vesica everted from the aedeagus is different. In *A. lutulenta*, a section of the vesica corresponding to half the length of the aedeagus is only slightly deflected from the longitudinal axis of the aedeagus; beyond that section, there is another slight flexure of the vesica. The shape of the everted vesica in *A. lueneburgensis* is quite different: immediately beyond the aedeagus it is strongly deflected from the former's longitudinal axis. Also, the cornuti present at the base of the vesica are distinctly different in the two species (Figure 3). In the case of the females of both species a slight difference is perceptible in the structure of the ostium bursae: in *A. lutulenta* it is shorter and connected with the ductus bursae by a short membranous section, while in *A. lueneburgensis* it is longer and connected with the ductus bursae by a longer membranous section. There are particularly conspicuous differences in the structure of the ductus bursae. In *A. lutulenta* this is longer and for most of its length is membranous with a few stronger ribs, and only the proximal and distal ends are strongly sclerotized. In *A. lueneburgensis*, however, the ductus bursae is shorter and strongly sclerotized with just a short membranous section in the middle part. A slight difference is also discernible in the appendix bursae. In *A. lutulenta* it is clearly shaped and takes the form of an extended oval bulge, from the middle part of which the ductus seminalis emerges. By contrast, the appendix bursae in *A. lueneburgensis* is

Table 1. Pairwise genetic distances between *Aporophyla* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
1 *A. lutulenta* Austria Lower Austria ABCL4504-14																																						
2 *A. lutulenta* Austria East Tyrol PHLAH565-12	0.003																																					
3 *A. lutulenta* Austria Kaernten LEASS938-17	0.005	0.005																																				
4 *A. lutulenta* Italy South Tyrol PHLAH566-12	0.003	0.000	0.005																																			
5 *A. lutulenta* Italy Basilicata KX045668	0.008	0.005	0.009	0.005																																		
6 *A. lutulenta* Greece Epirus KX046313	0.006	0.006	0.008	0.006	0.011																																	
7 *A. lutulenta* Poland Beskidy Mts. Umieszcz ad. Jasło AZ-451	0.013	0.016	0.018	0.016	0.022	0.018																																
8 *A. lutulenta* Poland Bieszczady Mts. Krzyve AZ-455	0.011	0.011	0.013	0.011	0.016	0.016	0.009																															
9 *A. lutulenta* Poland Pieniny Mts. Sromowce Niżne AZ-468	0.002	0.002	0.004	0.002	0.007	0.004	0.014	0.013																														
10 *A. lutulenta* Poland Bieszczady Mts. Krzyve AZ-486	0.000	0.004	0.005	0.004	0.009	0.005	0.013	0.011	0.002																													
11 *A. luenеburgensis* Denmark LEELIA322-11	0.026	0.026	0.027	0.026	0.024	0.032	0.034	0.025	0.023	0.022																												
12 *A. luenеburgensis* Latvia Pape KT782466	0.024	0.024	0.026	0.024	0.023	0.030	0.032	0.023	0.022	0.020	0.002																											
13 *A. luenеburgensis* Norway Vest-Agder LON6118-17	0.025	0.025	0.026	0.025	0.022	0.031	0.033	0.024	0.022	0.020	0.002	0.000																										
14 *A. luenеburgensis* Switzerland Vaud ABCLC143-16	0.024	0.024	0.026	0.024	0.023	0.030	0.032	0.023	0.022	0.020	0.002	0.000	0.000																									
15 *A. luenеburgensis* Netherlands South Holland KX049799	0.026	0.026	0.027	0.026	0.024	0.029	0.032	0.023	0.022	0.020	0.003	0.002	0.002	0.002																									
16 *A. luenеburgensis* Germany Brandenburg GBLAF582-14	0.024	0.024	0.026	0.024	0.023	0.030	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.002																								
17 *A. luenеburgensis* Germany Schleswig-Holstein GBLAA1249-15	0.026	0.026	0.027	0.026	0.024	0.032	0.034	0.025	0.022	0.020	0.002	0.002	0.002	0.002	0.003	0.002																						
18 *A. luenеburgensis* Germany Lower Saxony Luneburg KX047223	0.024	0.024	0.026	0.024	0.023	0.030	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.002	0.000	0.002																					
19 *A. luenеburgensis* England Essex CGUKA152-09	0.024	0.024	0.026	0.024	0.023	0.030	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.002	0.000	0.002	0.000																				
20 *A. luenеburgensis* Poland Polesie Maroszyn AZ-452	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000																			
21 *A. luenеburgensis* Poland Polesie Kosyń AZ-453	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000																		
22 *A. luenеburgensis* Poland Poznań AZ-456	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000																	
23 *A. luenеburgensis* Poland Bielinek AZ-457	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000																
24 *A. luenеburgensis* Poland Borne Sulinowo AZ-460	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000															
25 *A. luenеburgensis* Poland Polesie Wola Uhruska AZ-490	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000														
26 *A. luenеburgensis* Poland Polesie Wola Uhruska AZ-487	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000													
27 *A. luenеburgensis* Poland Poznań AZ-491	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000												
28 *A. luenеburgensis* Poland Strzeszyn AZ-492	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000											
29 *A. luenеburgensis* Poland Marianka ad Brody AZ-494	0.020	0.020	0.022	0.020	0.018	0.025	0.032	0.023	0.022	0.020	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
30 *A. luenеburgensis* Poland Podlasie Mielnik AZ-489	0.022	0.022	0.023	0.022	0.022	0.027	0.034	0.025	0.023	0.022	0.005	0.004	0.004	0.004	0.004	0.004	0.005	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004									
31 *A. luenеburgensis* France Vendee GU663049	0.026	0.026	0.027	0.026	0.024	0.032	0.034	0.025	0.023	0.022	0.003	0.002	0.002	0.002	0.003	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.005								
32 *A. luenеburgensis* France Isere GU663045	0.026	0.026	0.024	0.026	0.024	0.032	0.034	0.025	0.023	0.022	0.003	0.002	0.002	0.002	0.003	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.003							
33 *A. australis* France Aude NOCJH011-09	0.049	0.047	0.047	0.047	0.049	0.049	0.058	0.051	0.045	0.047	0.053	0.052	0.053	0.052	0.050	0.052	0.053	0.052	0.052	0.049	0.049	0.049	0.049	0.049	0.049	0.049	0.049	0.049	0.049	0.052	0.053	0.053						
34 *A. australis* France Pas-de-Calais NOCJH012-09	0.052	0.050	0.047	0.050	0.052	0.052	0.061	0.054	0.049	0.051	0.056	0.055	0.056	0.055	0.053	0.055	0.056	0.055	0.055	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.056	0.056	0.056	0.053	0.003				
35 *A. australis* England CGUKB670-09	0.050	0.049	0.049	0.049	0.050	0.050	0.060	0.052	0.047	0.049	0.055	0.053	0.054	0.053	0.052	0.053	0.055	0.053	0.053	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.054	0.055	0.055	0.055	0.002	0.002			
36 *A. nigra* France Provence Cote d'Azur PHLAE463-11	0.056	0.058	0.058	0.058	0.056	0.062	0.067	0.060	0.058	0.056	0.058	0.056	0.056	0.056	0.058	0.056	0.058	0.056	0.056	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.058	0.058	0.058	0.040	0.043	0.041		
37 *A. nigra* Norway Vest-Agder LON2750-16	0.056	0.058	0.058	0.058	0.056	0.062	0.067	0.060	0.058	0.056	0.062	0.061	0.061	0.061	0.061	0.062	0.061	0.062	0.061	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.063	0.062	0.062	0.043	0.046	0.044	0.005	
38 *A. nigra* Germany Brandenburg GBLAB745-13	0.055	0.056	0.056	0.056	0.055	0.061	0.065	0.058	0.056	0.054	0.059	0.058	0.057	0.058	0.059	0.058	0.059	0.058	0.058	0.056	0.056	0.056	0.056	0.056	0.056	0.056	0.056	0.056	0.056	0.060	0.059	0.059	0.059	0.028	0.041	0.040	0.002	0.005
39 *A. nigra* England Essex CGUKA1144-09	0.056	0.058	0.058	0.058	0.056	0.062	0.067	0.060	0.058	0.056	0.061	0.059	0.059	0.059	0.061	0.059	0.061	0.059	0.059	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.061	0.061	0.061	0.061	0.041	0.044	0.043	0.005	0.008	0.003

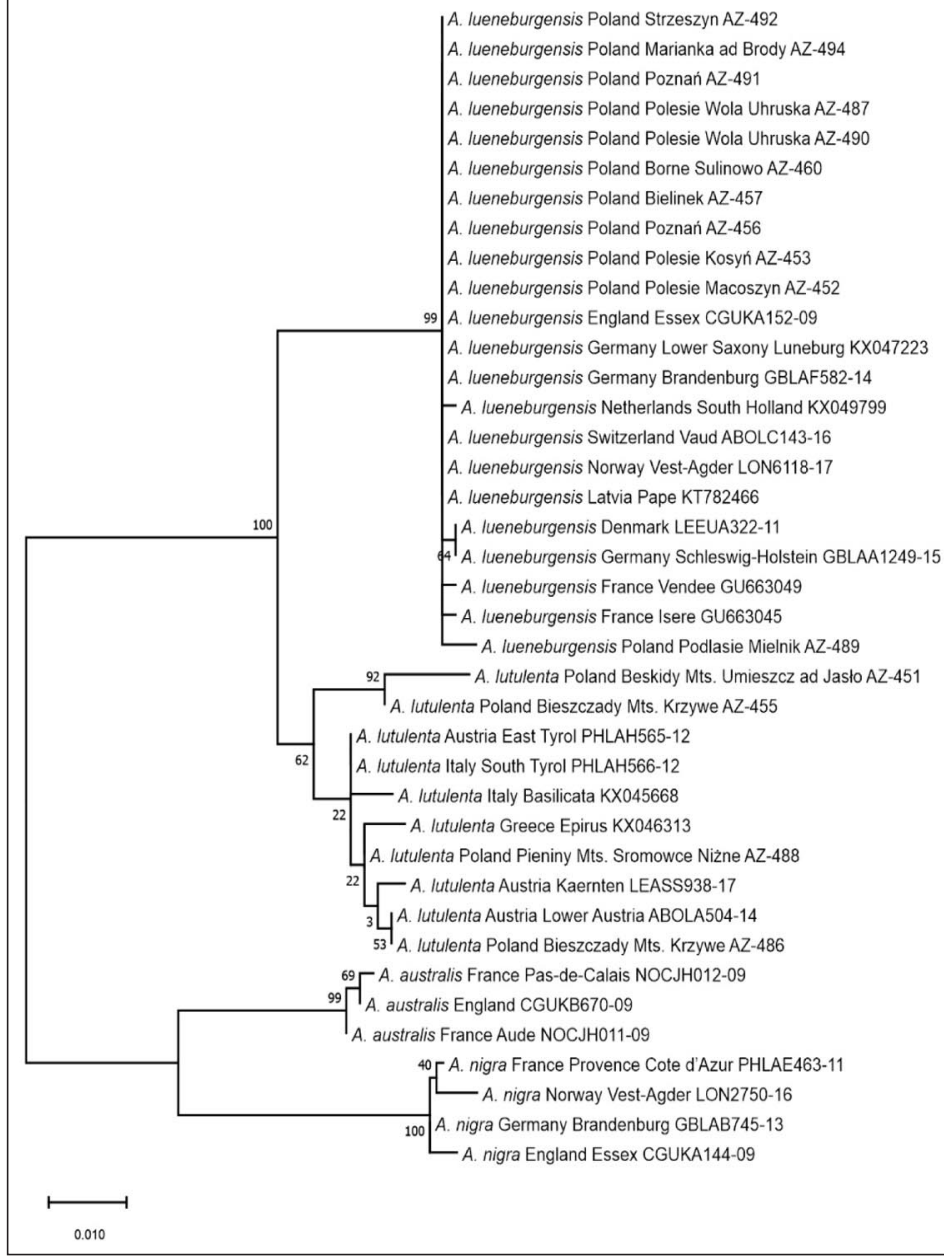

Figure 5. COI-based Maximum Likelihood phylogenetic tree of the genus *Aporophyla* with bootstrap branch support values.

slightly smaller, taking the form of a short, less regular bulge, with the ductus seminalis emerging from its distal end (Figure 4). Additionally, while it was not subject of analysis in this study, it is worth mentioning that differences between studied species are also noticeable in the adult caterpillars of said species (Wegner, 2021).

Genetic analysis

Genetic analysis of specimens of *A. lutulenta* and *A. lueneburgensis* from Poland showed unequivocally that all the moths (6 exx.) from the localities in western Poland, i. e. Bielinek, Borne Sulinowo, Marianka ad Brody, Poznań and Strzeszyn, belonged, as anticipated, to *A. lueneburgensis.* It turned out, moreover, that all the individuals from eastern Poland, i. e. Podlasie (1 ex.) and Polesie (4 exx.) from the localities at Mielnik, Macoszyn, Kosyń and Wola Uhruska, also belonged to *A. lueneburgensis.* In contrast, all the moths from southern Poland (4 exx.) from the localities at Krzywe (Bieszczady Mts.), Umieszcz (Beskid Mts.) and Sromowce Niżne (Pieniny Mts.) belonged to the sister species *A. lutulenta*.

The results of the phylogenetic analyses confirm that *A. lutulenta* and *A. lueneburgensis* belong to two distinct yet closely related species (Figure 5). The mean genetic distance between the *A. lutulenta* and *A. lueneburgensis* species groups was 0.02, whereas the distance between any other two of the examined species' groups was slightly greater, i. e. between 0.04 and 0.06. The mean within-group distance was the shortest in the *lueneburgensis* and the *australis* groups (0.002), longer in the *nigra* group (0.004) and the longest in the *lutulenta* group (0.008). The pairwise distances between all the specimens examined are shown in Table 1.

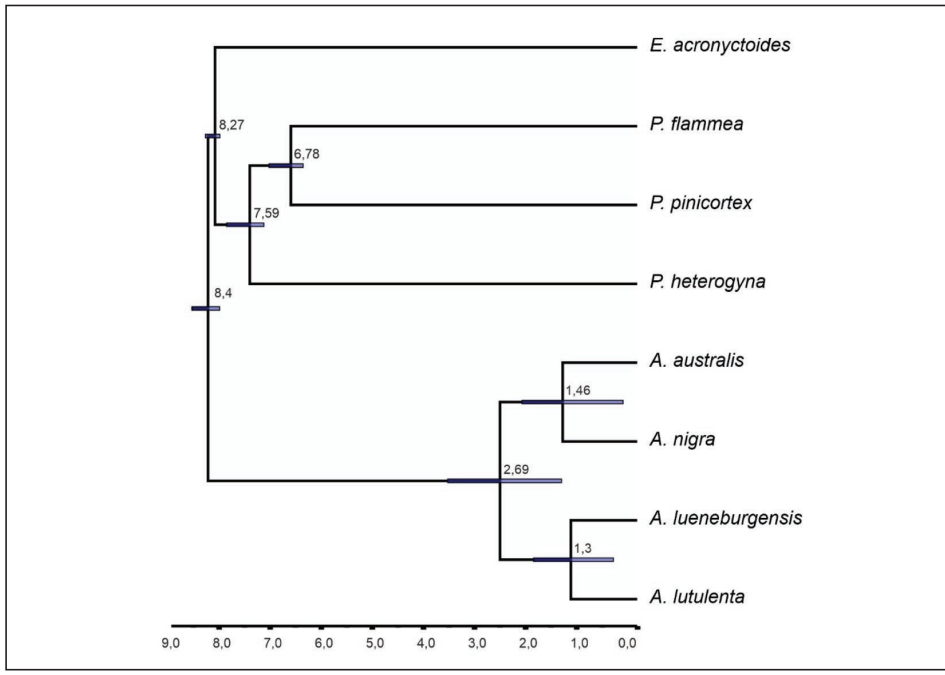

Figure 6. MCC tree of *Aporophyla* and the closest related genera derived from Bayesian analyses with divergence times shown in Myr.

The inference from the StarBEAST2 analysis is that the genus *Aporophyla* diverged during the Pleistocene (mean: 2.69 Mya, 95% HPD: 1.51-3.72) (Figure 6), which makes *Aporophyla* a relatively young genus within Noctuidae. Moreover, *A. australis* diverged from its sister species *A. nigra* 1.46 Mya (95% HPD: 0.3-2.32), and *A. lueneburgensis* from its sister species *A. lutulenta* 1.3 Mya (95% HPD: 0.5-2.12).

Discussion

The results of this research have confirmed that populations of the two sister species *A. lueneburgensis* and *A. lutulenta* in Poland are allopatric. It turned out, however, that the populations from eastern Poland (Podlasie and Polesie) belong not to *A. lutulenta*, as thought earlier (Nowacki, 2002), but to *A. lueneburgensis*. In the context of the latest records of *A. lueneburgensis* from Latvia and Lithuania (Aarvik et al. 2017), this result comes as no surprise. Morphological analysis of specimens of the two species has shown that they differ only slightly in shape, coloration and wingspan. But as there is considerable individual variation within each species, these morphological differences cannot be used for a reliable discrimination between them.

Figure 7. Map of the distributions of: **1.** *Aporophyla lueneburgensis* (Freyer, 1848). **2.** *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775) in Europe.

Similarly, analysis of the male and female genitalia revealed only slight differences in their overall structures. However, there were significant differences in the morphologies of the vesica everted from the aedeagus (male) and in the ostium bursae, ductus bursae and appendix bursae (female). These differences may effectively prevent spermatophore transfer to the female bursa copulatrix during the second stage of copulation. It seems, therefore, that the lock-and-key systems in the internal genitalia

can prevent successful copulation between individuals of the two species (Lafontaine & Mikkola, 1987; Mikkola, 1993).

Genetic analysis showed that individuals of *A. lueneburgensis* from western and eastern Poland differed distinctly from those of *A. lutulenta* obtained from the Bieszczady, Beskid and Pieniny mountain ranges in south-eastern Poland. The calculated genetic distance between individuals of these two sister species was 0.02, which confirms their taxonomic separateness. For comparison, the genetic distance calculated for pairs of the morphologically distinct species *A. australis* and *A. nigra* is slightly greater at 0.04. What is surprising is the relatively large difference between the mean intra-group distance in *A. lueneburgensis* and *A. lutulenta.* It was the smallest (0.002) among the specimens of *A. lueneburgensis*, which testifies to the minimal genetic differentiation of this species in Europe. This may be due to the distribution of *A. lueneburgensis* being contiguous in western Europe, where uplands and lowlands are dominant and there are no distinctive barriers separating particular populations. This stands in contrast to *A. lutulenta,* in which the mean intra-group distance was 0.008, the highest value of any of the four *Aporophyla* species. This indicates the considerable dispersion and isolation of *A. lutulenta*'s various populations in Europe, which is hardly surprising since it occurs in those parts of Europe dominated by mountains, from Italy and the eastern Alps and Carpathians to the south and east across the Balkan Peninsula and further as far as the Near East (Figure 7).

This is confirmed by the results of Orhant's (2012) comparison of a number of morphologically diverse specimens, superficially resembling both species, from western Europe, i. e. from Belgium and France to western Austria and Germany. He inferred from the results of his genetic study that all the specimens belonged to the same species, *A. lutulenta*, and that *A. lueneburgensis* was merely a synonym of the former. Orhant's (2012) diagnosis was correct in the sense that he was dealing with one species, but crucially, the specimens he examined came from areas of Europe potentially inhabited exclusively by *A. lueneburgensis.* Andrillo (2019) expressed exactly the opposite opinion: he compared the genetic code of the remains of *A. lueneburgensis* discovered in bat guano from the Geneva region in Switzerland with sequences deposited in the BOLD gene bank and found that it did indeed indicate the presence of that species. On this basis he carried out a revision of earlier faunistic data, removing *A. lutulenta* from the list of Swiss Lepidoptera and replacing it with *A. lueneburgensis*. At the same time, he acknowledged that *A. lutulenta* was a species occurring in eastern Europe (Andrillo, 2019). This hypothesis was supported by results relating to both species from Bavaria and Austria, where both species occur (Haslberger & Segerer, 2016; Huemer et al. 2019). It turns out, then, that the high mountain ranges crossing central Europe, from the Alps to the Sudetes and western Carpathians, form the boundary between the distributions of *A. lueneburgensis* and *A. lutulenta*. In summary, those areas of Europe lying to the north of these mountain ranges, from the Iberian Peninsula to southern Scandinavia, can be assumed to be inhabited by *A. lueneburgensis,* while those to the south and east are home to *A. lutulenta.*

The results of this research confirm our hypothesis regarding the phylogeny and interrelationships of *A. lueneburgensis* and *A. lutulenta*. This proposed that as a consequence of the historical breakup of the contiguous population of the precursor species in Europe, populations of these moths became isolated from each other for an extremely long time, eventually evolving into two distinct, sister species. We can assume that the distribution of the precursor species was disrupted during the Pleistocene as a result of the Günz glaciation 1.2 - 0.95 Mya. During that period, northern and eastern Europe was covered by the ice-sheet whereas the remainder of the continent from the Pyrenees to the Black Sea was a periglacial zone with long-term permafrost (Mojski, 1993). This hypothesis is confirmed by the approximate divergence time of *A. lueneburgensis* and *A. lutulenta*, calculated at c. 1.3 Mya. The precursor species' original distribution having been split up, the isolated populations will have survived in two refuges: the Pyrenean one along the Atlantic coast and the Near Eastern one to the south of the Black Sea. The speciation of the two present-day sister taxa gradually took place during the subsequent hundreds of thousands of years of glaciation in Europe. Once the ice-sheet had begun to retreat from Europe, during the Holocene, the two new species gradually started spreading into the ice-

free areas. *A. lueneburgensis*, being adapted to the Atlantic climate, moved mainly along the coasts of the Atlantic Ocean and North Sea, colonizing the whole of western Europe and progressively extending its range eastwards across Germany and into western Poland. In the late 20th century, it expanded rapidly into the areas around the Baltic Sea: records from southern Sweden and Finland, as well as Lithuania and Latvia testify to this (Aarvik et al. 2017). As our research has shown, it has also reached eastern Poland. *A. lutulenta*, on the other hand, evolved from that part of the precursor population isolated in the Near Eastern refuge and probably became adapted to the warmer Mediterranean climate. During the Holocene, it gradually spread westwards along Mediterranean coasts, eventually reaching the Alps and locally many sites to the south of the line formed by the Carpathians, Sudetes and Alps. This is a probable explanation for the allopatric distribution of the two species in Poland. It is certain that *A. lutulenta* occurs in southern parts of Poland, where it was recorded at Stuposiany (Bieszczady Mts.) (Bieszczady, 1973) and also during our field studies. The earlier literature data pertaining to the occurrence of *A. lutulenta* in Western Pomerania (Urbahn & Urbahn, 1939), Gdańsk (Speiser, 1903) or Lower Silesia (Wolf, 1935-1944) in fact relate to *A. lueneburgensis*, which has recently been recorded in eastern Poland as well.

Acknowledgments

We wish to thank Dr. Antonio Vives (Madrid, Spain) for translating the title and abstract into Spanish.

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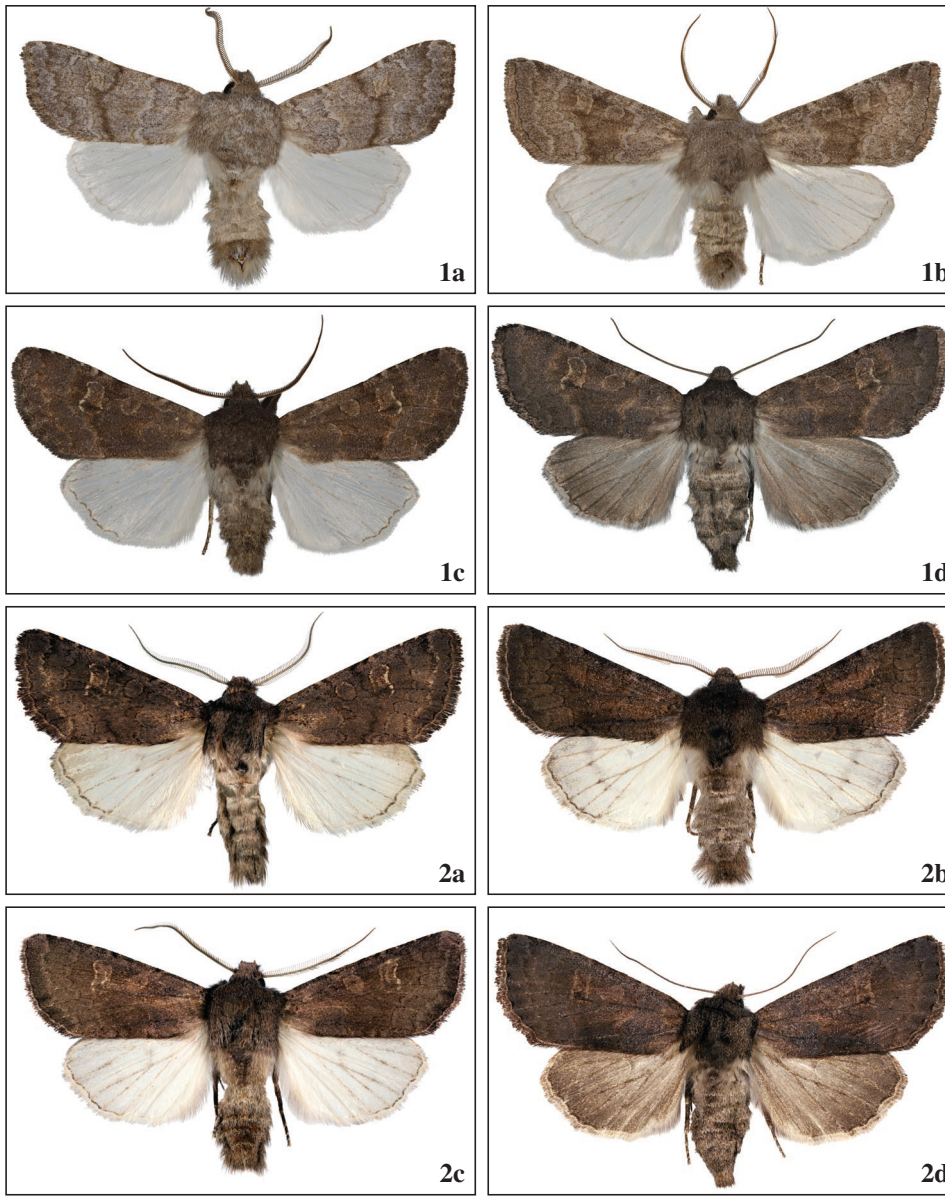
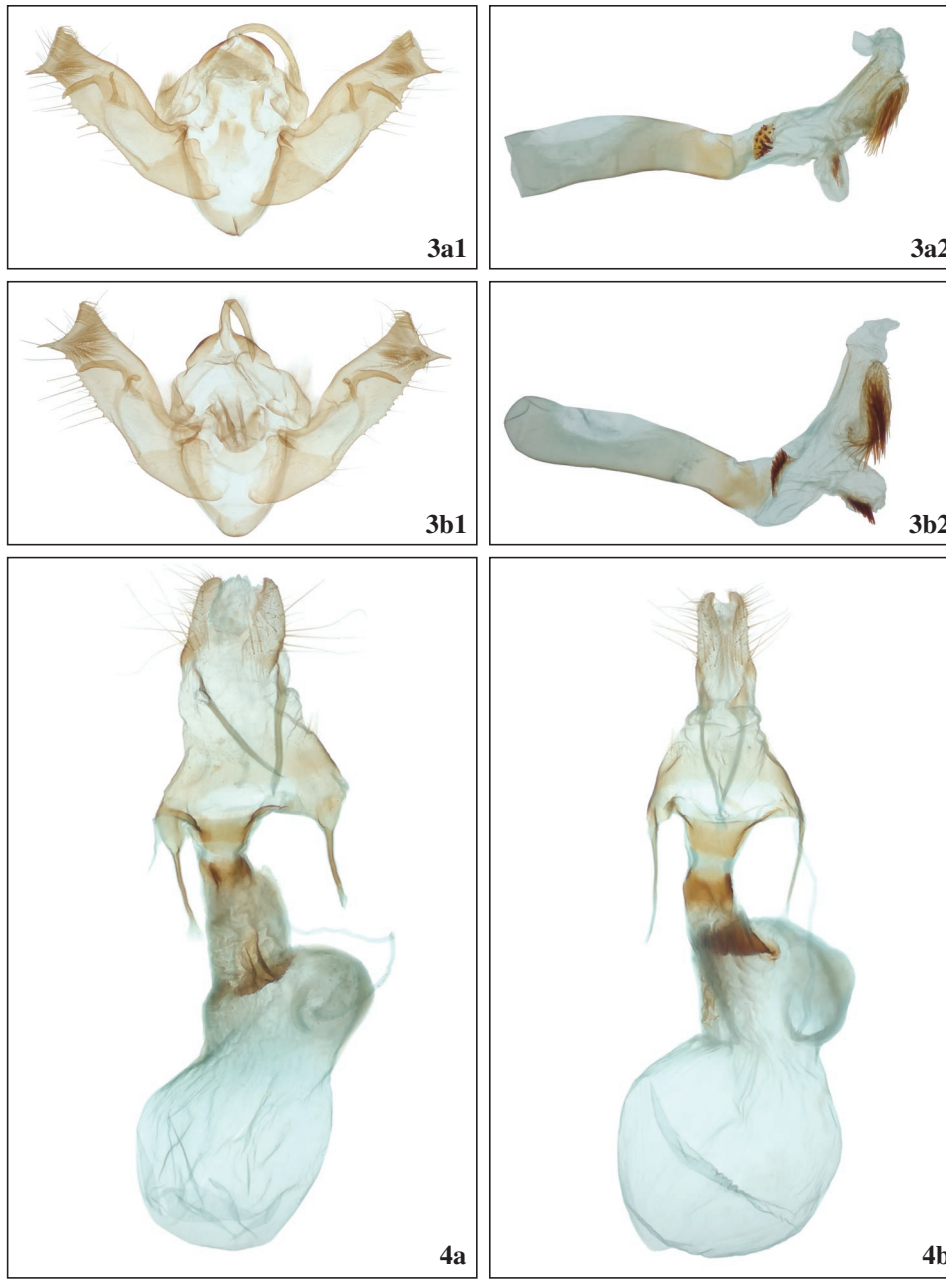
*Autor para correspondencia / *Corresponding author*

(Recibido para publicación / *Received for publication* 2-XII-2021)
(Revisado y aceptado / *Revised and accepted* 30-III-2022)
(Publicado / *Published* 30-III-2023)



Figures 1-2. 1. Imagines of *Aporophyla lueneburgensis* (Freyer, 1848) from Poland: **a.** Biedrusko, 12-IX-1999, leg. J. Nowacki, **b.** Biedrusko, 12-IX-1999, leg. J. Nowacki, **c.** Mielnik, 10-IX-2005, leg. D. Łupiński, D. Mielnik, 11-IX-2005, leg. D. Łupiński. **2.** Imagines of *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775) from Poland: **a.** Pieniny Mts., Zamczysko, 29-VIII-2003, leg. J. Nowacki, **b.** Bieszczady Mts., Krzywe, 10-IX-2013, leg. Ł. Matuszewski, **c.** Bieszczady Mts., Krzywe, 10-IX-2013, leg. Ł. Matuszewski, **d.** Bieszczady Mts., Krzywe, 10-IX-2013, leg. Ł. Matuszewski.



Figures 3-4. 3. Male genitalia: **a1, a2.** *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775), **b1, b2.** *Aporophyla lueneburgensis* (Freyer, 1848). **4.** Female genitalia: **a.** *Aporophyla lutulenta* ([Denis & Schiffermüller], 1775), **b.** *Aporophyla lueneburgensis* (Freyer, 1848).