Coexisting Cyclic Parthenogens Comprise a Holocene Species Flock in *Eubosmina*

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**Abstract**

**Background:** Mixed breeding systems with extended clonal phases and weak sexual recruitment are widespread in nature but often thought to impede the formation of discrete evolutionary clusters. Thus, cyclic parthenogens, such as cladocerans and rotifers, could be predisposed to “species problems” and a lack of discrete species. However, species flocks have been proposed for one cladoceran group, *Eubosmina*, where putative species are sympatric, and there is a detailed paleolimnological record indicating a Holocene age. These factors make the *Eubosmina* system suitable for testing the hypotheses that extended clonal phases and weak sexual recruitment inhibit speciation. Although common garden experiments have revealed a genetic component to the morphotypic variation, the evolutionary significance of the morphotypes remains controversial.

**Methodology/Principal Findings:** In the present study, we tested the hypothesis of a single polymorphic species (i.e., mixing occurs but selection maintains genes for morphology) in four northern European lakes where the morphotypes coexist. Our evidence is based on nuclear DNA sequence, mitochondrial DNA sequence, and morphometric analysis of coexisting morphotypes. We found significant genetic differentiation, genealogical exclusivity, and morphometric differentiation for coexisting morphotypes.

**Conclusions:** We conclude that the studied morphotypes represent a group of young species undergoing speciation with apparent reproductive barriers despite coexistence in the freshwater pelagic zone.

**Introduction**

Breeding systems are expected to play a central role in speciation [1–4]. In strictly sexual species, gene flow can unite populations, whereas ecological divergence and a lack of interbreeding can result in the formation of discrete evolutionary clusters. Likewise, in strictly asexual species, periodic selective sweeps of clones may unite populations and discontinuous ecological niches might lead to the appearance of discrete evolutionary clusters [5]. However, in groups with mostly asexual systems, extended clonal phases followed by weak sexual recruitment may prevent the formation of discrete lineages. In these mixed breeding systems recombination might be sufficient to prevent clonal selective sweeps but insufficient to homogenize populations by gene flow [2,6]. In addition, mixed breeding systems are often associated with small organisms and powerful dispersal abilities, potentially reducing the capacity for speciation [1,4]. Mixed breeding systems might also promote the formation (sexual phase) and stabilization (clonal phase) of hybrid products, further blurring the boundaries of evolutionary clusters. Finally, experimental results indicate that animals with mixed breeding systems suffer a reduced rate of adaptation compared to those with obligate outcrossing [7]. Thus, organisms with mixed breeding systems such as cyclic parthenogens (cladocerans, rotifers, aphids etc.) could be predisposed to slow rates of cladogenesis, and lack rapid radiations.

An opposing view is that mixed breeding systems, such as cyclical parthenogenesis, can imbue organisms with a capacity for quantum phenotypic evolution and potentially enhance speciation rates [8,9]. Here, the action of mutation and selection on multiple rounds of asexual reproduction results in a build up of unexpressed genetic variance. Infrequent sexual reproduction can then cause a flush of expressed genetic variance and enhanced capacity for adaptation. For cyclic parthenogens several rapid radiations have been proposed for allopatric forms, and there is indirect evidence for their rapidity [10]. However, only one group of lacustrine cladocerans, the *Eubosmina* group has an excellent continuous paleolimnological record of a radiation during the Holocene [11–28] and proposed sister taxa coexist in the same lakes. The recent emergence of the morphotypes is well documented in the fossil record; only the *longispina* morphotype has been recorded from the interglacial and late glacial sediments - all other morphotypes are restricted to postglacial sediments. Eleven taxa
are recognized in the most recent taxonomic treatment of the complex [29] but whether morphotypes represent real evolutionary lineages or merely polymorphisms remains a controversy [30].

The taxa are diagnosed by the shapes of the carapace, antennules, and paired posterior spines called mucros. Eubosmina are unique, among freshwater cladocerans, in the existence of putative species flocks - up to nine taxa can co-occur in the same lake [31]. Phenotypic plasticity is evident in the diagnostic characters [32,33], but common garden experiments have revealed that the morphological differences have a genetic component. That is, clones from different taxa cultured under identical conditions retain some of their characteristic morphology [34].

Ecological differentiation among Eubosmina morphotypes is most pronounced between E. longispina, which is usually found in oligotrophic waters, and the extreme forms e.g. berolinensis, gibbera and the rites, which are usually found in more eutrophic lakes. The morphological differences in antennules, brood chambers and posterior spines have been found to reduce vulnerability to invertebrate predation [34–38]. Thus, abundances of the extreme forms are correlated with invertebrate predators and the nutrient status of lakes seasonally, spatially, and temporally [18,39–42].

A critical evolutionary question remains: do the coexisting morphotypes of Eubosmina represent polymorphisms or discrete evolutionary lineages? The evolutionary status of the morphotypes of Eubosmina is a longstanding controversy [29,30,34,43]. Earlier genetic analysis using allozymes [34], and sequence variation in the nuclear rDNA array [34–38] found that the European morphs were closely related, but were unable to rule out random morphotype-genotype associations. Hellsten & Sundberg [44] reported genetic isolation based on non-statistical patterns (multidimensional scaling) of RAPD markers between coexisting E. longispina and E. coregoni in Östersjön Lake, Sweden. However, they also reported that over 15% of the RAPD bands were not repeatable and the contribution of non-cladoceran DNA (microbes, algae, symbionts) to the patterns with anonymous RAPD markers is unknown. Here, we explicitly test the hypothesis of evolutionary discreteness in the Eubosmina group using nuclear and mitochondrial DNA sequences and geometric morphometric analysis from the same individuals. We chose four lakes that contain different combinations of coexisting morphotypes. Testing for reproductive barriers in coexisting populations (rather than among allopatric populations) is straightforward but important because a small amount of interbreeding should homogenize coexisting lineages. Significant morphometric and genetic differentiation found in coexisting taxa is consistent with reproductive barriers but inconsistent with geographic isolation. Reciprocal monophyly is neither expected nor observed in young radiations [45–49]. If the morphotypes do represent young (postglacial) lineages, then we expect the genetic patterns found in haplotype networks to have the characteristic signature of young lineages. That is, we expect some sharing of haplotypes among coexisting species and low but significant measures of genetic divergence. Often presumed older central haplotypes are shared among recently diverged species [45,46,49]. Genetic divergence is therefore a continuum that proceeds with time from differentiation with sharing to monophyly. Reduced sharing of haplotypes, significant genetic divergence, and significant morphometric differentiation of coexisting forms are evidence of reproductive barriers.

Results

Genetic Analyses

Genetic differentiation was significant by most measures for coexisting morphotypes of Eubosmina (Table 1). Both ND2 and HSP90 showed significant genetic differentiation among coexisting morphs with the exception of those from Ragnerudsjön Lake. The mitochondrial gene ND2 exhibited less differentiation than the nuclear gene HSP90 analyses. FST values differed significantly from zero in all the cases for ND2 and for six out of seven comparisons for HSP90. Sequential FST values differed significantly from zero in nine out of eleven cases for ND2 and in eight of eleven cases for HSP90 haplotypes (Table 1). For individual lakes, both FST and FST values were significantly greater than zero for both analyzed genes of longispina and coregoni from Väner Lake, and longispina, cedrostomi and longiconis from Stora Fargen Lake. FST values for ND2 and HSP90 and FST for HSP90 of berolinensis, coregoni, and gibbera from Fleesensee Lake were significantly greater then zero as well. The FST for HSP90 (coregoni and gibbera) was, however, not significant. For Ragnerudsjön Lake, only FST and FST values of ND2 gene haplotypes belonging to longispina and kessleri and FST value of ND2 comparing longiconis and kessleri haplotypes were significantly higher than zero. Inspection of TCS haplotype networks (Fig. 1) revealed that morphotypes often shared the most common and interconnected haplotypes (the presumed ancestral haplotypes), but had several private haplotypes (the exception was HSP90 from Ragnerudsjön Lake (Fig. 1). Only 3 of 33 ND2 haplotypes and 9 of 28 HSP90 haplotypes were shared between longispina and coregoni from Väner Lake. 3 of 25 ND2 haplotypes and 3 of 22 HSP90 haplotypes belonging to longispina, cedrostomi and longiconis from Stora Fargen were shared. For Ragnerudsjön Lake, mtDNA showed significant differentiation as only 3 of 21 ND2 haplotypes were shared, nDNA haplotypes lacked differentiation as there were only four unique haplotypes from a total of nine closely related haplotypes. Also 3 of 13 ND2 haplotypes and 1 of 18 HSP90 haplotypes from berolinensis, coregoni, gibbera and berolinensis and gibbera morphotypes, respectively, were shared in the Fleesensee. Although we lack sequences of both genes for all of the analyzed specimens, it is obvious from our data that most individuals possessed morphotype-specific combined ND2-HSP90 haplotypes. Genealogical sorting index values (gsi) indicated that morphotypes within all lakes but Ragnerudsjön showed significant lineage divergence (Table 1). Among the three lakes with significant sorting only the HSP90 locus for longispina had a non-significant value.

Morphometric Analyses

The morphometric analysis (of size-free shape variation) mirrored the genetic analysis. Morphometric analyses of dorsal margins (Fig. 2) supported the discreteness of nearly all co-existing morphotypes. Goodall’s F test showed significant differences of the mean carapace shapes relative to within group variances in nine of ten comparisons (Table 1). The sole non-significant value involved the longispina and longiconis morphs from Ragnerudsjön Lake (F: 1.19, p < 0.316).

The CVA plot (Fig. 3) revealed the discreteness of the morphotypes in each lake with the first axis. The second axis showed separation only for the morphotypes of Stora Fargen Lake. The weakest differentiation in mean shapes was found in Ragnerudsjön Lake (Table 1).

Assignment analyses using jackknifed CVA assignments supported the discreteness of most morphotypes as assignment probabilities were greater than 91% in Väner, Stora Fargen and Fleesensee Lakes (Table 1). The degree of differentiation among morphotypes within a lake resulted in 43 correct assignments and 1 incorrect assignment in Väner Lake, 44 correct assignments and 4 incorrect assignments in Stora Fargen Lake, and in 16 correct assignments and 1 incorrect assignment in Fleesensee Lake. The longispina, kessleri and longiconis carapace morphotypes from Ragnerudsjön Lake
could not be effectively discriminated - jackknifed groupings produced 24 correct and 21 incorrect assignments.

**Discussion**

The statistically significant genetic and morphological differentiation of co-existing morphotypes of *Eubosmina* is inconsistent with the hypothesis of simple polymorphisms. Instead, because even very weak gene flow will homogenize coexisting taxa, the evidence supports the existence of reproductive barriers. The nature of the isolating mechanisms for the *Eubosmina* is unknown but taken together with existing paleolimnological, biogeographical, and ecological evidence, our results provide empirical evidence that animals with a mixed sexual and asexual breeding system are susceptible to the fastest of radiations. The diversification rate of *Eubosmina* is on par with another cyclic parthenogen, the pea aphid, that was estimated to have among the fastest diversification rates known for animals at 1 divergence event every 6,700 years [50–52]. Complete genealogical exclusivity is absent [50–52]. In the present case, continuity and coexistence of the morphotypes of *Eubosmina* for thousands of years is well documented in the paleolimnological record of many European lakes. We have shown that significant but not complete genealogical exclusivity is present among sympatric *Eubosmina*. Complete genealogical exclusivity is, of course, unexpected for Holocene-scale differentiation in animals. We observed the expected pattern of genetic differentiation with incompletely sorted lineages [45,46,49,53–55]. Significant genetic and morphological differentiation among co-existing morphotypes is consistent with a reproductive barrier (and difficult to explain by another process). The differentiation in genotype frequencies, morphology, and the continuous sediment record of coexistence in some lakes is undiminished by the expected existence of shared haplotypes. Indeed, denial of the existence of young species based on the presence of shared alleles precludes the study of recent adaptive radiations.

There are however, some lakes in which apparent intermediate morphotypes of *Eubosmina* emerge [17,18,22–24]. In one of the four lakes (Ragnerudsjön), morphometrics failed to clearly assign morphotypes based on carapace shape – and this was the sole lake that lacked significant lineage differentiation (gai) and genetic differentiation. The failure of the analysis in this lake could be due to the presence of intermediate forms between longispina and kessleri in Ragnerudsjön or simply a relatively recent divergence. Most of these “intermediates” are assigned to longicornis morphotype in this
A larger sampling of the genomes is necessary to determine if there is introgression beyond early generation hybrids. Hybridization among cladoceran lineages is well known, and often leads to a markedly reduced clonal diversity in the hybrid lineages compared to the parental lineages [56–58]. This pattern is clearly absent in the current study as coexisting morphotypes share similar haplotype diversity. We note that approximately 75% of the adult individuals in Ragnerudsjön can be distinguished using the shape and the size of antennules and mucros – characters that we excluded because they are used to define most morphotypes. Overall, our geometric morphometric analyses confirm the existence of distinct morphotypes within *Eubosmina* in all four lakes with some intermediates in Ragnerudsjön Lake. We conclude that when antennules and mucro shapes are included, identification according to Lieder’s system [29] can be safely applied.

There are several converging lines of evidence to support the very young age of the *Eubosmina* radiation. The detailed paleolimnological record of morphotype evolution from many lakes agrees on a postglacial origin for all morphotypes except *longispina*. Most morphotypes are restricted to non-refugial lakes that are less than 20,000 years old. Biogeographic evidence and

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**Figure 1. TCS networks of ND2 and HSP haplotypes.** ND2 haplotypes in the left column and the HSP haplotypes in the right column, representing haplotypes of specimens from lakes (from the top to the bottom): Vänern, Stora Färgen, Ragnerudsjön and Fleesensee. Area of circle is proportional to the number of individuals sharing the haplotype. Small, uncolored circles represent missing intermediate haplotypes. Circles with following numbers of individuals sharing the haplotype were suggested as central/ancestral haplotypes (from the top to the bottom): 20, 24, 18, 5 for ND2 and 5 (next 24), 43, 12, 15 for HSP. Colors correspond to different morphotypes: *coregoni*-red; *longispina*-green, *cederstroemi*-violet; *longicornis*-light blue; *kessleri*-yellow; *berolinensis*-dark blue; *gibbera*-pink; and are proportional to the numbers of specimens of the morphotypes sharing one haplotype. Apparent non random-low sharing of haplotypes among/between morphotypes reflects their significant genetic separation in amount expected for young species undergoing speciation.

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the association of morphotypes with cultural eutrophication also support a recent radiation. Finally, the patterns of haplotype divergence found in our study are also consistent with recent lineage formation, sharing is largely restricted to the more common central haplotypes. Further evidence of recent radiation could be provided by coalescent analyses, but the strong possibility of numerous parallel origins of morphotypes that are apparent from some of haplotype networks (Fig. 1) and in paleolimnological records complicates an approach that depends on lineages having the same common ancestor. The increased complexity of our observed networks from more compact starlike networks with one evident central haplotype [45,49] may be attributable to multiple origins of morphotypes.

If radiations in organisms with mixed breeding systems can be rapid, then why are so few known? Most organisms with mixed breeding systems are very small and lack a detailed fossil record. Studies of species diversity, the tempo of evolution, and morphological evolution are inherently difficult for these groups. Still, at least one other group of cyclic parthenogens, aphids, has been shown to undergo a very rapid adaptive radiation as evidenced by modelling bacterial endosymbiont divergence. Other groups with mixed breeding systems, such as protists and rotifers, appear to have many morphologically cryptic lineages, as well as some Cladocera [59–62]. Numerous candidate groups for recent radiations are apparent in Cladocera and Rotifera. The Ponto-Caspian for example, contains two potentially recent species flocks involving cyclic parthenogens from the order Onychopoda [63]. In Daphnia, several closely related forms found in neighbouring or connected waters could be the products of rapid radiations as well [64]. Recent radiations in cyclic parthenogens could have been overlooked because of the use of slowly evolving genetic markers [47,48]. Our study and the recent evidence from other systems challenge the proposals that organisms with mixed breeding systems suffer a reduced speciation capacity in organisms with mixed breeding systems.

are also inconsistent with the antiquity of clades such as the Cladocera. If mixed breeding systems lead to weak speciation potentials, then macroevolutionary processes would likely replace such lineages with strictly sexual lineages [65]. No such breeding system conversion is known in cyclic parthenogens.

We have little direct knowledge of the mechanism of origination. We still know little about the role of hybridization in the radiation or why so many extreme forms exist in the pelagic zone of eutrophic waters. More work is also needed to assess the role of human activities (deforestation and agriculture) on the radiation, particularly as there is an association of lineages to nutrient status and intensity of predation in these lakes. Our study does reveal, however, that even under a presumed worst-case scenario for speciation, with mixed breeding systems and strong vagility and a relatively homogenous limnetic habitat, rapid radiations happen.

Conclusions

We reject the hypothesis that morphotypes of Eubosmina lack morphological and genetic discreteness under sympatry. Instead, the results are consistent with the establishment of Holocene reproductive barriers as predicted by the detailed paleolimnological record. We observed the expected pattern of genetic differentiation with incomplete lineage sorting. We conclude that mixed breeding systems with weak sexual reproduction fail to preclude rapid radiations, but are instead, associated with some of the most rapid radiations known in animals.

Methods

Sample Collection

Bosminid taxonomy remains in a state of flux. We have therefore followed the latest taxonomy of Eubosmina with four species (B. (E.) coregoni, B. (E.) longispina, B. (E.) longicornis, B. (E.) crassicornis) and eleven subspecies (B. (E.) coreoni coregoni, B. (E.) c. gibbera, B. (E.) c. thersites, B. (E.) longispina longispina, B. (E.) l. reflexa, B. (E.) l. ruhei, B. (E.) longicornis longicornis, B. (E.) l. berolinensis, B. (E.) l. cederstroemi, B. (E.) l. kessleri, B. (E.) crassicornis) based on morphological traits [29]. Our study includes seven members out of those eleven subspecies belonging to three species and we use their subspecies names only and treated them as morphotypes.

The studied lakes were: Vanern (Sweden), N 27.5° 47.7′, E 12° 41.4′, with longispina (ls) and coreoni (cor) morphotypes, sampled on June 16, 2004; Stora Fargen (Sweden), N 56° 57.7′, E 13° 20.7′, with longispina, cederstroemi (ced) and longicornis (lc) morphotypes sampled on June 29, 2004; Ragnerudson (Sweden), N 58° 37.6′, E 12° 5.6′, with longispina, kessleri (kes) and longicornis morphotypes sampled on September 25, 2002; Fleesensee (Germany), N53°29′, E 12°28′, with berolinensis (ber), coreoni and gibbera (gibb) morphotypes, sampled on May 8, 2004. These lakes were chosen based on coexistence of morphotypes. We have a record of coexistence of longispina and coreoni morphotypes at our sampling site in Vanern Lake from 2002 and historic samples documenting the coexistence of longispina, cederstroemi and longicornis morphotypes as far back as in 1933 in Lake Stora Fargen. Specimens were preserved in 96% ethanol.

DNA extraction, PCR, sequencing and cloning

We extracted nucleic acid from single adult individuals by incubation in 15–20 μl of Quick-Extract (Epicentre) for 2 hours at 65°C followed by 10 min at 98°C. We performed PCR in a 30 μl total reaction using 3 μl of extract, 3 μl of 10× PCR buffer, 0.6 μl of each of 10 mM dNTPs solution, 0.9 μl of each 10 μM primer and 0.6 unit of Taq polymerase with 1.5 mM MgCl₂. The PCR conditions were 94°C for 30 s, 50°C for 30 s, 72°C for 1 min,
20 sec for 40 cycles, followed by 72°C for 7 min for the complete mitochondrially-encoded NADH dehydrogenase subunit 2 (ND2) gene sequences. The PCR conditions for the nuclear-encoded Heat Shock Protein 90 (HSP90) gene sequences were the same except for annealing time, which was just 1 minute. We designed the following primers: MetR4 (5’-GCTTCAGCTTCG GCCATCCTGTCAG-3’), R1 (5’-AATAAACCTAAGTGAGCGGTCGGCC-3’) for ND2 and HSP-R1 (5’-TCACCAACGTCTTGACGTTCGGTTCC-3’), HSP F2 (5’-ACAAGTTGGACAGTGGCAAGGAGCTG-3’) for HSP90. PCR products were sent for sequencing to Genaissance pharmaceuticals (Connecticut, USA). We cloned apparent heterozygous PCR products with the TOPO TA Cloning Kit for Sequencing (Invitrogen™) to obtain both alleles of HSP90. Clones were sequenced until both alleles could be differentiated. The average number of cloned sequences per one heterozygous PCR product was four.

DNA sequences alignments and analyses
We assembled and edited sequences using Sequencher 4.2. (Gene Codes Corporation) and manually aligned both ND2 and HSP90. Sequences were deposited in Genbank under the following accession numbers GU249620-GU250313.

We have analyzed following numbers of haplotypes and morphotypes on the genes under this study: Lake Vanern: ND2: 33 haplotypes (ls 39, cor 40), HSP: 28 haplotypes (ls 41, cor 40); Lake Stora Färgen ND2: 25 haplotypes (ced 35, ls 26, lc 7), HSP: 22 haplotypes (ced 27, ls 22, lc 7); Lake Ragnerudskjön ND2: 21 haplotypes (ls 19, kes 23, lc 2), HSP: 9 haplotypes (ls 23, kes 26, lc 3); Fleesensee ND2: 13 haplotypes (ber 19, cor 13, gibb 17), HSP: 18 haplotypes (ber 18, cor 2, gibb 18).

We used the TCS 1.21 program [66] to estimate haplotype networks with connections that have a 95% probability of being the most parsimonious. Indels were treated as a fifth character for the putative introns of HSP90 sequences. We calculated genetic differentiation (FST and ΦST values) in ARLEQUIN 3.01 [67], where FST’s are based on the frequencies of haplotypes and ΦST’s are based on the genetic distances among haplotypes. We tested statistical significance by a permutation procedure. To calculate FST’s we pooled all unshared haplotypes into one. We used the genealogical sorting index (gsi) to quantify the genealogical
exclusivity of coexisting morphotypes [68]. The gsi is an index that tracks genealogical differentiation in young species from polyphyly (gsi = 0) to monophyly (gsi = 1). The input trees were calculated using PHYML 3.0 [69] with a GTR substitution model, proportion of invariable sites and among site rate parameters estimated from the data. We used the most complex model but note that neither substitution model choice nor over-parameterization has much effect on analyses with very closely related sequences. Trees were outgroup rooted using specimens from the established sister group, Eubosmina sp. from North America. Significance of the gsi was calculated by permutation (10000 replicates). Because unbalanced comparisons can change the results, we excluded comparisons of the morphotypes with less than seven specimens (see Table 1).

Geometric morphometry
We carried out pairwise geometric morphometric comparisons between coexisting morphotypes (collected from the same planktonic sample) of adult Eubosmina to examine their discreteness based on carapace shape. We compared the shapes of the dorsal margin of the carapace from the point of the upper insertion of the antennules to the posterior point where the carapace opens (Fig. 2). The two endpoints represent homologous landmarks. The antennules and the mucro that are the main structures used to classify most of the morphotypes were not part of the morphometric analyses.

We digitized the outline of the dorsal margins of the carapaces using tpsDig [70] of following numbers of specimens: Lake Vänern: ls 22, cor 22; Lake Stora Färjen ced 22, ls 18, lc 8; Lake Ragnerudsjön: ls 18, kes 21, lc 6; Fleesensee: ber 5, cor 7, gibb 5.

We then aligned the digitized specimens in Chainman [71], reducing the curve to twenty landmarks. The semi-landmark alignment involved a generalized least squares superimposition followed by a distance minimizing procedure to mathematically remove differences between the curves attributable to random positioning of points along the curve [72–74]. We used Goodall’s F-test [75] with p-values determined by 900 random permutations of the data in TwoGroup6h [71,76] to test if different morphs within each lake have statistically significant pairwise differences in the average shape. We calculated Procrustes distances (the standardized measure of the distance between shapes used to e.g. test whether the distance between one pair of samples differs from the distance between another pair of analyzed samples) in TwoGroup6h to test how the magnitudes of differences between the mean forms vary among the morphs when there are meaningful differences in shapes. We also applied Canonical Variates Analysis (CVA) in CVAGen6 [71,76] to determine if we could assign specimens to morphotype based purely on the measured shape of the specimens when considering all morphotypes simultaneously, and we estimated the rate of correct assignments with a jackknife-test of assignments based on the CVA axes. In the jackknife procedure, each specimen is omitted in turn from the calculation of the CV axes, and then assigned to a morphospecies based on those axes. The jackknife rate of correct assignments is a reasonable estimate of how well the CVA would perform in assigning newly acquired specimens to the correct morphotype or morphotype discreteness.

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Author Contributions
Conceived and designed the experiments: MF HDS DJT. Performed the experiments: MF. Analyzed the data: MF VS HDS DJT. Contributed reagents/materials/analysis tools: JES DJT. Wrote the paper: MF VS HDS JES DJT.

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