Taxonomic recognition approach by morphometrical and molecular genetic methods of conchologically similar Helicellinae species (Helicoidea, Gastropoda)

MARKUS PFENNINGER, STEFFEN NAUMANN, DIRK STEINKE & BERND SEIDENDORF

Abteilung Ökologie & Evolution, Zoologisches Institut, J.W.Goethe-Universität Frankfurt am Main, BioCampus Siesmayerstraße, D-60054 Frankfurt, Germany.

Abstract. We studied the practicability of a genetic cluster approach for taxonomic recognition with six conchologically similar Helicellinae species, using 16S rDNA sequences and nine morphometric measurements of shape, size and shell surface structure. Specimens of at least two allopatric populations of each nominal species were used to include individual as well as geographic variation. Using the genetic cluster membership as grouping variable, a Canonical Discriminant Analysis on the morphologic variables obtained a highly significant discrimination model. Additionally, museum collection material was integrated into the analysis to confirm *a posteriori* the specific name to the recognised entities. Theoretical and practical issues and implications of the presented approach that places problematical species recognition into a single coherent statistical framework are discussed.

Kurzfassung. Taxonomischer Arterkennungsansatz durch morphometrische und molekulargenetische Methoden bei Helicellinae-Arten mit ähnlicher Schalenform. In dieser Arbeit wurde exemplarisch die Möglichkeiten eines statistischen Artidentifizierungsansatzes erkundet. Sechs Helicellinae-Arten, deren taxonomische Zuordnung aufgrund ihres ähnlichen Gehäuses immer wieder als problematisch dargestellt wurde, gingen in die Analyse ein. Individuen von wenigstens zwei allopatrischen Populationen jeder nominalen Art wurden verwendet, um sowohl individuelle als auch geographische Variation zu berücksichtigen. Es wurden von jedem Individuum 16S rDNA-Sequenzen erstellt und neun morphometrische Messgrößen der Form, Größe und Oberflächenstruktur der Schale erhoben. Die aus der 16S-Analyse erschlossene evolutionäre Linie der Individuen wurde als gruppierende Variable in einer kanonischen Diskriminanzanalyse verwendet. Dies ergab ein hochsignifikantes Diskriminierungsmodell. Um den so identifizierten Arten *a posteriori* einen existierenden taxonomischen Namen zuzuweisen, wurde Schalenmaterial aus der Malakologischen Sammlung des Senckenbergmuseums in die Analyse integriert. Die theoretischen und praktischen Implikationen des vorgeschlagenen Ansatzes, der die Artdiagnose von problematischen Taxa in einen kohärenten statistischen Rahmen stellt, werden diskutiert.

Key words. Taxonomic recognition, Helicellinae, Canonical Discriminant Analysis.

Introduction

Substantial variation in shell morphology can be observed in the Helicellinae land snails with high intraspecific conchological variation but small interspecific differences. They often occur in sympatry or neighbouring sympatry. Thus, conchological determination is often problematical, leading to a considerable uncertainty about species ranges and taxonomic validity of described taxa (GITTENBERGER 1993, PFENNINGER & MAGNIN 2001). These morphological differences between conspecific populations can be due to environmental modifications, adaptational modifications that are not reflected in the phylogenetic history of the species and morphological differences that are concordant with phylogenetic lineages. Only the latter are relevant for taxonomy. As a result, estimating existing biodiversity in Helicellinae over their distribution range has proven difficult. A similar situation is often encountered in malacology (e.g. ARMBRUSTER 1997, RENARD et al. 2000), rendering the recognition of species difficult and, hence, the taxonomy of certain groups. The problem is at least partly due

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to the widespread use of the typological species concept (TSC) in malacology that does not account for morphological variation among allopatric populations, leading to the designation of many species names to the same evolutionary entity. The biological species concept (BSC) on the other hand is problematical to apply to molluscs, because allopatric populations often do constitute reproductively isolated entities (BAUR & BAUR 1992, SCHILTHUIZEN & LOMBAERTS 1994, TURNER et al. 2000) that have nevertheless still genetic cohesion. One of the possible solutions to this practical and philosophical problem (discussed in HULL 1997) could be an updated, genetic version of Darwin's own pragmatical species definition, as advocated by MALLET (1995). Darwin's species definition is based on the common observation of many naturalists that the existing phenotypic diversity in nature is not continuous, but with gaps between the units that he called species. Mallet extends this observation with the experience of geneticists that there usually exists a greater gap in the amount of genetic variation between'good species' compared to within species variation. The inference of a species or taxonomically relevant unit is therefore based on the congruence of both phenotypic and genotypic variation.

To gain information about phylogenetic relations and population processes on the species level and beyond, effectively neutral genetic markers like allozymes, microsatellites, RFLPs or mitochondrial DNA sequences have been widely used in malacology (e.g. ARTER 1990, SCHILTHUIZEN et al. 1995, WILKE & DAVIES 2000). This molecular genetic information can be used as background against which the phenotypic variation can be compared.

While qualitative differences in shell colour and banding within Helicid snail species is well studied in conjunction with molecular markers (e.g. ARTER 1990, DAVISON & CLARKE 2000), quantitative shell variation was not yet explored in Helicoid snails for its usefulness to distinguish problematical taxa. Recent advances in morphometrics (ROHLF 1995) have provided methods of quantitative shape analysis. As an example, we want to explore the possibilities of one of the many possible implementations of the genetic cluster approach to discriminate statistically six Helicellinae species on the basis of quantitative morphological shell traits. We have studied six conchologically similar species that co-occur within or close to the species range of *Candidula unifasciata* (Poiret, 1801) and that have often been confused in collections and taxonomical studies (GITTENBERGER 1993).

Material and methods

For each species, at least two allopatric populations were sampled for genetic analysis (Table 1). The mitochondrial 16S rDNA was used as genetic marker. DNA isolation, fragment amplification, sequencing and alignment were performed as described in PFENNINGER & MAGNIN (2001). Sequences used in this analysis can be obtained from GeneBank (For accession numbers see Table 1). Genetic clusters were obtained by performing an UPGMA on the absolute number of differences between all sequences. A cluster was defined as the aggregation of terminal taxa whose distances to the node uniting them was small (<20%) against the distance between this node and the next node towards the root of the resulting tree. Computations were carried out with PAUP* 4.0b8 (SWOFFORD 1998).

The following character sets were measured by image analysis from each of the individuals in genetic analysis:

1. Shell shape was assessed through the use of the first three relative warp scores of a Thin Plate Spline analysis (ROHLF 1995). This geometrical analysis of shape was based on 55 homologous landmarks applied to an electronic image of the individual. The first relative warp score turned out to be a descriptor of a depressed vs. globular shell shape, the second opposed roundish vs. elliptical apertures and the third score represents a round vs. angular periphery of the last whorl.
2. Shell sculpture traits were measured through rib-spacing as average distance between ribs, coarseness as average distance between a base line and perimeter of ribs and regularity of ribs as the coefficient of variation of inter-rib distances.
3. Shell size was quantified as overall breadth and length.

These shell morphology variables were used to perform a Canonical Discriminant Analysis (CDA) with the genetic cluster membership as grouping variable. To break a potential circu-

Tab. 1. Taxa used in analysis with sampling location. Material from the Senckenberg Museum (Frankfurt/Germany) is designated by the collection number.

Taxon	Sampling location	*Collection No.*	N	GenBank Accession No.
Candidula unifasciata (Poiret 1801)	Sisteron / France		6	AF408011-16
	Dierdorf / Germany		6	AF407882-87
	Grasse / France	60827	6	-
	Budapest / Hungary	278316	7	-
Candidula gigaxii (L. Pfeiffer 1850)	Caderousse / France		5	AF407836-40
	Maussane / France		3	AF407833-35
	Verdun / France		4	AF407832-30, AF113841
	Boulbon / France	60929	9	-
Helicella conspurcata (Draparnaud 1805)	Caderousse / France		4	AF458910-13
	Sete / France		4	AF458914-17
	Banyuls / France	217615	7	-
	Marseille / France	217613	7	-
Trochoidea geyeri (Soós 1926)	Schlüchtern / Germany		5	AF407811-16
	Luberon / France		5	AF407785-89
	Metz / France	60212	5	-
	Weimar / Germany	63981	6	-
Candidula spadae (Calcara 1845)	Bolognola / Italy		5	AF458918-22
	Presta / Italy		5	AF458923-27
	Monte Vettore / Italy	98019	6	-
	Morte / Italy	98007	4	-
Candidula intersecta (Poiret 1801)	Oostende / Belgium		5	AF458928-32
	Middelkerke / Belgium		5	AF458933-37
	Wendugne /Belgium	60605	11	-
	Kiel / Germany	217550	10	-

larity and to confirm a specific name for each morphometrically and genetically defined group, samples for each nominal species from the Malacological Collection of the Senckenberg-Museum were included in the shell morphological analysis (Table 1). CDA was computed with STATISTICA 5.5 (StatSoft).

Tab. 2. Means and standard deviations of meaningful variables for the groupings used in CDA. The taxa designations are *a posteriori* classifications.

classification	coarseness [mm]	rib spacing [mm]	irregularity [coeff. of var.]	shell height [mm]	shell breadth [mm]	*N*
C. spadae	0.012±0.001	0.139±0.025	0.260±0.078	5.52±0.42	8.96±0.31	10
C. unifasciata	0.019±0.004	0.154±0.021	0.233±0.096	3.72±0.20	6.27±0.39	12
C. intersecta	0.035±0.024	0.206±0.029	0.324±0.094	5.44±0.60	8.17±0.72	10
C. gigaxii	0.029±0.003	0.104±0.027	0.022±0.006	4.14±0.39	6.69±0.77	12
T. geyeri	0.090±0.007	0.141±0.027	0.044±0.062	4.06±0.43	6.01±0.59	10
H. conspurcata	0.021±0.001	0.101±0.005	0.019±0.008	3.42±0.24	5.42±0.38	8
All	0.035±0.029	0.153±0.044	0.187±0.146	4.55±0.94	7.12±1.36	62

Results

Application of the UPGMA algorithm on the matrix of absolute number of differences between 16S rDNA sequences resulted in six clusters as defined above (Figure 1). The means, s.d., and range for the meaningful variables (i.e. not the shape variables that are dimensionless) are given in Table 2. The original data matrix is available from M.P. upon request. Using the cluster membership for each individual as grouping variable in CDA resulted in a highly significant discriminant model (Wilk's lambda: 0.0007614 approx. $F_{(45,226)} = 47.7$ $p < 0.0000$). Five roots were extracted from the data. Examination of the Factor Structure Matrix showed that root 1 was mainly correlated with the coarseness of the ribs; root 2 was a descriptor of overall size (breadth and length), whereas the shell shape determined root 3. Distances between all group centres were significantly large. Almost all (99.28%) of the classifications were correct; only one specimen (*C. gigaxii* as *H. conspurcata*) was assigned to an other group than predicted. The *a posteriori* classification of the museum specimens gave a similar result. Except two specimens, all individuals presumed to belong to the same species were classified to one group, respectively. This finding allowed confirming the traditional taxonomic names of the genetic clusters (Figure 1). A plot of the canonical scores of the first two roots is given in Figure 2.

Discussion

The results show that the presented implementation of the genetic cluster approach can be successfully applied to discriminate conchologically similar Helicellinae land snail species on the basis of shell traits. The presented approach overcomes both the problems associated with the typological species concept and the biological species concept by placing the process of species recognition into a rigorous statistical framework. This is achieved by integrating individual and geographic variation of shell morphology on the basis of the objectively definable genetic clusters into statistical discrimination models. However, the additional integration of type or topotype material is desirable. It is encouraging to see that even relatively few individuals per species were enough to find a discrimination model that was sufficiently powerful to correctly assign the great majority of the museum specimens from different locations.

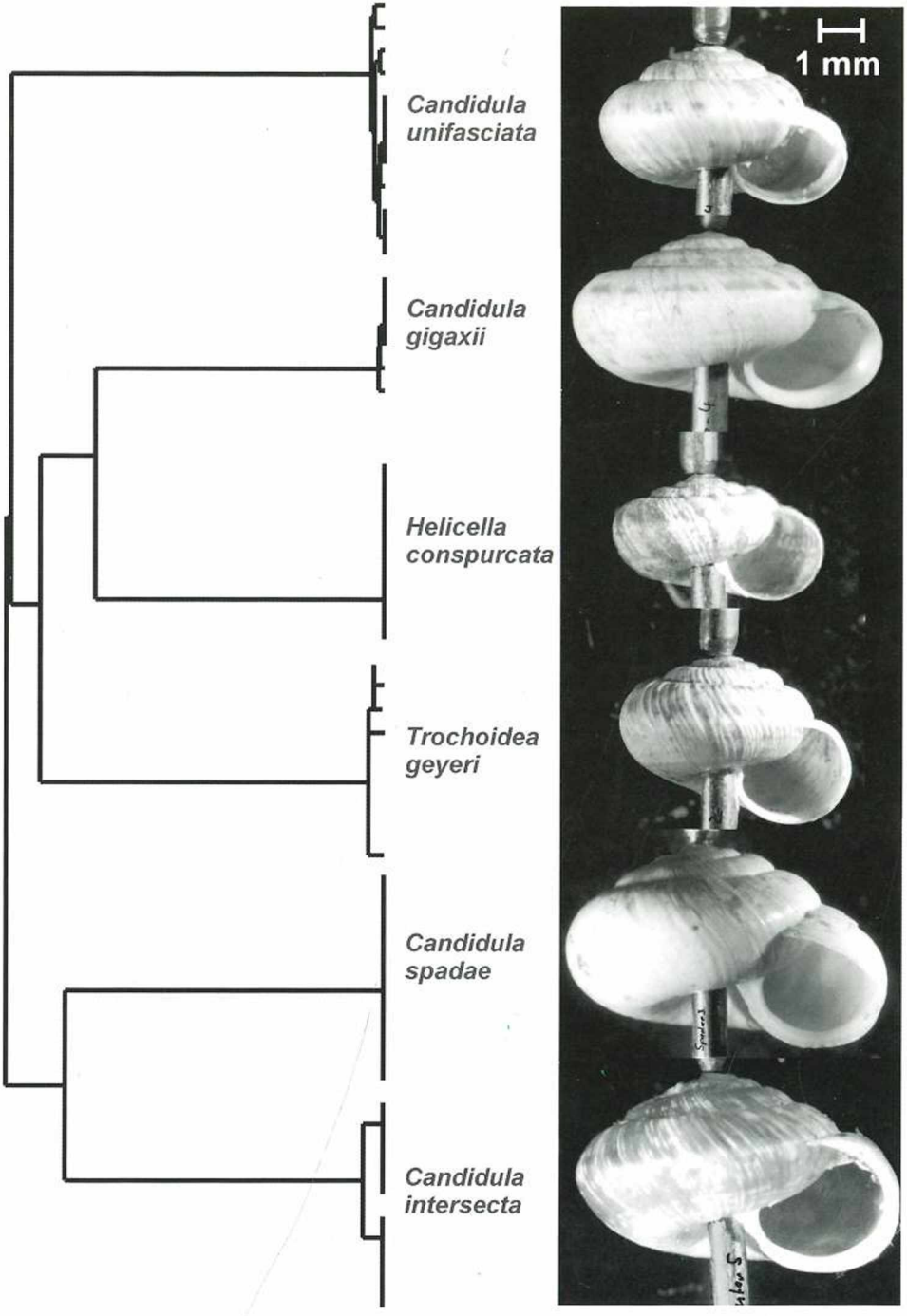

Fig. 1. UPGMA-tree of pairwise differences between 16S rDNA sequences of Helicellinae land snails. The resulting clusters were used as grouping variable for the Canonical Discrimination Analysis (CDA) of shell feature variables. The names were assigned posterior to the CDA. Note that the tree does not depict phylogenetic relationships but phenetic similarity of sequences.

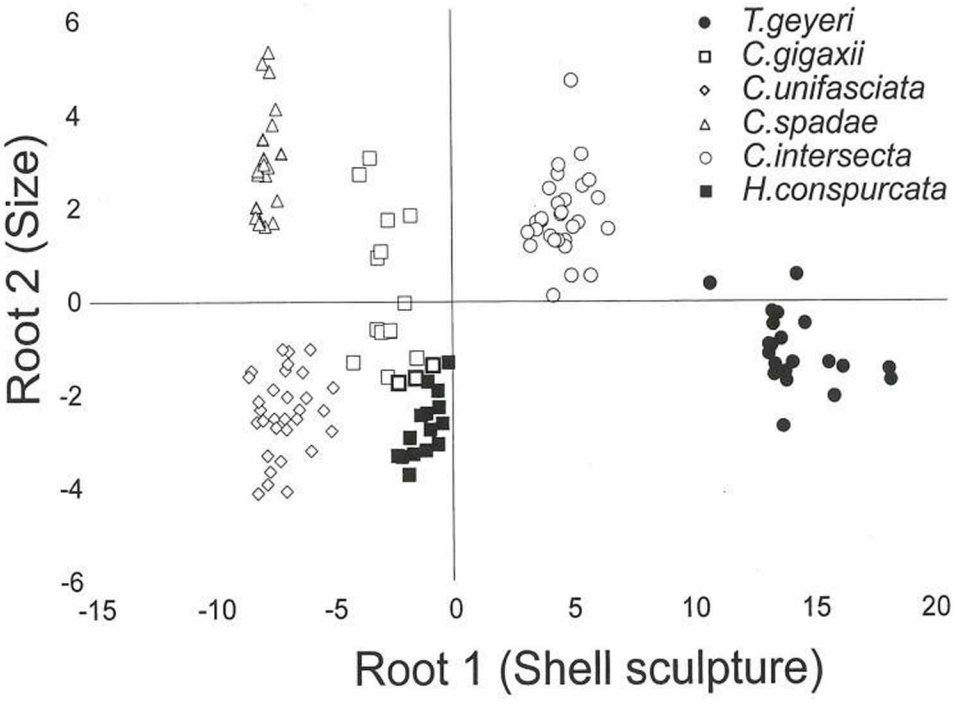

Fig. 2. Scatterplot of the first two canonical scores derived from Canonical Discrimination Analysis. Species designation is given after the designation of CDA. Assignments not in concordance with genetic cluster membership or museum classification occurred only at the border between *C. gigaxii* and *H. conspurcata*. The symbols for these specimen have a bold outline.

Upon closer microscopical inspection of the few misassigned specimens (*C. gigaxii* as *H. conspurcata*), it became clear that these individuals were erroneously classified by the original collectors and not by the CDA, because they possessed the characteristic hair pits of the latter. This shows that more variables could have been used to obtain a better discrimination model. What could be seen as a weakness of the study illustrates an advantage of the presented technique of species delimitation. It is an open method in the sense that integration of more information, both about more variables and more specimens, is likely to yield better shell morphologic species models.

The limitation on shell characteristics, however, is not necessary. Anatomical features could also be used. However, restriction to shell characteristics offers the opportunity to integrate material into the analysis where soft body parts are not available anymore. Statistically based inferences of taxonomic identity are possible with materials from collections, field trips or even adequate photographs from publications.

We have used a phenetic approach to derive genetic clusters, because an estimation of evolutionary relationships between examined taxa was not intended and will be published elsewhere. This was a deliberate choice, although other, explicitly phylogenetic methods like bootstrap support of phylogenetic tree clades, Nested Clade Analysis (TEMPLETON 1998) or pairwise sequence divergence distributions can also be used to define genetic clusters. The common objective of these approaches should be to define objective genetic entities whose most recent common ancestor lived more recently than the one between the taxa in question. The inference of evolutionary lineages depends also on the sensible choice of genetic markers employed. With more closely related species, more and faster evolving genes or multilocus approaches will be the tool of choice.

Situations may occur where the proposed method fails to discriminate taxa despite of an extensive database, because of the lack of congruence between genetic and phenotypic data. Such situations can include interspecific hybridisation with morphological and/or genetic intermediates, cryptic speciation with morphological stasis or speciation processes where the gene-flow between the emerging entities has not yet completely ceased in the contact area. All these cases of incongruence present not a problem to the genetic cluster approach as long as we see species cohesion and speciation as a temporally extended process and take such inferences as an opportunity to study the evolutionary processes involved.

Our results show that the statistical rather than philosophical genetic cluster approach to delimit species (MALLET 1995) is practical and may be able to overcome the difficulties of taxonomic recognition in malacology associated with other concepts. A few other studies (VAN MOORSEL et al. 2000, RENARD et al. 2000, PFENNINGER & MAGNIN 2001) have already shown that the application of both molecular genetic and morphological methods can be successfully used in inferring species status and evolutionary processes. However, the presented study shows how both methods can be integrated into a single coherent statistical framework. The approach presented here is relatively easy to perform and can be extended to other problematic taxa. Moreover, aside from providing a convenient tool to delimit problematic species, the approach promises to be sensitive enough to study the evolutionary processes that underlie the observed morphological and genetic divergence.

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