A systematic review of the land snail *Euglandina singleyana* (Binney, 1892) (Mollusca: Gastropoda: Spiraxidae)

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Abstract.—A systematic review of *Euglandina singleyana* was undertaken to thoroughly examine shell morphology, allozyme, and mtDNA variation in specimens of *E. singleyana* endemic to central Texas. Allozyme similarity ranged from 95% in the most geographically proximal individuals of *E. singleyana* (Val Verde and Real counties) to 82% between the most distant individuals (Comal and Val Verde counties). DNA sequence similarity, based on a 397 bp partial 16S mtDNA sequence, ranged from 98% in eastern specimens (Comal and Kerr counties) to 95% in individuals from Kerr, Real, and Val Verde counties. Analysis of variation in shell morphology, allozyme similarity, and mtDNA sequences supports the existence of a single, highly variable, widespread species of *Euglandina* in central Texas. This study also examines the validity of *E. immemorata*, using morphometric and DNA sequence data and *E. exesa*, using morphometric data. The morphometric analysis showed that *E. immemorata* and *E. singleyana* differ significantly in shape. The current status of *E. immemorata* and *E. exesa* are also examined.

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*Euglandina singleyana* (Binney, 1892) is found in a wide variety of habitats along the southern margin of the Edwards Plateau in Texas, from Terrell County in the west to Fayette County in the east, and south to Refugio County (Fig. 1; Singley 1893, Pilsbry 1946, Fullington & Pratt 1974, Huhrich 1985). In the eastern part of its range it is found under rocks and logs in wooded stream valleys in the limestone of the Edwards Plateau. In the clay and sandy areas of the Balcones Escarpment, it is restricted to wooded lowlands. The range of this species extends to the western Stockton Plateau where it is found under fallen *Yucca* and in rock crevices in desert shrub habitat dominated by *Lechuguilla* cactus (Fullington & Pratt 1974).

A great deal of the historical taxonomic confusion regarding this species appears to be related to its relatively wide geographical range of ~250 km, compared to the median range of land snail distribution of 50 km reported by Solem (1984) and high level of variation in shell morphology. Shells collected in the eastern part of the range can readily be distinguished from those collected in the western part of the range. One purpose of this study was to examine variation in shell morphology of specimens from throughout the range of the species in central Texas to determine if there are distinct differences in western versus eastern shell morphology or if there is continuous (clinal) change in shell shape and size across the range of the species. The purpose of this study was also to examine allozyme and DNA sequence variation in individuals from across the range of the species (Comal, Kerr, Real, and Val Verde counties) to determine if specimens conforming to the description of *E. singleyana* formed a monophyletic group. This study addresses the taxonomy of this species from a phy-
logenetic species concept approach using both monophyly (sequence analysis) and diagnosability (shell morphometrics) criteria (Minton & Lydeard 2003).

The carnivorous land snail genus *Euglandina* has a problematic taxonomic history. Von Martens (1901:47) noted that "many species have been described only from one or a few examples, and not figured. In this genus ... it seems to be very difficult, or rather impossible, to draw a clear line of distinction between local variations and nearly allied species." In addition to these problems, Thompson (1987) also noted that many original descriptions are scattered among nineteenth century journals in several languages and often lack critical details of sculpture of the adult and embryonic shells.

*Euglandina singleyana* from central Texas appears typical of this group in having a history of confusion regarding its identity. Initially, Roemer (1849) identified specimens from New Braunfels as *Glandina truncata* (Gmelin, 1788). Binney & Bland (1869) later considered Texas specimens to be *Glandina corneola* Pfeiffer, 1857. Binney (1892) described and named this Texas snail *Glandina singleyana*. Pilsbry later (1907) noted that in the Binney (1885) monograph, shells from Texas were listed with the name *G. decussata* (Deshayes,
1850), the description and figure were of *G. corneola*, and the locality and anatomical descriptions were those of *G. singleyana*. Singley (1893) listed this species as *G. decussata* var. *singleyana*. Pilsbry (1907; p. 175) removed all Mexican and mainland members of *Glandina* to the genus *Euglandina* due to taxonomic confusion regarding the name and types of *Glandina* and subsequent workers (Pilsbry & Ferriss 1906, Pilsbry 1907, 1946, Fullington & Pratt 1974, Hubricht 1985) have retained this generic name.

*Euglandina immemorata* Pilsbry, 1907 is another species associated with some degree of taxonomic uncertainty. This species was described by Pilsbry (1907) on the basis of two shells from “Texas” (exact locality unknown). Fullington & Pratt (1974) stated that the holotype of *E. immemorata* is not especially distinct from *E. singleyana* but could not be precisely matched by any material they had seen. They concluded that *E. immemorata* is probably *E. singleyana* or, if distinct (based on the improved knowledge of the Texas fauna since 1946) is probably not a Texas species.

*Euglandina exesa* Cockerell, 1930 was described from a single shell found in a limestone deposit in a cinnabar mine at Terlingua, Brewster County, Texas. Fullington & Pratt (1974) state that the holotype does not differ from many western specimens of *E. singleyana*. They consider *E. exesa* to be simply a western range extension of *E. singleyana*.

A systematic review of *E. singleyana* was undertaken to thoroughly examine the observed morphological differences and add further evidence using genetic techniques. This study also tests the conclusions of Fullington & Pratt (1974) by examining the validity of *E. immemorata* using morphometric and DNA sequence data and *E. exesa* using morphometric data.

Materials and Methods

Morphometric analysis.—Programs used for morphometric analysis are part of the Integrated Morphometrics Package (www.canisius.edu/~sheets/morphsoft.html) made available by David Sheets, Miriam Zelditch, and Donald Swiderski. Specimens of *Euglandina singleyana* (64), *E. immemorata* (six—including both type specimens), and *E. exesa* (holotype) were examined (Appendix 1). Twelve landmarks on each shell were digitized from photographs using the program tpsDig version 1.31 (F. J. Rohlf. tpsDig32: Digitize coordinates of landmarks and capture outlines. http://life.bio.sunysb.edu/morph/index.html) (Fig. 2). The landmarks were chosen to be repeat-
able and homologous (Swiderski 1993) across all shells, and most landmarks represent shell sutures (Stone 1998). For each specimen, digitized landmark coordinates were transformed to procrustes distances using partial procrustes superimposition methods. This was carried out in CoordGen6 (H. D. Sheets, Dept. of Physics, Canisius College, 2001 Main St. Buffalo, New York 14208, sheets@gort.canisius.edu). A principal component analysis was performed using partial procrustes distances (PCAGen6c; H. D. Sheets). This program computes partial warp scores for each specimen, using a procrustes mean specimen based on all data for comparison. The principal components (eigenvectors of the covariance matrix) are then calculated based on the covariance matrix derived from the partial warp scores. Landmarks 1 and 12 were designated end points and all specimens were standardized according to this baseline. This transformation to partial warp scores accomplishes standardization so that the principal component analysis examines variation in shape, excluding variation due to scale, rotation, and translation (Swiderski 1993, Stone 1998).

A canonical variates analysis was performed using the program CVAGen6 (H. D. Sheets) to determine the set of axes that allows for the greatest possible ability to discriminate between two or more groups. This program computes partial warp scores with reference to a common mean specimen then performs a multivariate analysis of variance followed by a canonical variates analysis. It determines how many distinct axes there are in the data, ($p = 0.05$) and computes the canonical variates scores of all the specimens. It also uses Mahalanobis distances to assign specimens to one of the groups. The single specimen of *E. exesa* was coded as an unknown and assigned to a group based on the canonical variates axes formed in the prior analysis (CVAGen6; H. D. Sheets). Lastly, a comparison was performed to determine if there was a significant difference in shape between *E. singleyana* and *E. immemorata* (Two-Group6c; H. D. Sheets).

**Allozyme analysis.**—Each collection locality of *Euglandina singleyana* was represented by one or two specimens. A total of seven specimens from five collection localities at ~50 km intervals throughout the geographic range of this species were examined (Appendix 1).

After collection, individuals were held without feeding for 7–10 days, then frozen in cryotubes in liquid nitrogen and stored in an ultracold freezer (−80°C) until analysis. Samples were homogenized in two volumes of distilled water using a glass rod and centrifuged to obtain an aqueous extract. Procedures for cellulose acetate electrophoresis and staining followed those of Hebert & Beaton (1993). Gels were purchased from Helena Laboratories Inc. (Beaumont, Texas), and the buffer used was tris-glycine pH 8.5. To examine variation within *E. singleyana*, scorable data for 19 loci (Table 1) were obtained and analyzed using Tools for Population Genetic Analysis 1.3 (M. P. Miller. Tools for Population Genetic Analysis (TFPGA 1.3): A windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by the author.). To determine genetic similarity, Nei's unbiased genetic identity was calculated (Nei 1978). An unweighted pair group method using arithmetic averages (UPGMA) cluster analysis was then performed using the genetic identity matrix.

**DNA sequence analysis.**—Twelve tissue samples were either preserved in 70% ethanol or frozen in liquid nitrogen and then stored in an ultracold freezer at −80°C. The outgroup taxa chosen were the closest relatives of *Euglandina* with sequences available on GenBank, relationships from Wade et al. (2001). Total genomic DNA was extracted from several milligrams of foot tissue by digestion with lysis buffer and Proteinase K and then purified by phenol: chloroform extraction according to standard procedures (see Palumbi, S., A. Martin, S.
Table 1.—Presumptive enzymatic loci resolved. Tris-glycine (pH 8.5) was used as the buffer system for all of the enzymes listed. All stain and buffer recipes are from Hebert & Beaton (1993).

<table>
<thead>
<tr>
<th>Enzyme system and abbreviation</th>
<th>Enzyme commission number</th>
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<tr>
<td>Adenylate Kinase (ADK)</td>
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<tr>
<td>Alcohol Dehydrogenase (ADH)</td>
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<td>Alcohol Oxidase (AOX)</td>
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</table>

Results and Discussion

Morphometric analysis.—In the examination of shell variation within *Euglandina* *singlyana* the first principal component (PC1) accounted for 67.42% of the variation present in the measurements and PC2 accounted for 9.66% of the variation present. A scatterplot, with specimens grouped by county, comparing the first two principal component axes, does not show any distinct groups within *E. singlyana* (Fig. 3). Shell variation described by principal component 1 appears to be continuous and the results of this analysis do not allow *E. singlyana* to be separated into groups based on shell characteristics.

The canonical variates analysis (Fig. 4) to determine if *E. immemorata* could be distinguished from *E. singlyana* was significant, ($df = 20, p = 3.70685 \times 10^{-5}$) with 100% correct reclassification of both species. A two-group comparison showed that the shapes of the two species are significantly different (Hotelling’s $T^2 f = 6.52$, $df = 24, 45, p = 3.8316 \times 10^{-8}$). This result indicates that *E. immemorata* is morphologically distinct from *E. singlyana*. This re-


Mitochondrial DNA sequences were obtained for an amplified segment of the 16S rDNA gene using 16sar and 16sbr primers (see Palumbi above). Approximately 10 ng of genomic DNA provided templates for double-stranded reactions via the polymerase chain reaction (PCR). PCR reactions were done in a 50 µL solution containing each dNTP at 0.22 µM, each primer at 0.1 µM, 1.5 mM MgCl₂, 1 unit Taq DNA polymerase, and 1X PCR reaction buffer. Reactions were amplified for 30 cycles of 92°C for 45 sec, 50°C for 45 sec, and 68°C for 2 min. Samples were purified and double-stranded DNA provided the template for cycle-sequencing using BigDye (ABI) chemistry followed by analysis on an ABI3100 automated sequencer.

Contigs were assembled in Sequencher® 4.0.5 (Gene Codes Corporation, Ann Arbor, Michigan and aligned by eye using BioEdit (Hall 1999) with reference to secondary structure models to refine the alignment and identify regions corresponding to loops and stems (Lydeard et al. 2000). Sequences were deposited with Genbank (Accession Numbers: AF405235—AF405241, AY149279, AY167887—AY167889). Aligned sequences were analyzed using maximum parsimony with PAUP*4.0b10 (Swofford 2002) using a heuristic search (10 addition replicates). The following options were used: uninformative characters were ignored, only minimal trees were kept, gaps were treated as missing, and zero length branches were collapsed. A bootstrap analysis with 1000 iterations was conducted (Felsenstein 1985). Bremer support values (Bremer 1994) were calculated using the Decay function of MacClade 4.03 (Maddison & Maddison 2000).
result, along with the discovery of specimens of *E. immemorata* in Nuevo Leon, Mexico (Correto-Sandoval 1993), supports the removal of *E. immemorata* from Texas faunal listings.

The unknown assignment test to determine the placement of *E. exesa* placed the single specimen in a cluster formed by *E. singleyana* (Fig. 4); however, this result was not significant (*p* ≥ 0.001). This species is represented by only one specimen. Therefore, this test is not replicable and has little statistical power. It is interesting that the CVA placed the specimen of *E. exesa* within *E. singleyana*, supporting the statements of Fullington & Pratt (1974) about this species. However, without more evidence, nothing conclusive can be stated about the validity of this species.

**Allozyme analysis.**—The results of this study reveal a moderate to high degree of genetic similarity in all specimens of *Euglandina singleyana* examined across its range. Seven of 19 loci (MDH-2, MDHP-2, IDH, LDH, SOD, G6PDH, PGM) were monomorphic for all specimens. The Val Verde County specimens exhibited polymorphisms at nine enzymatic loci (MDH-1, MDHP-1, PGD, AOX, AAT-1, ADK, IDDHH, ADH, HK), the Real County specimens exhibited polymorphism at six (MDH-1, MDHP-1, AOX, HK, NSP, ADH). In the specimens examined, there were no fixed allelic differences among populations of *E. singleyana* across its geographic range.

Allozyme variation appeared to follow a general geographic pattern with specimens having the smallest genetic distance from their most proximal geographic neighbors (Fig. 5). Genetic divergence calculated using Nei’s unbiased genetic identity resulted
in the most western specimens from Val Verde County and the Real County specimens displaying a genetic similarity of 94.5%. The Val Verde+Real cluster was genetically similar to the more centrally located Kerr County specimen at a level of 89.7%. This cluster is 85.2% similar to the most eastern specimen from Comal County.

Perez & Streth (2002) found that specimens of *Euglandina texasiana* (Pfeiffer, 1857) from collection localities 150 km distant, in south Texas and northern Tamaulipas, had a similarity of 94.5%. In comparison, this study observed 94.5% similarity between specimens of *E. singleyana* located 157 km apart, and 85.2% similarity between the most geographically distant specimens (246 km) from Comal and Val Verde Counties. *Euglandina singleyana* was found to display enzyme polymorphism at 12 of 19 loci examined. This level of allozyme variability is more similar to the results of studies on *Helix aspersa* (Selander & Kauffman 1975) and differs from results found in *Liguus* by Hillis et al. (1991) and *Rumina decollata* by Selander & Kaufman (1975), which were notably less variable.

The levels of genetic distance among the widely separated populations of *Euglandina singleyana* are within the range found in other organisms for genetic divergence between subspecies (Quicke 1993). However, there are no fixed differences among the populations and genetic distance is low between geographically proximal populations. This analysis indicates that the specimens of *E. singleyana* examined represent a single species.

**DNA sequence analysis.**—The region of 16S mtDNA that was sequenced resulted in
an aligned data matrix of 397 base pairs of which 191 were phylogenetically informative. Phylogenetic analysis of the data using maximum-parsimony analysis resulted in three equally parsimonious trees of 480 steps (CI = 0.8646, gI = −2.024336). These three trees differed only in the placement of the Comal County (eastern) specimen relative to the Kerr County specimen. One topology places the Kerr County specimen (central) sister to the Real+Devil's River clade (Central & Western). An alternate topology places the Comal County specimen in this position, and the final topology describes Comal and Kerr County specimens as each others' closest relative. Bootstrap analysis (1000 pseudoreplicates) of the aligned data matrix using maximum-parsimony produced the tree shown in Fig. 6. *Euglandina singleyana* forms a monophyletic group. The sister taxon to *E. singleyana* is the specimen of *Euglandina* from Northern Coahuila. Also outside this grouping is *E. corneola* from Tamaulipas, Mexico. Pairwise sequence identity was calculated for each clade. Within the Val Verde County cluster (Devil's River and Comstock) there is 98–100% sequence similarity. The two Real County specimens had identical 16S sequences (=100% similarity). Sequence similarity between Kerr County and Comal County was 97%. All possible combinations of pairwise comparisons were performed with a minimum similarity of 95% among specimens from Kerr and Real County. The two specimens of *E. immemorata* formed a group apparently not closely related to *E. singleyana*.

Both allozyme and sequence analyses show an interesting geographic pattern with populations most closely related to their geographically proximal neighbors. This pattern of strong geographic structuring is often seen in land snail species (Thomaz et al. 1996, Schilthuizen et al. 1999). Thomaz et al. (1996) examined geographic variation within *Cepea nemoralis* and *Helix aspersa* and found very high levels of sequence divergence (12%) within these species of land snails. The authors conclude that the most likely explanation for the observed levels of divergence is the population structure of land snails with low dispersal and large populations divided into infrequently interacting demes.

Avise et al. (1987) presents several tests of this hypothesis, one of which is: phylogenetic differentiation between long separated demes should be reflected in nuclear as well as mitochondrial assays. The congruence of the allozyme and mitochondrial data in the present study appear to fulfill
this requirement, lending credence to the idea that the specimens of *E. singleyana* examined in this study represent a single species. The present study does not have enough samples to conclusively examine geographic partitioning within this species; however, there does appear to be some geographic structure in the data.

Although sample sizes were low, they appear to be adequate to address the questions posed in this research. *Euglandina singleyana* is considered to be an uncommon species (Singley 1893; Neck 1984, 1988), and the area where they are found is rapidly being disturbed ecologically due to a growing human population. As a result of their very specialized feeding habits, rarity, habitat preferences, and human activities,
the live specimens examined during this study represent a significant collection of living specimens of *Euglandina singleyana*.

**Conclusions**

All of the analyses, including morphometric, allozyme, and mtDNA sequences, support the premise that *Euglandina singleyana* is a single, widespread, highly variable species. Both allozyme and DNA sequence results indicate that there are some detectable genetic geographic patterns within *E. singleyana*, as well as the observable morphological gradient across the range of this species. The geographically most distant specimens are the most genetically dissimilar and geographically proximal specimens are more similar.

The results of the morphometric and sequence analyses indicate that *E. immemorata* is distinct from *E. singleyana*. This result, combined with the recent discovery of specimens of *E. immemorata* from Nuevo Leon, Mexico leads to the conclusion that *E. immemorata* should be removed from Texas faunal listings. The taxonomic placement of *Euglandina exesa* could not be definitively determined.

**Acknowledgments**

We thank the following for assistance with collections and research: Dr. Robert Dowler, Dr. J. Kelly McCoy, Joel Brant, Jim Campbell, Dr. Brad Henry, Jerry F. Husak, Shailaja Marion, Mark Kitson, Jochen Gerber, Gene Hall, Jeanne Serb, Dr. Russ Minton, Dr. Charles Lydeard, Lynn McCutchen, and Bob Howells. Dr. Alfonso Correo-Sandoval was invaluable in obtaining specimens and permits in Mexico, Permit #NOM-126-ECOL-2001. We also thank Texas Parks and Wildlife Department for permits and access to Devils River State Natural Area. An NSF equipment grant at the University of Alabama (DBI-0070351) contributed to part of this research. Support was provided by an NSF-IGERT fellowship in the Freshwater Sciences program (DGE-9972810) and University of Alabama, Ecology & Systematics Enhancement fellowship. Thanks are also due the Beta Beta Beta association for two research awards that contributed to part of this research.

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Pfeiffer, von L. 1857. Novitates conchologicae. 8. p. 82, pl. xxii, fig. 11, 12.


Strecker, J. K. 1935. Land and fresh-water snails of Texas.—Transactions of the Texas Academy of Science 17:4–50.


Appendix 1

Material Examined and Distribution

Morphometric Analysis

Euglandina singleyana (64): The number of specimens of each lot that were used follows the museum catalog number in parenthesis (all undamaged shells with 5 or more whorls were used). Field Museum of Natural History: 29 specimens: FMNH 22347 (1),
30850 (2), 36309 (5), 50011 (4), 58295 (3), 62416 (6),
74849 (1), 78560 (2), 98199 (2), 109097 (1), and
175899 (1). The Academy of Natural Sciences of Phila-
delphia: 22 specimens: ANSP 186729 (1), 186731
(1), 346372 (3), 84622 (2), 104753 (1), 4312 (1),
84637 (1), 158379 (1), 134180 (4), 76849 (3), 150798
(1), 76837 (1) topotype, 186733 (1), and 87425 (1).
Angelo State Natural History Collection: 13 speci-
mens, individually numbered: (ASNHC 0008–0014,
042, 044–048).

*Euglandina immemorata* (6): FMNH 11777 (1),
4359 (1) locality unknown, types; Florida Museum of
Natural History 189621 (1), Nuevo Leon, Mexico,
Santiago, 1 km. N of Laguna de Sanchez. University
of Alabama Gastropod Collection, 3 specimens, indi-
vidually numbered: 632–634, Nuevo Leon, Mexico
25°23′00.9″N; 100°14′28.6″W, 1 km N of Lag-
una de Sanchez.

Allozyme Analysis

*Euglandina singleyana* (7): Specimens used in this
analysis are deposited in the Strecker Museum of Nat-
ural History, (SMNH) Baylor University (Accession
Number 2001-A-1-1; Catalog Numbers SM32439-
SM32446). (1) Landa Park, New Braunfels, Comal
County, Texas; (1) Kerrville, Kerr County, Texas; (2)
9 miles N of Leakey, Real County, Texas; (2) Devil’s
River State Natural Area (DRSNA), Val Verde County,
Texas; (1) Comstock, Val Verde County, Texas. *Rad-
dottus alternatus* from Val Verde County was selected
as an outgroup.

DNA Sequence Analysis

The same specimens listed in the above section on
allozyme analysis provided the tissue samples which
were used in the sequence analysis. Additionally in-
cluded were a specimen of an unidentified *Euglandina*
from La Cuesta in Northern Coahuila; *Euglandina cor-
neola* from 4 miles SW of Mante, Tamaulipas, Mexico;
2 specimens of *E. immemorata* from Nuevo Leon,
Mexico 25°23′00.9″N; 100°14′28.6″W, 1 km N of La-
guna de Sanchez; and one specimen of *E. rosea* from
Lake County, Florida 28°30′53″N; 81°44′15″W.

*Euhadra amaliae* (AF098712), *Phaeohelix phaeo-
gramma* (AF098714), and *Nesiohelix omphalina*
(AF098713) sequences from Chiba (1999) were used
as outgroups.

Distribution

Strecker (1935) listed *Euglandina singleyana* as oc-
curring in Bexar, Caldwell, Comal, Goliad, Hays,
Travis, Victoria, Atascosa, Frio, Gonzales, Guadalupe,
and Wilson counties. McGee (1971) added Bandera,
Fayette, Kendall, Kerr, Kinney, Val Verde, Medina,
and Real counties. Cheatum et al. (1972) added Terrell
County to the distribution. Collections by the authors
and examinations of museum specimens from the
Academy of Natural Sciences in Philadelphia and the
Field Museum of Natural History during this study add
Uvalde, Kimble, Chambers, Coryell, and Blanco coun-
ties to the known distribution (Fig. 1).

*County records.*—FMNH 78560, Horse Creek, Cor-
yell Co., Texas; FMNH 98199, Galveston, Chambers
Co., Texas; FMNH 210, 103/1, River Drift, Pedernales
Falls State Park, Blanco Co., Texas; ANSP 186731,
Garner State Park, Uvalde Co., Texas; ANSP 186727,
Roadside Park, Nueces River W of Uvalde, Uvalde
Co., Texas; ANSP 186733, Llano River, Highway 29,
12 miles S of Junction, Kimble Co., Texas.