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Variation in mitochondrial sequences and shell morphology of *Clausilia dubia* Draparnaud, 1805 (Gastropoda: Clausiliidae) in eastern Austria

Katharina Mason[1,2], Helmut Sattmann[1,3], Anita Eschner[1], Michael Duda[1,3], Josef Harl[3,4], Luise Kruckenhauser[2,3], Christoph Leeb[3] & Elisabeth Haring[2,3]

[1] *3rd Zoological Department, Natural History Museum Vienna, Burgring 7, 1010 Vienna, Austria*
[2] *Department of Evolutionary Biology, University of Vienna, Althanstraße 14, 1090 Vienna, Austria*
[3] *Central Research Laboratories, Natural History Museum Vienna, Burgring 7. 1010 Vienna, Austria*
[4] *Institute of Pathology, University of Veterinary Medicine, Vienna, Veterinärplatz 1, 1210 Vienna, Austria*

Correspondence: katharina.mason@nhm-wien.ac.a

Abstract: A molecular systematic analysis based on the mitochondrial *cytochrome c oxidase subunit 1* (*COI*) gene as well as a morphometric analysis was performed in *Clausilia dubia* in eastern Austria. Altogether 170 individuals of *C. dubia* were analysed (142 included in the morphometric analysis, 143 in the molecular genetic analyses) representing 12 subspecies occurring in eastern Austria (Lower Austria and Vienna). The observed intraspecific diversity in the mitochondrial marker sequence was high, with *p* distances up to 22.2 %. In the phylogenetic tree *C. dubia* was divided into four clades, none of them, however, corresponding to any of the subspecies which appeared to be randomly distributed within the tree. Subspecific assignment was possible by combining several morphological characters including the qualitative ones. Yet, subspecies distribution ranges overlapped considerably and some morphotypes occurred sporadically in distant places. The PCA analysis of shell measurements did not differentiate the subspecies. The mean values of measurements of several subspecies differed, but there was a considerable overlap in the ranges of measurements in most subspecies. The only correlation detected was that shells tend to be smaller, more densely ribbed, and in general less variable in higher elevations. These findings imply that environmental factors (e.g., altitude) could partly explain the high conchological variability within *C. dubia*. Based on the present data there are no good arguments for the current subspecific classification of *C. dubia*, at least not in the region in Lower Austria and Vienna covered. Future investigations should include qualitative characters into morphological analyses. Moreover, population genetic analyses including also nuclear markers and comprising all subspecies over their entire distribution ranges should be performed.

Key words: Clausiliidae, subspecies, mitochondrial variation, shell measurements, conchological variation, Austria

Zusammenfassung: An ost-österreichischen Populationen der Gitterstreifigen Schließmundschnecke *Clausilia dubia* wurden sowohl molekularbiologische Untersuchungen basierend auf dem Cytochrom-C-Oxidase-Untereinheit-1-Gen (*COI*) als auch morphometrische Analysen durchgeführt. Insgesamt wurden 170 Individuen der Art untersucht, die 12 in Ost-Österreich (Niederösterreich und Wien) vorkommende Unterarten repräsentierten. Davon wurden 142 in die morphometrische Analyse einbezogen, 143 in die DNA-Analyse. Die beobachtete intraspezifische Diversität der mitochondriellen Markersequenz war hoch, mit p-Distanzen bis zu 22,2 %. Im phylogenetischen Baum war *C. dubia* in mehrere Linien (Clades 1 bis 4) unterteilt, von denen allerdings keine einer der Unterarten entsprach. Diese schienen zufällig innerhalb des Baumes verteilt zu sein. Eine subspezifische Zuordnung war möglich, indem mehrere morphologische Merkmale, einschließlich der qualitativen, kombiniert wurden. Die Verbreitungsgebiete der Unterarten überlappten jedoch erheblich, und einige Morphotypen traten sporadisch in von der Hauptverbreitung entfernten Gebieten auf. Eine Hauptkomponentenanalyse der Schalenmaße differenzierte die Unterarten nicht. Die Mittelwerte der Messungen mehrerer Unterarten waren unterschiedlich, bei den meisten Unterarten gab es jedoch erhebliche Überschneidungen der Messbereiche. Die einzige festgestellte Korrelation bestand darin, dass die Schalen mit steigender Meereshöhe tendenziell kleiner, dichter gerippt und im Allgemeinen weniger variabel waren. Diese Ergebnisse deuten darauf hin, dass Umweltfaktoren (z. B. Seehöhe) teilweise die hohe Schalenvariabilität innerhalb von *C. dubia* erklären könnten. Die momentan vorhandenen Daten unterstützen die Unterarten-Klassifizierung von *C. dubia* nicht, zumindest nicht im untersuchten Gebiet in Niederösterreich und Wien. Zukünftige Untersuchungen sollten qualitative Merkmale in die morphologischen Analysen einbeziehen sowie populationsgenetische Analysen, auch mit nukleären Markern und über das gesamte Verbreitungsgebiet von *C. dubia* umfassen.

Schlüsselwörter: Clausiliidae, Unterarten, mitochondriale Variation, Schalenmaße, Variation der Schalenmorphologie, Österreich

Introduction

The family Clausiliidae, belonging to the pulmonate land snails (Stylommatophora) is characterised by a complex clausiliar apparatus in the aperture, a unique feature among gastropods protecting the animals against dehydration and predation (Solem 1972, Gittenberger 1995). Clausiliidae are easily distinguishable from other land gastropods by their tower-shaped shell, which is usually left-coiled. But there are also exceptions, such as the subfamily Alopiinae with predominantly species with right-handed shell coiling. The family Clausiliidae currently includes nine subfamilies, 155 genera, and 1278 species (Nordsieck 2007) with a high number of subspecies per species compared to other gastropod families (Páll-Gergely et al. 2019). Clausiliidae are mainly distributed in Europe, Asia, and South America, but occur also in Africa (Kerney et al. 1983, Nordsieck 2007). Many clausiliid species prefer rocky habitats for which the slender shell provides a perfect adaptation allowing them to retreat into narrow rock crevices. The present study deals with *Clausilia dubia* Draparnaud, 1805, one of the four species of the genus *Clausilia* currently recognised for Austria. While in earlier literature mainly morphological features of the outer shell of the animals were used for the description and differentiation of *Clausilia* species (Ehrmann 1933, Ložek 1964, Kerney et al. 1983), the most recent overview of Nordsieck & Neubert (2002) considered almost exclusively differences in the clausiliar structures. The Craven door snail, *Clausilia dubia,* is a mainly calciphilous species preferably living on damp and shady rocks and stone walls (Kerney et al. 1983). In Austria it occurs from lowlands to high elevations (220 – 2260 m above sea level) (Klemm 1960).

The species has been known from numerous fossil findings since the Pliocene, where it occurred in roughly the same distribution area in Western and Central Europe as it does today (Kerney et al. 1983, Frank 2006). The high morphological variation and the considerable number of subspecies described within *C. dubia* [21 according to Bank & Neubert (2017)] make this species taxonomically challenging. The number of subspecies occurring in Austria differs depending on the literature: both in the CLECOM/MolluscaBase list (Falkner et al. 2000, Bank & Neubert 2017), as well as in the list of Klemm (1960) there are 14 subspecies recorded for Austria, while the 'Checklist of Austrian Molluscs' (Reischütz 1998, supplemented by Fischer 2015) lists 16 subspecies. Presently, altogether 17 subspecies occurring in Austria are mentioned in the available literature, 13 of which occur in Eastern Austria (Lower Austria and Vienna). These subspecies can be distinguished by the characters described in the literature (Klemm 1960, 1974) which are summarised comprehensively in Jaksch (2012). Yet, subspecies classification in Eastern Austria is problematic as in this relatively small area the distribution ranges of subspecies partially overlap (Klemm 1974). This is contradictory to the definition of subspecies as the combination of phenotypically similar populations of a species that inhabit a geographical subarea (of the species' distribution range) and differ morphologically from other populations of the species (Mayr 1969). Therefore, their delimitation appears doubtful.

Previous studies on *C. dubia*, depending on various morphological criteria, had led to different conclusions regarding the validity of the subspecies (Klemm 1960, Edlinger 1997, Frank 1997, Edlinger 2000, Edlinger & Fischer 2000, Nordsieck 2002). A critical reevaluation of the subspecies of *C. dubia* was done by Nordsieck (2002), who emphasised that clausiliids can mainly be determined based on clausiliar structures (Nordsieck & Neubert 2002, Nordsieck 2007). According to his new system, *C. dubia* is divided in two "major subspecies" only, *C. d. dubia* s. l. in the eastern range of the species and *C. d. vindobonensis* s. l. in the western part of the distribution. According to Nordsieck (2002), all other subspecies should be included into one of these two or should take an intermediate position between them. Indeed, Nordsieck (2002) classified some of the subspecies/populations treated here as intermediate.

In the present study, we reanalysed the results of Jaksch (2012) concerning morphological subspecies differentiation and phylogeography of *C. dubia* in Eastern Austria, the region where the majority of subspecies occur. Besides a morphometric analysis including twelve of the 13 occurring subspecies, we reanalysed the mitochondrial *cytochrome c oxidase subunit 1* (*COI*) data and present here the updated results. The following questions were addressed: (1) Is the subspecies division of *C. dubia* in Eastern Austria reflected in the mitochondrial gene tree? (2) Is there a geographic pattern in the genetic tree? (3) Can the subspecies be differentiated by a morphometric analysis? (4) If the morphometric analysis revealed any other groups, are they differentiated genetically?

Material and Methods

Samples of *C. dubia* were collected in the years 2005–2011 between May and October at 59 sampling sites (Appendix 1). These were located in seven mountainous regions at the margins of the Eastern Alps: Rax-Schneeberg Group, Gutenstein Alps, Ybbstal Alps, Prealps east of the Mur, Wienerwald, Waldviertel, and Wachau. Sampling sites were mostly limestone rocks, situated between 276 and 2024 m asl in Lower Austria and Vienna (Appendix 2). Twelve out of the 13 subspecies listed for Lower Austria were included in the present study: *C. d. dubia* Draparnaud, 1805; *C. d. bucculenta* Klemm, 1960; *C. d. gracilior* Clessin, 1887; *C. d. huettneri* Klemm, 1960; *C. d. kaeufeli*

Klemm, 1960; *C. d. moldanubica* Klemm, 1960; *C. d. obsoleta* Schmidt, 1857; *C. d. runensis* Tschapeck, 1883; *C. d. schlechtii* A. Schmidt, 1857; *C. d. speciosa* A. Schmidt, 1857; *C. d. steinbergensis* Edlinger, 2000; *C. d. tettelbachiana* Rossmässler, 1838; *C. d. vindobonensis* A. Schmidt, 1857. Only *C. d. steinbergensis* Edlinger, 2000 could not be included due to a lack of material. Examples of the subspecies are provided in Plates 1 and 2 in the Appendix. Whenever possible, type localities were sampled. The specimen list (Appendix 1) provides sampling sites, geographic regions, lab codes as well as inventory numbers of the specimens stored in the Mollusc Collection at the Natural History Museum Vienna (NHMW). Altogether 170 individuals of *C. dubia* were analysed, 142 of which were included in the morphometric analysis (only individuals with completely intact shells were investigated) and 143 in the molecular genetic analyses (in most cases, three individuals per locality; five for type localities). *Neostyriaca corynodes* (Held, 1836) served as outgroup in the DNA analysis. This sample was also collected in the course of our field excursions in Lower Austria (Appendix 1, Fig. 1).

Taxonomic classification

Since the characters used in the original descriptions and determination keys of *C. dubia* (Ehrmann 1933, Klemm 1960, Kerney et al. 1983, Ložek 1964) varied and also differed partially from Nordsieck's (2002) system emphasising on clausiliar structures, a comprehensive determination key was established by Jaksch (2012) including all available qualitative shell traits. Specimens were determined accordingly, and material (empty shells) of the Mollusc Collection of the NHMW was used for comparison. Of particular importance were specimens of the "Klemm Collection", and type material of the nominate form of *C. dubia* Draparnaud, 1805 and from several subspecies.

Molecular genetic analyses

For DNA extraction, the shell was carefully broken at the apex, as the soft body of the animals was in most cases withdrawn into the shell. A small piece of the foot was removed with sterile tweezers and used for DNA extraction, which was carried out in most cases with the GEN-

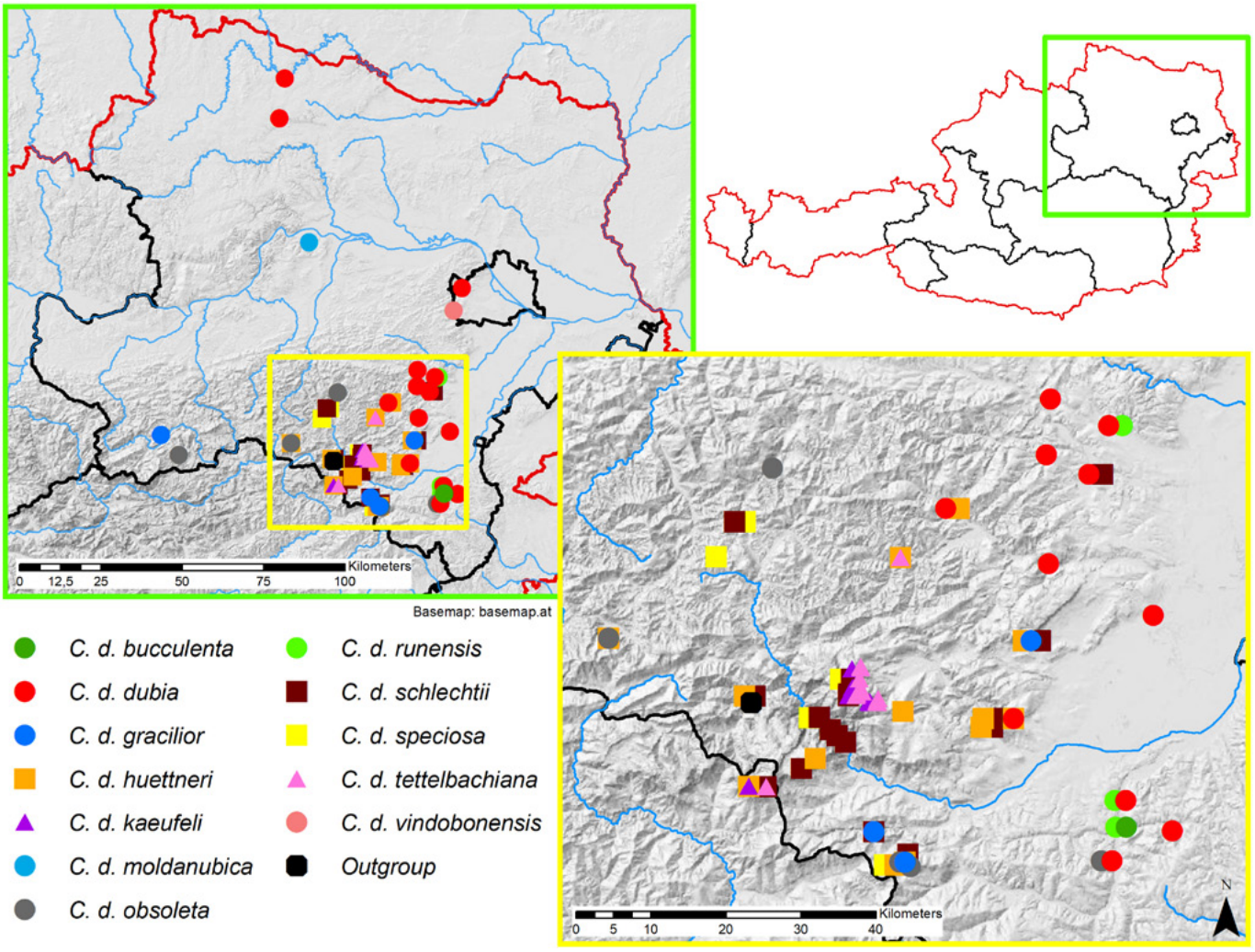

Fig. 1: Sampling localities of *Clausilia dubia* and *Neostyriaca corynodes* (outgroup).

IAL First DNA all tissue DNA extraction kit (GEN-IAL, Troisdorf, Germany) according to the manufacturers' instructions. After the final precipitation, the DNA pellet was dried overnight and eluted in 30 µl elution buffer. DNA was stored at -20 °C. In some cases, the QIAGEN DNeasy Blood & Tissue Kit (QUIAGEN, Germany) was used for DNA extraction (standard protocol). With this method, the purified DNA was finally eluted from the column in 40 µl elution buffer. Remaining DNA was transferred to the NHMW DNA and Tissue Bank. For all extractions, negative controls were carried out (without tissue). PCR amplification of a partial region of the mt *cytochrome c oxidase subunit 1* gene (*COI*) (the DNA barcoding region) was carried out with various primers listed in Table 1. As amplification of *COI* with primers already known from the literature (COI_folmer_fwd, COI_schneck_rev, L1490-Alb) mostly led to very poor results, new forward primers were constructed based on complete mitochondrial genome sequences of *Albinaria caerulea* (Deshayes, 1835) and other gastropods. The forward primer was placed in the *tRNA-Lysine* gene (Alb_Lys1+; alternatively: Alb_Lys2+). In combination with the reverse primer ClausCOI_rev1, which binds to the middle part of the *COI* gene, it was possible to amplify a 900 bp amplicon. For the outgroup, another reverse primer was designed (ClausCOI_rev2). Besides, we tested the mt *16S rRNA* gene (*16S*) as a marker sequence using the primers 16S_schn_fwd / 16S_schn_rev, which allow amplification of a 426 bp-fragment (sequenced section 387 bp) (Table 1). Yet, initial phylogenetic calculations showed that there is too little information in this short fragment and therefore the *16S* marker sequence was not further analysed (data not shown).

PCR was carried out in a volume of 25 µl containing 1.25 units Taq Polymerase (QIAGEN, Hilden, Germany), 1 µM of each primer, and 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 5 µl Q-Solution, 2.5 µl 10x PCR buffer and 1 µl of template DNA. The following PCR protocol was used: initial denaturation 94 °C (3 min); 35 cycles: 94 °C (30 s) / 50 °C (30 s) / 72 °C (60 s); final extension at 72 °C (10 min). Negative PCR controls were carried out to screen for contaminated reagents. After checking on agarose gels, PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) prior to sequencing. In some cases, PCR yielded weak bands which were excised from 1 % agarose gels, purified using the QIAquick Gel Extraction Kit (Qiagen) and reamplified using 1 µl of the purified PCR product. If this did not improve the amount of PCR product, PCR was repeated using the Phusion® High Fidelity DNA Polymerase (Finnzymes, Espoo, Finnland). Gel-purified amplicons were then extended by adenylation of PCR products using DyNAzyme II© DNA polymerase (Finnzymes) and then cloned with the TOPO-TA© cloning kit (Invitrogen, Carlsbad, CA, USA). Sequencing (both directions) was performed at LGC Genomics (Berlin, Germany) using the PCR primes (direct sequencing of PCR products). Cloned PCR products were sequenced with universal M13 primers. Sequences obtained in the present study are deposited in the BOLD database under the accession numbers listed in Appendix 1.

The raw sequences were edited manually using Bioedit v.7.1.3 (Hall 1999). The alignment of the *COI* sequences was straightforward since there were no insertions or deletions. The final *COI* alignment comprised 844 bp as the short 5´section coding for the tRNALys was excluded from the analysis. The start codon of the *COI* gene departs from the mt code for invertebrates being TTG instead of ATG or ATA. *P* distances were calculated with MEGA (version 5.05; Tamura et al. 2011). Bayesian inference analyses (BI) were done in MrBayes (version 3.2.1 x64; Ronquist & Huelsenbeck 2003) and a Maximum-Likelihood (ML) was calculated with IQ-TREE (v.1.6.12; Nguyen et al. 2015). Prior to the tree calculations, a model test was performed with MEGA, resulting in T92+G+I as best-fit substitution model. However, since MrBayes does not feature the model this model, the BI and ML bootstrap (1000 replicates) trees were both calculated with the next complex model

Table 1: PCR primers used in the present study.

Primer name	Sequence (5´- 3´)	Reference
ClausCOI_rev1	ACT GTA AAC ATA TGA TGA GCC CAA	this study
ClausCOI_rev2	GAT GAG CCC AAA CAA TAA ACC C	this study
Alb_Lys1+	CCT AAT TTT TTA TGG CCG AG	this study
Alb_Lys2+	GCA TCA AAT TTT TAA TTT GAA TTA CG	this study
L1490-Alb	ACT CAA CGA ATC ATA AAG ATA TTG G	Gittenberger et al. 2004
COIfolmer_fwd	GGT CAA CAA TCA TAA AGA TAT TGG	Duda et al. 2011
COIschneck_rev	TAT ACT TCT GGA TGA CCA AAA AAT CA	Duda et al. 2011
16S_schn_fwd	CGC AGT ACT CTG ACT GTG C	Pfenninger et al. 2003
16S_schn_rev	CGC CGG TCT GAA CTC AGA TC	Pfenninger et al. 2003

GTR+G+I. The BI analyses were run for $5x10^6$ generations (2 runs each with 4 chains, one of which was heated), sampling every hundredth tree. Tracer (Version 1.5.0; Rambaut & Drummont 2007) was used to assess whether the two runs had converged and when the stationary phase was reached, which was the case already after several thousand generations. The first 10 % of trees were discarded as burn-in and a 50 % majority-rule consensus tree was calculated from the remaining trees. A Neighbor Joining (NJ) bootstrap tree (1000 replicates) was calculated using the model TN92+G, the next-complex model applicable. Median Joining networks were calculated with Network v.4.6.0.0 (Fluxus Technology Ltd., Colchester, UK) applying the default settings. In order to reduce unnecessary median vectors, the networks were then post-processed with the MP (Maximum Parsimony) option.

Morphometric analysis

Several photographs of each specimen were taken under a stereomicroscope (Leica MZ12 5, Wild macroscope M420) with a microscope camera (Nikon DS-L2): total images of the front and the back (8x magnification) and several images of the aperture (20x magnification). To depict the details of the clausiliar structures, five images of different focal levels were made per specimen. These images were then combined using Helicon Focus (version 5.2; Helicon Soft Ltd., 2000–2011) to form a deep-focus image. In addition, of at least one individual per locality, the palatum was carefully removed to allow inspection of the lamellae, as well as the clausiliar plate, which often proved helpful in the determination. From this lateral position (turned to the right), a deep-focus image was computed from five images.

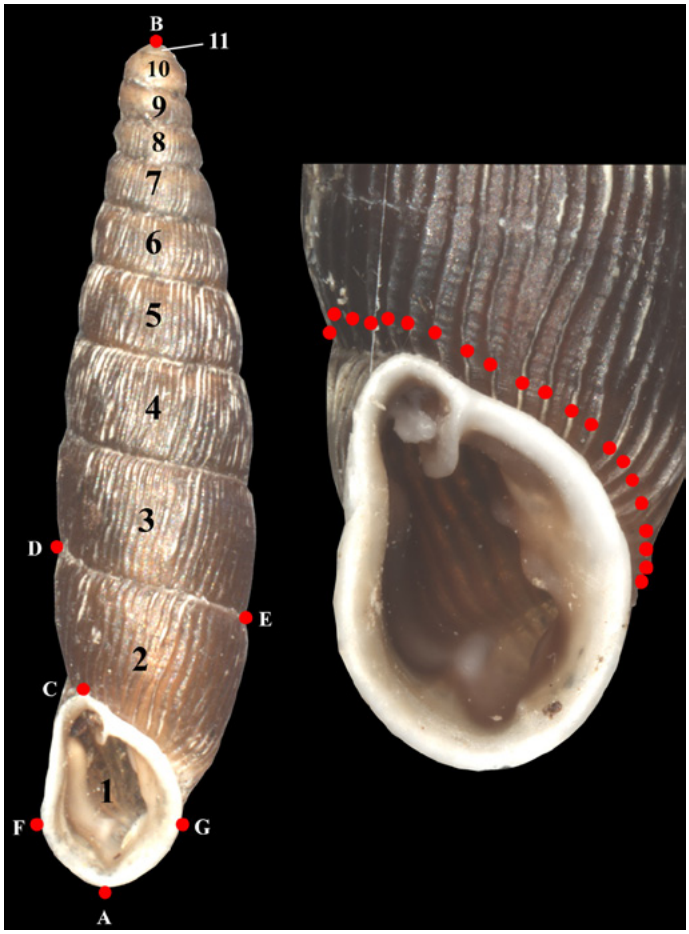

Fig. 2. Landmarks for morphometric measurements (red dots; for better visibility enlarged). Left: numbering of whorls (*C. d. gracilior*). A–B: shell height, A–C: aperture height, D–E: shell width, F–G: aperture width. right: marking of ribs along the aperture (*C. d. dubia*).

All measurements were made based on the photographs of the shells in stable orientation using tpsDig, version 2.16 (Rohlf 2010) to place anatomical measuring points (landmarks) in the corresponding places. For measuring shell height, landmarks were set at the apex and the lower margin of the aperture, for the shell width on both sides of the suture between first and second whorl. For the aperture height and width, the outermost points of the aperture were selected. In addition, the number of whorls (W) and the number of ribs (R) were counted (Fig. 2). Whorls of elongated shells are usually counted in frontal view, but there is no consensus on how to make it, and therefore different methods (Fehér & Szekeres 2016: Fig. 2 vs. Welter-Schultes 2012: Plate A1) give different results. Here we followed the method of Welter-Schultes (2012). Ribs were manually counted from images of the mouth using GSA Image Analyzer, version 3.8.1. by placing a dot on each rib. The program then counts the number of points. The ribs of the penultimate whorl (the first above the mouth) were counted, as close as possible along the mouth edge (Fig. 2).

Measurement error was determined in three subspecies, two individuals of each were photographed five times independently and measurements were taken as described above. The measurement error was 0.58 % (i.e., 0.11 mm).

For statistical analysis, all individuals with intact shell were measured, regardless of whether they were also included in the molecular genetic analysis or not. In total, nine parameters were evaluated in 142 individuals: Measurements: shell height (SH), shell width (SW), aperture height (AH), aperture width (AW), number of whorls (W), and number of ribs (R). In addition, the shell size (SH x SW x SW) as well as the relative shell shape (SH/SW) (see Welter-Schultes 2010) and the ratio between the shell height and aperture height (RSA = SH/AH) was calculated. By calculating the ratio between the number of whorls and the shell height (W/lnSH), the factor of exponential growth of the shell was eliminated. Thus, it could be shown whether individuals are exceptionally large/small or have an exceptionally high/low number of whorls. Since the number of

ribs correlates strongly with the shell width, the rib density (R/πSB) was calculated according to Welter-Schultes (2010).

We used a Principal Component Analysis (PCA) to analyse the morphological data. To investigate the effect of the sea level on morphological traits we calculated linear mixed models (LMM) with principal components (PC) with an Eigenvalue > 1 as dependent variable and the sea level as fixed factor. To take general differences between sampling sites into account, the sampling locality and region were used as random factors in the LMMs. To verify the assumptions of Boettger (1932) and Kempermann & Gittenberger (1988) that the rib density increases with seal level, we additionally calculated a LMM with the log-transformed rib density as dependent variable. Shapiro-Wilk tests were used to test the residuals of the LMMs for normality. Statistical analyses were carried out in R (R Core Team 2020). LMMs were calculated with the function "lmer" in the R-package "lme4" (Bates et al. 2015) and p-values were calculated with the Satterthwaite's method implemented in the package "lmerTest" (Kuznetsova et al. 2017).

Results

Distribution of subspecies

The subspecies assignment of individuals (using all available morphological characters; Jaksch 2012) was straightforward and consistent with the taxonomic assignment of museum specimens. Yet, individuals of some subspecies of *C. dubia* were found at localities that were not within the distribution range according to the literature (Klemm 1960). E.g., according to Klemm (1960) *C. d. obsoleta* should occur only in southwestern Lower Austria, however, we found individuals from southeastern Lower Austria clearly corresponding to this subspecies. Furthermore, individuals clearly assigned to *C. d. gracilior* were found much further to the north and northwest of the known range in southeastern Lower Austria, and also some *C. d. runensis* individuals were collected further to the north than reported by Klemm (1960). The distribution of samples (Fig. 1) indicates that the distribution areas of the subspecies overlap strongly.

Molecular genetic analysis

The 844 bp alignment comprising 144 *COI* sequences was used for the phylogenetic reconstructions calculated with BI, ML and NJ algorithms. The trees showed the same overall topology with one difference: In the ML tree, clade 1 is the sister clade to clades 2A, 2B, and 3, however with extremely low bootstrap support (48 %). Designation of clade numbers 2A and 2B was done to remain comparable with the tree presented by Jaksch (2012), where Clade 2 was found in all analyses. This difference between the two studies is probably due to slightly different outgroup species used in the present study. Nevertheless, this uncertainty only reflects the unclear phylogenetic relationships between these lineages, which is also underlined by low support values. A second change with respect to Jaksch (2012) is the re-designation of Clades 4 and 5 (in Jaksch 2012), which we treated here as only one clade (Clade 4) due to the low intra-clade divergences.

Table 2. *P* distances (in %) of *COI* sequences within and between clades.

Clade	1	2A	2B	3	4
Mean within clades	5.2	4.5	2.4	5.8	2.1
Maximal within clades	11.1	6.0	2.4	8.8	4.6
Mean between clades					
1					
2A	19.7				
2B	19.4	9.4			
3	20.5	12.3	13.9		
4	19.7	9.8	11.8	9.2	
outgroup	23.5	20.1	19.9	21.4	20.8

Fig. 3 shows the BI tree with ML and NJ bootstrap values indicated for most nodes. Except clade 2A, all clades received high support values. The same is true for the node combining Clades 3+4, as well as for the node combining Clades 2A, 2B, 3+4. Mean *p* distances between and within clades are shown in Table 2. The mean distances between clades ranged from 9.4 % (2A vs. 2B) to 20.5 % (1 vs. 3). The maximum *p* distance within *C. dubia* was 22.2 %.

The subspecies appear to be randomly distributed among clades as illustrated by the colour coding of sequences in the tree. At least four subspecies are found in each clade. Clade 1 contains six subspecies and Clade 4 even ten (only *C. d. runensis* and *C. d. vindobonensis* are missing). Three subspecies are present in single clades only (but together with other subspecies): *C. d. runensis*, (Clade 1), *C. d. vindobonensis* (Clade 4), and *C. d. moldanubica* (Clade 4). *C. d. dubia* is present in all clades.

For the closely related sequences of the large Clade 4, a Median Joining network was constructed which did not reveal any pattern, neither when highlighting the subspecies nor the seven geographic regions (Appendix 1) (Fig. 4). Thus, even within one clade there is no geographic pattern.

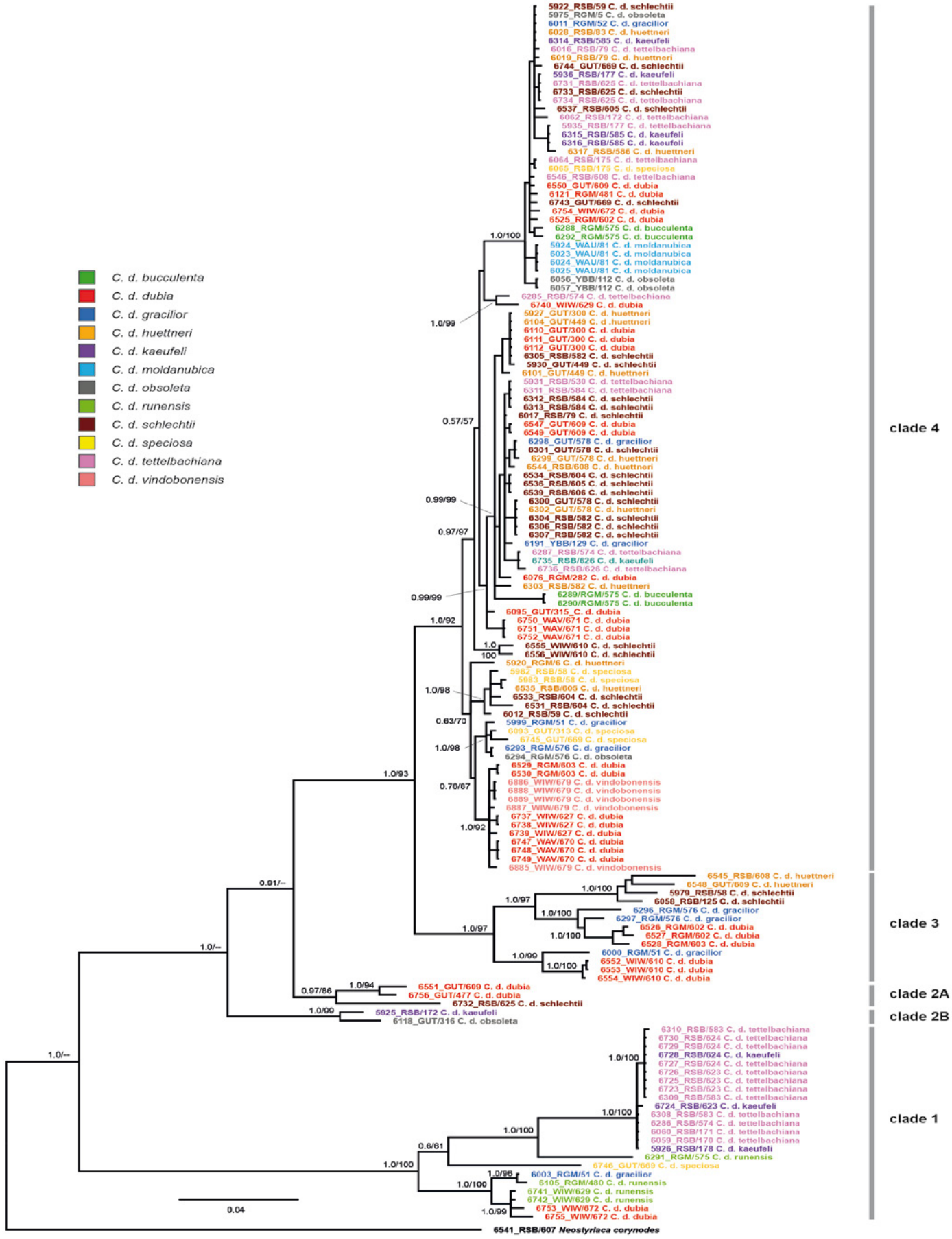

Fig. 3. Bayesian inference tree of *COI* sequences. BI posterior probabilities, and bootstrap values (ML, NJ) are indicated at most nodes. The scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied. Outgroup: *Neostyriaca corynodes*.

Table 3. Measurements of subspecies of *Clausilia dubia* investigated. SH = Shell height; SW = Shell width; For SH and SW numbers reported by Klemm (1960) are given (except for *C. d. vindobonensis* because Klemm did not consider this subspecies). W/lnSH = ratio between the number of whorls and the shell height.

	***C. d. bucculenta* (n=4)**			***C. d. dubia* (n=25)**			***C. d. gracilior* (n=12)**			***C. d. huettneri* (n=13)**		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Shell height [mm]	11.72	0.70	11.3–12.8	11.66	0.67	10.3–12.9	11.67	0.56	10.9–12.7	11.08	0.91	9.9–13.4
SH Klemm (1960)			9.5–12.5			11.8–14.8			9.3–12.8			9.9–12.2
Shell width [mm]	2.69	0.06	2.6–2.7	2.61	0.16	2.4–3.1	2.36	0.18	2.0–2.6	2.40	0.16	2.2–2.7
SW Klemm (1960)			2.5–3.0			2.8–3.2			2.0–2.6			2.4–2.7
Aperture height [mm]	2.66	0.24	2.5–3.0	2.64	0.19	2.4–3.1	2.40	0.19	2.1–2.7	2.44	0.16	2.2–2.7
Aperture width [mm]	1.90	0.13	1.8–2.1	1.89	0.10	1.7–2.1	1.69	0.12	1.5–1.9	1.76	0.11	1.6–1.9
Idealized shell volume	84.68	7.45	77.5–95.0	79.97	14.45	60.5–117.7	65.75	12.03	43.3–85.9	65.68	13.38	49.6–83.0
W/lnSH	11.65	0.71	10.9–12.5	11.75	1.07	9.8–13.7	14.24	1.78	11.7–17.6	12.51	1.11	11.1–14.9
Rib density	2.52	0.43	2.1–3.0	3.79	0.86	2.4–6.1	3.97	0.87	3.0–6.1	5.08	0.68	3.6–5.9

	***C. d. kaeufeli* (n=9)**			***C. d. moldanubica* (n=5)**			***C. d. obsoleta* (n=7)**			***C. d. runensis* (n=4)**		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Shell height [mm]	9.72	0.45	9.2–10.6	11.93	0.51	11.0–12.5	12.25	1.52	10.7–14.9	10.49	0.63	9.9–11.4
SH Klemm (1960)			9.7–11.7			9.3–12.3			9.3–12.3			7.0–10.0
Shell width [mm]	2.51	0.16	2.3–2.8	2.68	0.15	2.5–2.9	2.51	0.17	2.3–2.8	2.57	0.14	2.4–2.7
SW Klemm (1960)			2.6–3.1			2.5–3.1			2.6–3.0			2.2–3.0
Aperture height [mm]	2.27	0.11	2.0–2.4	2.61	0.12	2.5–2.8	2.62	0.14	2.5–2.9	2.51	0.07	2.4–2.6
Aperture width [mm]	1.70	0.07	1.6–1.8	1.95	0.09	1.9–2.1	1.83	0.10	1.7–2.0	1.84	0.09	1.7–1.9
Idealized shell volume	61.43	10.37	49.4–78.0	88.15	9.70	80.1–102.7	79.81	13.53	59.5–95.9	69.40	8.13	59.4–77.6
W/lnSH	10.91	0.78	9.8–12.0	11.96	0.96	10.7–12.9	12.29	1.23	10.6–14.0	11.43	0.94	10.5–12.7
Rib density	5.68	0.80	4.0–6.6	3.16	0.33	2.6–3.4	4.33	0.72	3.2–5.4	3.44	0.91	2.1–4.0

(n=5)	***C. d. schlechtii* (n=26)**			***C. d. speciosa* (n=5)**			***C. d. tettelbach.* (n=27)**			***C. d. vindobonensis***		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Shell height [mm]	11.75	1.01	9.9–14.4	11.80	1.18	10.3–13.9	9.98	0.73	8.7–11.4	13.55	0.69	12.9–14.4
SH Klemm (1960)			9.8–11.2			10.6–17.2			8.4–11.4			-
Shell width [mm]	2.50	0.20	2.1–3.0	2.48	0.16	2.3–2.7	2.43	0.16	2.1–2.8	3.05	0.11	2.9–3.2
SW Klemm (1960)			2.0–2.5			2.6–3.7			2.3–2.8			-
Aperture height [mm]	2.51	0.22	2.1–3.1	2.47	0.18	2.3–2.8	2.25	0.14	1.8–2.5	3.04	0.05	3.0–3.1
Aperture width [mm]	1.77	0.14	1.5–2.1	1.78	0.15	1.6–2.1	1.67	0.10	1.5–1.9	2.22	0.10	2.1–2.4
Idealized shell volume	74.16	16.83	47.5–125.4	76.00	13.99	63.1–98.0	58.56	11.53	42.0–81.6	126.58	11.46	107.7–137.0
W/lnSH	12.48	1.29	10.0–15.7	13.43	1.04	11.7–14.3	11.72	1.02	10.2–14.0	10.59	0.62	9.7–11.4
Rib density	4.82	0.85	3.4–6.5	5.18	0.64	4.4–5.9	5.73	0.86	4.0–7.8	2.85	0.47	2.4–3.4

Morphometric analysis

Table 3 provides an overview of shell measurements of 142 individuals. Comparing shell height and shell width to the values given by Klemm (1960) a rough concordance is found. Looking at the mean values several subspecies differed considerably, *C. d. vindobonensis* had the largest mean shell height (13.55 mm) and *C. d. kaeufeli* the lowest (9.72 mm). The highest shell width was found in *C. d. vindobonensis* (3.05 mm), while *C. d. gracilior* and *C. d. huettneri* had the lowest shell widths (2.36 and 2.40 mm, respectively). The considerable differences in shell shape and size among subspecies becomes also apparent comparing the shell volumes. For example, the mean volume of *C. d. vindobonensis* (126.58 mm^3) is more than double the size of the smallest form *C. d. kaeufeli* (61.43 mm^3). Yet, considering the overlapping ranges, an unambiguous assignment to a subspecies based only on measurements is not possible (Table 3).

In the PCA the subspecies were not well differentiated (Fig. 5) and most subspecies overlapped with several other subspecies. The first and the second PC of the PCA with the morphological data had an Eigenvalue > 1 and were therefore considered to be relevant. The first PC (PC1; explaining 54.4 % of the variance) described larger snails (high and wide shell and aperture) and a rather low rib density, while PC2 (26.7 %) was influenced by a small shell shape and a low whorl density (Table 4). The LMM revealed a significant negative correlation between PC1 and the sea level (of localities where individuals were found) and a positive correlation between PC2 and sea level (both $p < 0.001$). Thus, individuals found in high altitudes are generally smaller, have a higher rib density, a lower relative shell shape and a lower whorl density. An

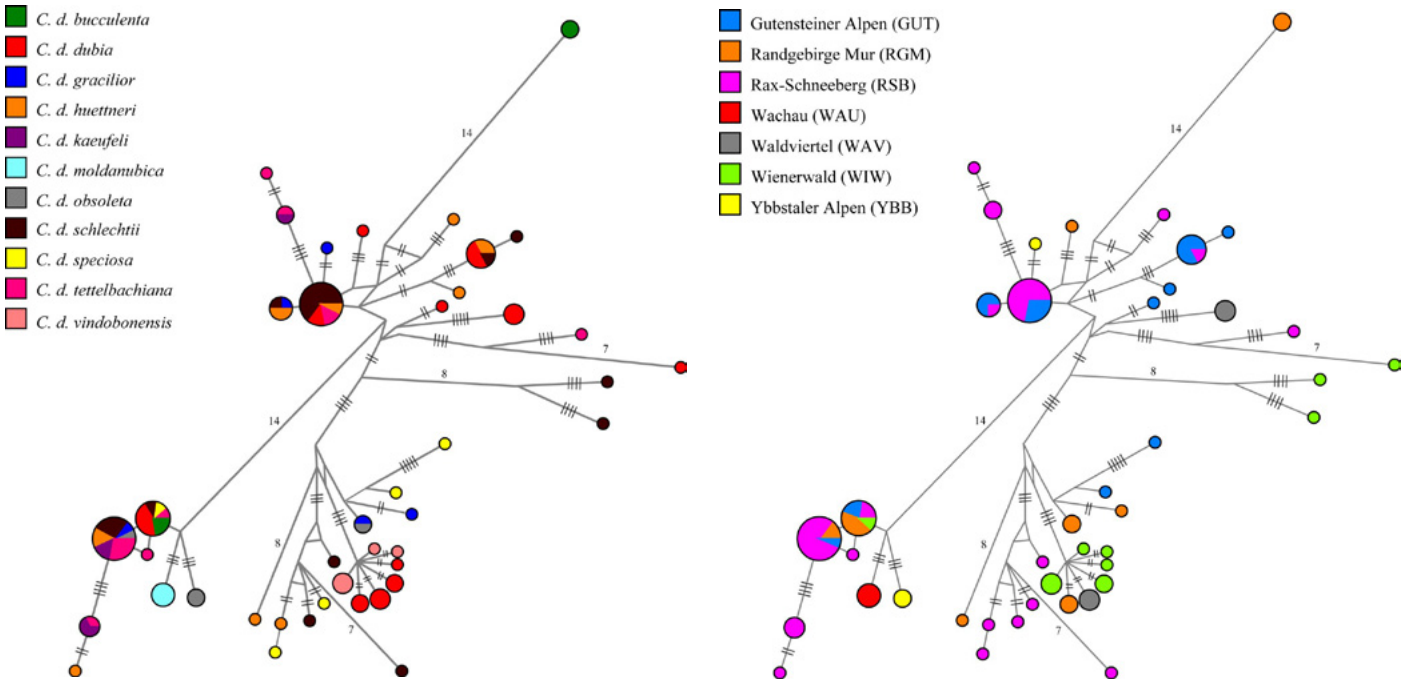

Fig. 4. Median Joining network of Clade 4. Colours indicate subspecies (left) and regions (right) respectively. Regions: Randgebirge Mur = Prealps east of the Mur.

increased rib density at higher altitudes was also confirmed by the LMM with rib density as dependent variable (estimate = 0.00015; $p < 0.001$). Considering the seven geographic regions (Appendix 1) a slight differentiation is visible in the PCA (Fig. 6). Three regions (WIW, RSB, RGM) are reaching to the periphery of the plot, while all regions overlap in the center. The best differentiation is found concerning the sea level of the localities (Fig. 6). Concerning the question whether the clades might be differentiated morphologically, this was not confirmed in the PCA (Fig. 7).

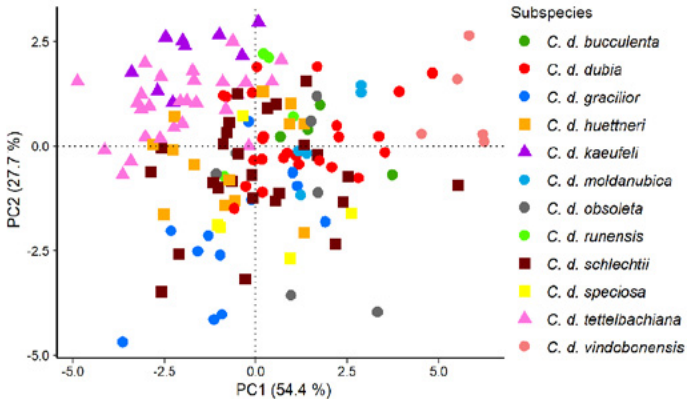

Fig. 5. PCA of morphometric measurements and parameters of 142 individuals of *Clausilia dubia* highlighting the subspecies.

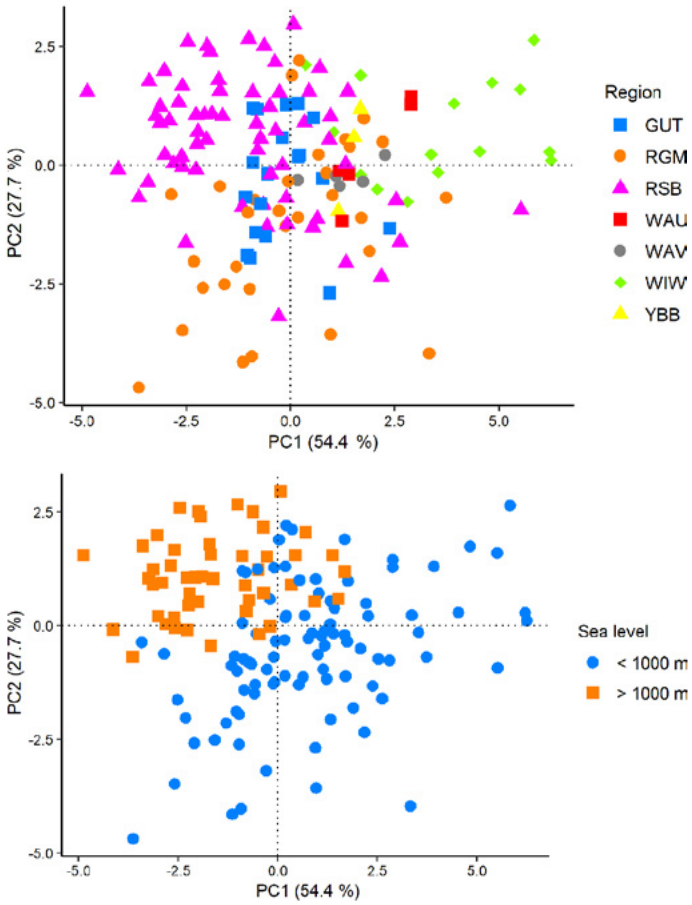

Fig. 6. PCA of morphometric measurements and parameters of 142 individuals of *Clausilia dubia* highlighting geographic regions (above) and sea level (below). GUT = Gutenstein Alps, RSG = Rax-Schneeberg Group, RGM = Prealps east of the Mur, WAU = Wachau, WAV = Waldviertel, WIW = Wienerwald, YBB = Ybbstal Alps.

Table 4. Contribution of variables to principal components (PC), Eigenvalues and proportion of explained variance for each PC.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Shell height	0.384	-0.296	-0.180	0.183	-0.274	0.264	0.308	0.650	0.193
Shell width	0.400	0.264	0.025	0.051	-0.445	-0.225	0.551	-0.429	-0.174
Aperture height	0.432	-0.054	0.142	0.133	0.497	-0.174	-0.022	0.212	-0.670
Aperture width	0.414	0.125	-0.265	0.104	0.549	-0.152	0.056	-0.203	0.603
Relative shell shape	0.088	-0.603	-0.243	0.150	0.047	0.469	-0.026	-0.542	-0.173
Shell size	0.438	0.057	-0.064	0.134	-0.410	-0.133	-0.770	-0.051	0.031
Whorl density	-0.176	-0.541	-0.302	-0.042	-0.085	-0.756	0.052	0.032	0.000
Rib density	-0.298	0.134	0.002	0.941	-0.006	-0.079	0.021	-0.007	-0.003
RSA	0.123	-0.383	0.850	0.083	-0.005	-0.100	0.023	-0.095	0.299
Eigenvalue	2.212	1.551	0.886	0.766	0.443	0.348	0.087	0.046	0.028
Proportion of explained variance	0.544	0.267	0.087	0.065	0.022	0.013	0.001	0.000	0.000

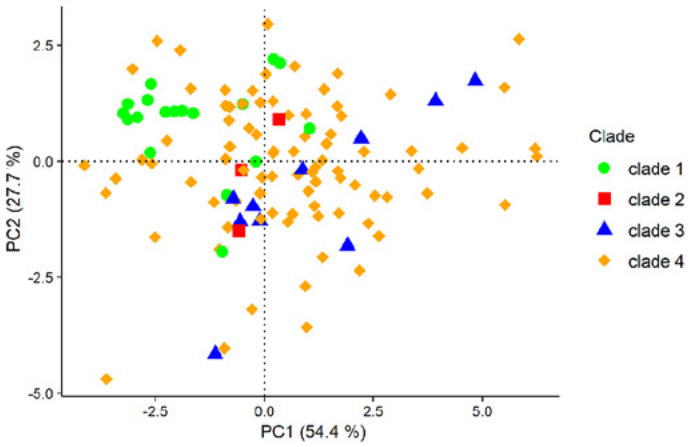

Fig. 7. PCA of morphometric measurements and parameters of 142 individuals of *Clausilia dubia* highlighting 4 clades (clades 2A and 2B were combined).

Discussion

The present study provides first insights into the intraspecific variation in *C. dubia* concerning morphological and mtDNA sequence data. The total number of individuals examined was quite high and the localities covered the distribution of *C. dubia* in Lower Austria, where the majority of its subspecies occur. Yet, some subspecies were represented by a few specimens only (e.g., of *C. d. bucculenta*, *C. d. moldanubica, C. d. runensis* and *C. d. vindobonensis*). This was, however, partly due to the small known distribution areas and scarce findings of these subspecies in Lower Austria and Vienna.

The major outcome of the study was that neither the phylogenetic analysis based on *COI* nor the morphometric analysis (based on measurements) reflected differentiation of the subspecies (as defined by shell-morphology including qualitative characters). The clades observed in the mt tree do not correspond with any of the subspecies, which appear to be randomly distributed within the tree. Even the proposed division into two major subspecies postulated by Nordsieck (2002) – *C. d. dubia* in the east and *C. d. vindobonnensis* in the west – is not at all reflected in the mt tree. The only clear correlation found was that shells tend to be smaller, more densely ribbed, and in general less variable in higher elevations. The infraspecific taxonomy is discussed below in the light of these findings.

Genetic distances

The observed intraspecific *p* distances are remarkably high (up to 22.2 %), but not unusual (Davison 2002). For several land snail species, high intraspecific genetic variation in mt genes has been reported (e.g. Thomaz et al. 1996, Chiba 1999, Guiller et al. 2001, Duda et al. 2011, Kruckenhauser et al. 2014, Kirchner et al. 2016, Mason et al. 2020).

Concerning the genetic differentiation of clades, mean *p* distances between the *C. dubia* clades ranged from 9.4 to 20.5 %. An explanation for the high genetic diversity within *C. dubia* might be the fragmented distribution range, partly due to the limestone habitat requirements. Most land snails are presumed to be rather restricted in their mobility. Studies on *Albinaria*, for example, showed that the range of action in the course of an adult animal's life is between 2 and 5 m (Schilthuizen & Lombaerts 1994). Similar low mobility values for clausiliids have been reported for *Montenegrina subcristata* (Pfeiffer, 1848) by Bulatovic et al. (2019) and by Junker (2015) for *Cochlodina laminata* (Montagu, 1803). Yet, it has to be emphasized that both *Albinaria* and *Montenegrina* (occurring exclusively on limestone walls) are much more stenoecious than *C. dubia* and *C. laminata*. The high intraspecific distances of *C. dubia* and the incongruence between morphological diversity and mitochondrial clades is similar to what was found in *Arianta arbustorum* (Linnaeus, 1758) (Haase et al. 2003, Haase & Misof 2009, Bondareva et al. 2020), or

within the *Trochulus hispidus* (Linnaeus, 1758) complex (Kruckenhauser et al. 2014). Yet, while in *T. hispidus* the distinct clades are distributed in a geographic pattern, this is not the case in *C. dubia*. The fact that (like in *A. arbustorum*) its mt clades do not correspond to geographic areas, implies that ranges of populations might have shifted repeatedly (e.g., in the course of Pleistocene climate oscillations) allowing substantial gene flow over time (Haase & Misof 2009). As in *A. arbustorum* and *T. hispidus*, the findings in *C. dubia* may be well interpreted by assuming long-term persistence of large populations in large distribution ranges with sporadically mixing of populations.

Subspecies differentiation and morphological variation

One major question was whether the differentiation of subspecies as mainly described in Klemm (1960) is reflected in the morphometric or genetic data. Approaching these questions, the determination of individuals to subspecific level was a prerequisite. Although important traits as, e.g., of the clausiliar plates, as used by Nordsieck (2002) and shell measurements provided in the literature were not diagnostic, taxonomic assignment was possible by combining several characters including the qualitative ones. The results of the PCA showed that with shell measurements alone subspecies were not clearly differentiated. The finding that shells tended to be smaller in higher elevations, e.g., the largest individuals (assigned to *C. d. vindobonensis*) occurred in the Vienna Woods (425 m asl.) confirmed the results of Edlinger (1997). In contrast, *C. d. kaeufeli* from the highest locations in our sample featured the smallest individuals and possessed a theoretical shell volume of about half of *C. d. vindobonensis*. A similar phenomenon of shell size reduction with increasing altitude has been described, e.g., in the helicid land snail *Arianta arbustorum* by Baur & Raboud (1988), Arter (1990) and Baminger (1997). On the other hand, Edlinger (1997) detected in *C. dubia* only a low negative correlation of shell height with higher elevations and stated for *C. dubia* that the suggestion of Klemm (1960) of a succession of shell height with altitude is not generally convincing. In contrast, Welter-Schultes (2000) recorded an increasing shell height with increasing altitude in *Albinaria* populations in Crete. These controversial observations were also discussed by Hausdorf (2003). An impact of environmental factors on morphology has already been presumed in numerous snails examined, but only in some species a clear correlation with ecological conditions could be evidenced (Nica et al. 2011). As biotic factors, average population density has been put forward (Anderson et al. 2007). Crucial abiotic factors are, besides temperature or precipitation (be it average or minimum and maximum values), the calcium content of the substrate. Goodfriend (1986) reported that a higher calcium content results in higher observed shell heights of snails and that individuals in a humid environment had larger shell height than those from dry regions. A correlation between humidity and the relative number of whorls (and thus shell height) was suspected and it was assumed that individuals with more whorls were better adapted to dryness as they may retreat deeper into the shell and thus would be better protected. In the present investigation, the majority of the lowland forms actually had a rather small number of whorls, albeit whorl numbers in some high-altitude populations were likewise very low or even lower. Thus, the assumptions of Goodfriend (1986) were not confirmed.

The rib density also showed a trend to increase with sea level in *C. dubia.* Subspecies of the lowest regions in Lower Austria possessed the fewest ribs (*C. d. bucculenta*, *C. d. vindobonensis*), while the two summit forms had highest rib densities. These findings confirm earlier observations about the difference in rib density (Boettger 1932, Kempermann & Gittenberger 1988). Studies in species of another rock-dwelling snail genus, *Albinaria*, showed that ribbing evolved several times independently (Giokas 2008) suggesting either functional features (e.g., water retain capability) of ribbing or homoplasy (Fehér et al. 2018). As with increasing rib density, the thickness of the shell also increases, it might be even assumed that ribs could provide mechanical stability and better thermal insulation. Yet, a certain level of phenotypical plasticity may be assumed as individuals with larger numbers of ribs were also found in the lowlands. The generally higher morphological variation in lowland populations (also exemplified by syntopic occurrence of subspecies) could be explained by repeated admixture of formerly isolated populations as well as by phenotypic plasticity of certain (e.g., qualitative) characters. In *C. dubia*, oviposition usually occurs in spring as well as in autumn. This, in addition, might cause seasonal differences resulting in variation of shell morphology.

Taxonomic considerations

One might ask whether the clades in the mt tree would represent species. Yet, on the basis of the present morphological data, there is no indication for such an assumption. To address this question from a genetic point of view, nuclear data would be necessary, a challenge for future investigations. The main questions in the present study regarded to subspecific differentiation. According to Mayr (1967), subspecies are defined as phenotypically similar populations (i.e, groups of actually or potentially interbreeding populations) of a species that inhabit a geographical part of the area of the species and are morphologically different from other populations of the species. He thus emphasised the geographic aspect leaving open

the question of the amount of morphological differentiation. According to Amadon (1949) the so-called 75 % rule states that at least 75 % of a population should be morphologically clearly distinguishable from another population to be considered as a valid subspecies. Similarly, Sudhaus & Rehfeld (1992) considered 80 % as considerable difference. Irrespective of which subspecies concept is preferred, our genetic results neither confirmed the subspecies classification of *C. dubia* of Klemm (1960) nor the subdivision into two major subspecies as proposed by Nordsieck (2002). Yet, although neither morphometric measurements nor mt sequences discriminated the subspecies clearly, it has to be kept in mind that – combining all characters (also qualitative, e.g., the shape of the ribs) – the determination of subspecies was straightforward. For example, *C. d. bucculenta* and *C. d. vindobonensis*, which were clearly distinguishable by their ribbing, were neither differentiated in the morphometric landmark analysis from each other nor from other subspecies. Thus, the results of the morphometric analysis alone are not a good argument to question the validity of the subspecies as qualitative characters were not included into the analyses.

Two aspects have to be considered: (1) In the phylogenetic tree (Fig. 3) there is no phylogeographic pattern and the subspecies appear randomly distributed throughout the clades (which is also illustrated in the network, Fig. 4A). Yet, the finding of no differentiation in a marker sequence cannot be regarded as evidence against validity of subspecies (Patten 2015). Subspecies, in the perception of species in *statu nascendi*, are not necessarily expected to have genetically diverged or to demonstrate reciprocal monophyly in the phylogenetic marker sequence used (which is expected to be quasi-neutral and to reflect time). Gene flow may contribute to incomplete lineage sorting of quite diverged lineages and result in a picture observed in the present study. Anyhow, the lack of any phylogeographic structure is only one part of the reasoning that the currently accepted subspecies are fairly elusive. (2) According to the distribution of the subspecies as represented in the present sample, subspecies ranges would be overlapping considerably and some morphotypes occur sporadically at distant places. It has to be kept in mind, that the distribution ranges of several subspecies analysed in Lower Austria extend much further. Some regions in Lower Austria might be zones of admixture of several formerly separated populations.

In any case, understanding subspecies as entities with a defined geographic range and certain morphological (and possibly genetic) properties, there are no good arguments for the current subspecific classification of *C. dubia*, at least concerning those examined in the present work. The question arises whether some of the subspecies could be united. Jaksch (2012) tested for such "morphogroups", yet without meaningful results. They were neither differentiated in the morphometric nor in the genetic analyses. Nevertheless, the high similarity between some of the subspecies suggests that such an approach could eliminate major inconsistencies of the present intraspecific taxonomy of *C. dubia*. For example, *C. d. dubia*, *C. d. moldanubica* and *C. d. vindobonensis* are very similar and their ranges overlap widely. Likewise, the distribution ranges of the quite similar *C. d. kaeufeli* and *C. d. tettelbachiana* overlap to some extent and they occurred frequently at the same locality. Thus, uniting them into one subspecies might be considered. The same is true for *C. d. huettneri*, *C. d. schlechtii* and *C. d. speciosa* (at least in Lower Austria). *C. d. bucculenta* and *C. d. runensis* are morphologically very similar and easy to distinguish from the other subspecies by rib density (Edlinger & Fischer 1997). The distribution range of *C. d. bucculenta* hardly overlaps with any of the other subspecies except *C. d. runensis*, which itself has a very small distribution range within the range of *C. d. bucculenta.*

Future analyses should comprise all subspecies over their entire distribution ranges and include qualitative characters in the statistical analyses as well as population genetic analyses of nuclear markers to evaluate whether there is any genetic structure and whether the genetic diversity and differentiation of the mt genome is reflected in the nuclear genome. Eventually, considering ecological factors possibly shaping shell morphology, it might be considered, whether and to which extent for such highly polymorphic taxa, terms like "variety", "forma" or "morphotype" would be more appropriate.

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Plate 1. Subspecies of *Clausilia dubia. C. d. dubia* (NHMW 109000/AL/01653/6747), *C. d. bucculenta* (NHMW 79000/K/11390), *C. d. gracilior* (NHMW 79000/K/11275), *C. d. huettneri* (NHMW 79000/K/11228), *C. d. kaufeli* (Paratype, NHMW 40036), *C. d. moldanubica* (Paratype, NHMW 44957).

Plate 2. Subspecies of *Clausilia dubia. C. d. obsoleta* (NHMW 79000/K/35698), *C. d. runensis* (NHMW 109000/AL/0211/6291), *C. d. schlechtii* (NHMW 109000/AL/01621/6305), *C. d. s peciosa* (NHMW 109000/AL/01652/6746), *C. d. tettelbachiana* (NHMW 109000/AL/01643/6729), *C. d. vindobonensis* (Paratype, NHWM 31080).

Appendix 1. Specimens investigated. IndID refers to the collection number in the Mollusc Collection at the NHMW (the complete numbers contain, besides the numbers provided in the table, the prefix "Mollusca NHMW 109000/AL"; e.g., Mollusca NHMW 109000/AL /02117/6288). Region codes: RSB = Rax-Schneeberg Region, GUT = Gutenstein Alps, YBB = Ybbstal Alps, RGM = Prealps east of the Mur, WIW = Wienerwald, WAV = Waldviertel, WAU = Wachau. Within each geographic area, sometimes several sampling localities were sampled, which are defined by their Sample ID. Details for Sample IDs are given in Appendix 2. Morph.: + = included in morphological analysis; *COI:* BOLD Accession numbers.

IndID	Region	Area / Sample ID	Clade	*COI*	Morph.
C. d. bucculenta					
02117/6288	RGM	Schlattenbachtal, Scheiblingkirchen / 575	4	ALNHM414-20	+
02117/6289	RGM	Schlattenbachtal, Scheiblingkirchen / 575	4	ALNHM415-20	+
02117/6290	RGM	Schlattenbachtal, Scheiblingkirchen / 575	4	ALNHM416-20	+
02117/6292	RGM	Schlattenbachtal, Scheiblingkirchen / 575	4	ALNHM418-20	+
C. d. dubia					
01596/6076	RGM	Thernberg, Ruine Thernberg / 282	4	ALNHM396-20	
01601/6095	GUT	Gutensteineralpen, Berndorf / 315	4	ALNHM402-20	
01599/6110	GUT	Ternitz, Gösing, Flatzerwand / 300	4	ALNHM398-20	+
01599/6111	GUT	Ternitz, Gösing, Flatzerwand / 300	4	ALNHM399-20	+
01599/6112	GUT	Ternitz, Gösing, Flatzerwand / 300	4	ALNHM400-20	+
01610/6121	RGM	Burgruine Grimmenstein / 481	4	ALNHM409-20	+
01627/6525	RGM	Schlattenbachtal, Neustift / 602	4	ALNHM443-20	
01627/6526	RGM	Schlattenbachtal, Neustift / 602	3	ALNHM444-20	+
01627/6527	RGM	Schlattenbachtal, Neustift / 602	3	ALNHM445-20	+
02118/6528	RGM	Seebenstein, Türkensturz / 603	3	ALNHM446-20	+
02118/6529	RGM	Seebenstein, Türkensturz / 603	4	ALNHM447-20	+
02118/6530	RGM	Seebenstein, Türkensturz / 603	4	ALNHM448-20	+
01636/6547	GUT	Piestingtal, Pernitz, Hirschwände / 609	4	ALNHM461-20	+
01636/6549	GUT	Piestingtal, Pernitz, Hirschwände / 609	4	ALNHM463-20	+
01636/6550	GUT	Piestingtal, Pernitz, Hirschwände / 609	4	ALNHM464-20	+
01636/6551	GUT	Piestingtal, Pernitz, Hirschwände / 609	2A	ALNHM465-20	+
01638/6552	WIW	Triestingtal, Berndorf / 610	3	ALNHM466-20	+
01638/6553	WIW	Triestingtal, Berndorf / 610	3	ALNHM467-20	
01638/6554	WIW	Triestingtal, Berndorf / 610	3	ALNHM468-20	+
01648/6737	WIW	Halterbachtal, Spitalwiese / 627	4	ALNHM485-20	+
01648/6738	WIW	Halterbachtal, Spitalwiese/ 627	4	ALNHM486-20	+
01648/6739	WIW	Halterbachtal, Spitalwiese / 627	4	ALNHM487-20	+
01649/6740	WIW	Gainfarn, Ruine Merkenstein / 629	4	ALNHM488-20	+
01653/6747	WAV	Raabs/Thaya, Ruine Kollmitz / 670	4	ALNHM495-20	+
01653/6748	WAV	Raabs/Thaya, Ruine Kollmitz / 670	4	ALNHM496-20	
01653/6749	WAV	Raabs/Thaya, Ruine Kollmitz / 670	4	ALNHM497-20	+
01655/6750	WAV	Irnfritz-Messern, Ruine Grub / 671	4	ALNHM498-20	+
01655/6751	WAV	Irnfritz-Messern, Ruine Grub / 671	4	ALNHM499-20	+
01655/6752	WAV	Irnfritz-Messern, Ruine Grub / 671	4	ALNHM500-20	+
02096/6753	WIW	Triestingtal, Peilstein, Peilsteinwände / 672	1	ALNHM501-20	
02096/6754	WIW	Triestingtal, Peilstein, Peilsteinwände / 672	4	ALNHM502-20	+
02096/6755	WIW	Triestingtal, Peilstein, Peilsteinwände / 672	1	ALNHM503-20	
01608/6756	GUT	Piestingtal, Waldegg / 477	2A	ALNHM407-20	
C. d. gracilior					
01575/5999	RGM	Semmering, Breitenstein, Adlitzgraben / 51	4	ALNHM366-20	+
01575/6000	RGM	Semmering, Breitenstein, Adlitzgraben / 51	3	ALNHM367-20	+
02112/6001	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
01575/6003	RGM	Semmering, Breitenstein, Adlitzgraben / 51	1	ALNHM368-20	

02112/6004	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
02112/6005	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
02112/6006	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
01576/6011	RGM	Semmering, Breitenstein, Adlitzgraben / 52	4	ALNHM369-20	+
01584/6191	YBB	Lunz, Herdenglhöhle / 129	4	ALNHM386-20	+
01614/6293	RGM	Semmering, Maria Schutz / 576	4	ALNHM419-20	+
01614/6296	RGM	Semmering, Maria Schutz / 576	3	ALNHM421-20	+
01614/6297	RGM	Semmering, Maria Schutz / 576	3	ALNHM422-20	+
01617/6298	GUT	Hohe Wand, Große Kanzel, Springlessteig / 578	4	ALNHM423-20	+
C. d. huettneri					
02111/5920	RGM	Semmering, Sonnwendstein, Pollereshütte / 6	4	ALNHM365-20	+
01577/5923	RSB	Rax, Bismarcksteig / 79			+
01598/5927	GUT	Ternitz, Gösing, Flatzerwand / 300	4	ALNHM397-20	
02115/6018	RSB	Rax, Bismarcksteig / 79			+
01577/6019	RSB	Rax, Bismarcksteig / 79	4	ALNHM377-20	+
01581/6028	RSB	Rax, Thörlweg / 83	4	ALNHM382-20	
01606/6101	GUT	Ternitz, Gösing / 449	4	ALNHM405-20	+
01606/6104	GUT	Ternitz, Gösing /449	4	ALNHM406-20	
01616/6295	RGM	Semmering, Maria Schutz / 576			+
01618/6299	GUT	Hohe Wand, Große Kanzel, Springlessteig / 578	4	ALNHM424-20	+
01618/6302	GUT	Hohe Wand, Große Kanzel, Springlessteig / 578	4	ALNHM427-20	
01620/6303	RSB	Sierningtal, Stixenstein, Schlosspark / 582	4	ALNHM428-20	+
01626/6317	RSB	Schneeberg, Waxriegel, Niederer Hengst / 586	4	ALNHM442-20	+
01630/6535	RSB	Höllental, Naßwald / 605	4	ALNHM452-20	+
01634/6544	RSB	Piestingtal, Gutenstein / 608	4	ALNHM458-20	+
01634/6545	RSB	Piestingtal, Gutenstein / 608	3	ALNHM459-20	+
01637/6548	GUT	Piestingtal, Pernitz, Hirschwände / 609	3	ALNHM462-20	+
C. d. kaeufeli					
01587/5925	RSB	Schneeberg, Fadensteig, Fadenwände / 172	2B	ALNHM389-20	
01595/5926	RSB	Schneeberg, Waxriegel / 178	1	ALNHM395-20	
01594/5936	RSB	Schneeberg, Hochschneeberg, Ochsenboden / 177	4	ALNHM394-20	
02115/6014	RSB	Rax, Bismarcksteig / 79			+
02116/6068	RSB	Schneeberg, Klosterwappen / 176			+
02116/6069	RSB	Schneeberg, Klosterwappen / 176			+
01595/6072	RSB	Schneeberg, Waxriegel / 178			+
01625/6314	RSB	Schneeberg, Fischerhütte / 585	4	ALNHM439-20	+
01625/6315	RSB	Schneeberg, Fischerhütte / 585	4	ALNHM440-20	
01625/6316	RSB	Schneeberg, Fischerhütte / 585	4	ALNHM441-20	+
01641/6724	RSB	Schneeberg, Waxriegel / 623	1	ALNHM472-20	+
01642/6728	RSB	Schneeberg, Waxriegel / 624	1	ALNHM476-20	+
01646/6735	RSB	Schneeberg, Kaiserstein / 626	4	ALNHM483-20	+
C. d. moldanubica					
01580/5924	WAU	Wachau, Mautern, Stift Göttweig / 81	4	ALNHM378-20	+
01580/6023	WAU	Wachau, Mautern, Stift Göttweig / 81	4	ALNHM379-20	
01580/6024	WAU	Wachau, Mautern, Stift Göttweig / 81	4	ALNHM380-20	+
01580/6025	WAU	Wachau, Mautern, Stift Göttweig / 81	4	ALNHM381-20	+
01580/6026	WAU	Wachau, Mautern, Stift Göttweig / 81			+
01580/6027	WAU	Wachau, Mautern, Stift Göttweig / 81			+

C. d. obsoleta					
01574/5975	RGM	Semmering, Maria Schutz / 5	4	ALNHM364-20	+
01582/6056	YBB	Dürrenstein, Gipfelregion / 112	4	ALNHM383-20	+
01582/6057	YBB	Dürrenstein, Gipfelregion / 112	4	ALNHM384-20	+
01602/6116	GUT	Gutensteiner Alpen, Halbachtal, Kleinzell / 316			+
01602/6118	GUT	Gutensteiner Alpen, Halbachtal, Kleinzell / 316	2B	ALNHM403-20	+
01610/6123	RGM	Grimmenstein, Burgruine / 481			+
01615/6294	RGM	Semmering, Maria Schutz / 576	4	ALNHM420-20	+
C. d. runensis					
01609/6105	RGM	Seebenstein, Türkensturz / 480	1	ALNHM408-20	+
02117/6291	RGM	Schlattenbachtal, Scheiblingkirchen / 575	1	ALNHM417-20	+
01650/6741	WIW	Gainfarn, Ruine Merkenstein / 629	1	ALNHM489-20	+
01650/6742	WIW	Gainfarn, Ruine Merkenstein / 629	1	ALNHM490-20	+
C. d. schlechtii					
02114/5922	RSB	Höllental, Krummbachgraben / 59	4	ALNHM373-20	+
01605/5930	GUT	Ternitz, Gösing / 449	4	ALNHM404-20	
02113/5979	RSB	Höllental, Weichtalklamm / 58	3	ALNHM370-20	
01573/5996	RGM	Semmering, Maria Schutz / 4			+
02112/6002	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
02112/6006	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
02112/6007	RGM	Semmering, Breitenstein, Adlitzgraben / 51			+
02114/6012	RSB	Höllental, Krummbachgraben / 59	4	ALNHM374-20	+
01579/6017	RSB	Rax, Bismarcksteig / 79	4	ALNHM376-20	+
01583/6058	RSB	Rax, Jakobskogel, Große Kanzel / 125	3	ALNHM385-20	
01589/6066	RSB	Schneeberg, Kaiserstein / 175			+
01619/6300	GUT	Hohe Wand, Große Kanzel, Springlessteig / 578	4	ALNHM425-20	+
01619/6301	GUT	Hohe Wand, Große Kanzel, Springlessteig / 578	4	ALNHM426-20	+
01621/6304	RSB	Sierningtal, Stixenstein, Schlosspark / 582	4	ALNHM429-20	+
01621/6305	RSB	Sierningtal, Stixenstein, Schlosspark / 582	4	ALNHM430-20	+
01621/6306	RSB	Sierningtal, Stixenstein, Schlosspark / 582	4	ALNHM431-20	+
01621/6307	RSB	Sierningtal, Stixenstein, Schlosspark / 582	4	ALNHM432-20	+
01624/6312	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 584	4	ALNHM437-20	+
01624/6313	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 584	4	ALNHM438-20	+
01628/6531	RSB	Höllental, Abbrennbrücke / 604	4	ALNHM449-20	+
01628/6533	RSB	Höllental, Abbrennbrücke / 604	4	ALNHM450-20	+
01628/6534	RSB	Höllental, Abbrennbrücke / 604	4	ALNHM451-20	+
01629/6536	RSB	Höllental, Naßwald / 605	4	ALNHM453-20	+
01629/6537	RSB	Höllental, Naßwald / 605	4	ALNHM454-20	+
01631/6539	RSB	Höllental, Kornbrandmauer / 606	4	ALNHM455-20	+
01639/6555	WIW	Triestingtal, Berndorf / 610	4	ALNHM469-20	
01639/6556	WIW	Triestingtal, Berndorf / 610	4	ALNHM470-20	+
01644/6732	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 625	2A	ALNHM480-20	+
01645/6733	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 625	4	ALNHM481-20	+
01651/6743	GUT	Gutensteiner Alpen, Halbachtal, Rossbachklamm / 669	4	ALNHM491-20	+
01651/6744	GUT	Gutensteiner Alpen, Halbachtal, Rossbachklamm / 669	4	ALNHM492-20	
C. d. speciosa					
02113/5982	RSB	Höllental, Weichtalklamm / 58	4	ALNHM371-20	
02113/5983	RSB	Höllental, Weichtalklamm / 58	4	ALNHM372-20	+
01591/6065	RSB	Schneeberg, Fadensteig, Kaiserstein / 175	4	ALNHM392-20	+
01600/6093	GUT	Schwarzatal, Tiefental / 313	4	ALNHM401-20	+

01652/6745	GUT	Gutensteiner Alpen, Halbachtal, Rossbachklamm / 669	4	ALNHM493-20	+
01652/6746	GUT	Gutensteiner Alpen, Halbachtal, Rossbachklamm / 669	1	ALNHM494-20	+
C. d. tettelbachiana					
01612/5931	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 530	4	ALNHM410-20	
01593/5935	RSB	Schneeberg, Hochschneeberg, Ochsenboden / 177	4	ALNHM393-20	
02115/6013	RSB	Rax, Bismarcksteig / 79			+
01578/6016	RSB	Rax, Bismarcksteig / 79	4	ALNHM375-20	+
02115/6021	RSB	Rax, Bismarcksteig / 79			+
02115/6022	RSB	Rax, Bismarcksteig / 79			+
01585/6059	RSB	Schneeberg, Fadensteig, Fadenwände / 170	1	ALNHM387-20	+
01586/6060	RSB	Schneeberg, Fadensteig, Fadenwände / 171	1	ALNHM388-20	+
01588/6062	RSB	Schneeberg, Fadensteig, Fadenwände / 172	4	ALNHM390-20	+
01590/6064	RSB	Schneeberg, Fadensteig, Kaiserstein / 175	4	ALNHM391-20	+
02116/6067	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 176			+
01592/6070	RSB	Schneeberg, Hochschneeberg, Ochsenboden / 177			+
01612/6106	RSB	Schneeberg, Hochschneeberg, Ochsenboden / 530			+
01613/6285	RSB	Schneeberg, Hochschneeberg, Hahnriegel / 574	4	ALNHM411-20	+
01613/6286	RSB	Schneeberg, Hochschneeberg, Hahnriegel / 574	1	ALNHM412-20	+
01613/6287	RSB	Schneeberg, Hochschneeberg, Hahnriegel / 574	4	ALNHM413-20	+
01622/6308	RSB	Schneeberg, Waxriegel / 583	1	ALNHM433-20	+
01622/6309	RSB	Schneeberg, Waxriegel / 583	1	ALNHM434-20	+
01622/6310	RSB	Schneeberg, Waxriegel / 583	1	ALNHM435-20	+
01623/6311	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 584	4	ALNHM436-20	+
01635/6546	RSB	Piestingtal, Gutenstein / 608	4	ALNHM460-20	+
01640/6723	RSB	Schneeberg, Waxriegel / 623	1	ALNHM471-20	+
01640/6725	RSB	Schneeberg, Waxriegel / 623	1	ALNHM473-20	+
01640/6726	RSB	Schneeberg, Waxriegel / 623	1	ALNHM474-20	+
01643/6727	RSB	Schneeberg, Waxriegel / 624	1	ALNHM475-20	+
01643/6729	RSB	Schneeberg, Waxriegel / 624	1	ALNHM477-20	
01643/6730	RSB	Schneeberg, Waxriegel / 624	1	ALNHM478-20	+
01644/6731	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 625	4	ALNHM479-20	+
01644/6734	RSB	Schneeberg, Hochschneeberg, Klosterwappen / 625	4	ALNHM482-20	+
01647/6736	RSB	Schneeberg, Kaiserstein, Fischerhütte / 626	4	ALNHM484-20	+
C. d. vindobonensis					
02119/6885	WIW	Wienerwald, Wien, Leopoldsberg / 679	4	ALNHM504-20	+
02119/6886	WIW	Wienerwald, Wien, Leopoldsberg / 679	4	ALNHM505-20	+
02119/6887	WIW	Wienerwald, Wien, Leopoldsberg / 679	4	ALNHM506-20	+
02119/6888	WIW	Wienerwald, Wien, Leopoldsberg / 679	4	ALNHM507-20	+
02119/6889	WIW	Wienerwald, Wien, Leopoldsberg / 679	4	ALNHM508-20	+
Neostyriaca corynodes					
01632/6541	RSB	Höllental, Saurüsselbrücke / 607		ALNHM456-20	

Appendix 2. Coordinates and altitude (meters above sea level) of sampling localities (Sample ID). Sample IDs are the same as in Appendix 1.

Sample ID	Latitude	Longitude	Elevation
2	48.14	16.90	144
4	47.64	15.88	788
5	47.63	15.88	871
6	47.63	15.86	1477
51	47.66	15.84	650
52	47.66	15.84	650
58	47.75	15.77	592
59	47.73	15.79	556
79	47.69	15.71	1787
81	48.37	15.61	407
83	47.71	15.77	1592
112	47.79	15.06	1786
125	47.71	15.75	1734
129	47.84	14.98	850
170	47.79	15.81	1393
171	47.79	15.81	1525
172	47.79	15.81	1562
175	47.78	15.81	1910
176	47.77	15.81	1994
177	47.77	15.81	1905
178	47.76	15.83	1873
282	47.66	16.19	583
300	47.75	16.00	671
313	47.88	15.65	739
315	47.96	16.04	412
316	47.95	15.71	507
449	47.74	15.98	356
477	47.87	16.05	412
480	47.68	16.14	550
481	47.63	16.12	677
504	48.34	15.92	185

Sample ID	Latitude	Longitude	Elevation
530	47.77	15.81	2024
574	47.76	15.83	1804
575	47.66	16.14	382
576	47.63	15.87	771
578	47.81	16.02	744
582	47.75	15.98	448
583	47.76	15.83	1858
584	47.77	15.81	1994
585	47.77	15.81	1980
586	47.75	15.87	1197
602	47.66	16.16	395
603	47.68	16.14	550
604	47.73	15.80	508
605	47.77	15.69	626
606	47.74	15.78	517
607	47.76	15.69	630
608	47.88	15.87	489
609	47.92	15.94	640
610	47.94	16.11	321
623	47.76	15.83	1870
624	47.76	15.83	1860
625	47.77	15.81	2024
626	47.77	15.81	2005
627	48.23	16.24	276
629	47.98	16.13	457
669	47.91	15.68	914
670	48.82	15.53	440
671	48.71	15.51	460
672	48.00	16.05	560
679	48.27	16.34	425