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DETECTING A COMPLEX OF CRYPTIC SPECIES WITHIN *NEOECHINORHYNCHUS GOLVANI* (ACANTHOCEPHALA: NEOECHINORHYNCHIDAE) INFERRED FROM ITSs AND LSU rDNA GENE SEQUENCES

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ABSTRACT: *Neoechinorhynchus golvani* is an intestinal parasite of freshwater and brackish water fishes distributed in Mexico. The genetic variability of 40 samples representing 12 populations from north, south, and central Mexico, and 1 from Costa Rica, was estimated by sequencing 2 nuclear genes (ITS1, 5.8S, ITS2, and LSU rDNA, including the domain D2 + D3). The length of both genes ranged from 700 to 779 base pairs (bp) and from 813 to 821 bp, for ITSs and LSU, respectively. The genetic divergence among populations ranged from 19.5 to 35.3% with ITSs and from 9.28 to 19.58% with LSU. Maximum likelihood and maximum parsimony analyses were performed for each data set and also for 2 combined data sets (ITSs + LSU rDNA with and without outgroups), showing strong similarities among trees, with high bootstrap support in all cases. Genetic divergence, in combination with phylogenetic analyses, suggested that the acanthocephalan *N. golvani* represents a complex of cryptic species, which is composed of at least 3 lineages. The first lineage, corresponding with *N. golvani*, shows a wide distribution, including localities from northeastern Mexico, southwards through central and southeastern Mexico, and further down to Costa Rica. This lineage is associated with cichlid fishes in strictly freshwater environments. Lineages 2 and 3 are distributed in brackish water systems along the Gulf of Mexico and Pacific slopes, respectively; both are associated with eleotrid fishes, and apparently represent 2 cryptic species. The diversification of the eleotrid and cichlid lineages seems to be the result of independent host-switching events from the ancestral population.

Neoechinorhynchus Stiles and Hassall, 1905 is 1 of the most speciose genera within Acanthocephala, and it is classified into 2 subgenera based on egg anatomy, i.e., *Hebesoma* Van Cleave, 1928 and *Neoechinorhynchus* (Amin, 2002). Variation and combination of morphological traits such as proboscis shape, number of hooks, proboscis receptacle shape, testes shape and location, kind of cement gland, and egg shape with or without prolongations has been traditionally used to diagnose and delimit the 92 congeneric species (Aho et al., 1992; Amin, 2002; Amin et al., 2003; Barger et al., 2004; Barger and Nickol, 2004; Amin and Christison, 2005; Mikhailova and Atrashkevich, 2008). Additionally, species of *Neoechinorhynchus* exhibit an indirect life cycle, involving ostracods as intermediate hosts, and either marine, freshwater, and brackish water fish, or freshwater turtles and frogs, as definitive hosts, and occur mostly in the Nearctic region (Bullock, 1970; Schmidt, 1985; Kennedy, 2006). However, some species have been found in South America, Europe, India, Asia, and Australia (see Bullock, 1970). In Mexico, only 4 species of *Neoechinorhynchus* have been described, mostly from the Neotropical region; 2 of these species occur in freshwater turtles, i.e., *Neoechinorhynchus schmidti* Barger, Thatcher and Nickol, 2004 and *Neoechinorhynchus emyditoides* Fisher, 1960, and 2 other species occur in brackish and freshwater water fishes, i.e., *Neoechinorhynchus roseum* Salgado-Maldonado, 1978 and *Neoechinorhynchus golvani*, Salgado-Maldonado, 1978, respectively.

Neoechinorhynchus golvani is widely distributed in central and southern Mexico, where is primarily associated with cichlid fishes (Vidal-Martínez et al., 2001; Salgado-Maldonado, 2006), and sporadically in 10 other families of fishes (Pérez-Ponce de León et al., 1996; Salgado-Maldonado, 2006; Violante-González et al., 2007). Additionally, even though specimens of *N. golvani* show some morphological variation in terms of body size, several other diagnostic characters (Salgado-Maldonado, 1978) are exhibited by individuals from all populations irrespective of body size. Moreover, the geographic distribution of *N. golvani* is wide and fragmented, and therefore it is possible their populations may show genetic variation due to isolation and suppression of gene flow.

The main objective of the present research was to estimate the genetic divergence among some populations of *N. golvani* by using 2 nuclear genes, ITS1, 5.8S, ITS2 (ITSs), and the large subunit (LSU) of ribosomal DNA, including the D2 + D3 domains, as molecular markers, and to test for the possible presence of cryptic species.

MATERIALS AND METHODS

Specimens and DNA isolation

Adult acanthocephalans were collected from the intestines of their definitive hosts in 12 localities of Mexico and 1 in Costa Rica (Table I; Fig. 1). Worms were washed 3 times in 0.9% (w/v) saline, preserved in absolute ethanol, and stored at 4 C. For taxonomic identification, some specimens were stained with Mayer's paracarmine, cleared with methyl salicylate, and mounted on permanent slides with Canada balsam. The acanthocephalans were identified by conventional morphological criteria following keys of Amin (2002) and were allocated to the species *N. golvani*. In addition, original and revised descriptions of the species (Salgado-Maldonado, 1978; Barger et al., 2004) were consulted as needed. Voucher specimens were deposited at the Colección Nacional de Helmintos (CNHE), Instituto de Biología, UNAM, Mexico City, México (Table I).

Morphometry

For morphometry, 33 acanthocephalans from 9 populations were examined. Body length and width; proboscis length and width; as well as the length and width of anterior, middle, and posterior hooks of proboscis were taken following Petrochenko (1956). Each hook was measured from the tip to the base according with Mikhailova and Atrashkevich (2008). A total of 10 characters were considered. Descriptive univariate statistics (mean values, standard deviations, range) for all variables were calculated and Student's *t*-tests ($P < 0.005$) were used to test the equality of means for each variable. Statistical analyses were performed with the use of Microsoft Excel and PAST version 1.90 (Hammer et al., 2001).

Amplification and sequencing of DNA

Several specimens from each population were digested overnight at 56 C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl,

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TABLE I. Specimen information. Sample number, specimens analyzed (S), host species, collection sites (CS), locality name, geographical coordinates, GenBank accession number, and catalog number (CNHE) for specimens studied in this work. Sequences marked with an asterisk were obtained in this study. Family of freshwater and brackish fishes host of *Neoechinorhynchus golvani* (C) = Cichlidae; (E) = Eleotridae; Nd = not determined. The sample number for each locality corresponds with the same numbers as in Figures 2 and 3.

Sample no.	S	Host	CS	Locality	Coordinates		GenBank accession no.		Vouchers (CNHE)
					North	West	ITSs	LSU	
1–3	3	*Cichlasoma urophthalmus* (C)	1	Carrizal River, Tabasco	18°1′45″	92°55′00″	FJ388974* FJ968121* FJ968122*	FJ388993* FJ968134* FJ968135*	6754
4–7	4	*Vieja pearsei* (C)	2	Chicoasen Dam, Chiapas	16°56′02″	93°05′16″	FJ388976* FJ968118* FJ968119* FJ968120*	FJ388995* FJ968136* FJ968137* FJ968138*	6755
8–10	3	*V. pearsei* (C)	3	Nezahualcoyolt Dam, Malpaso, Chiapas	17°10′49″	93°36′49″	FJ388977* FJ968130* FJ968131*	FJ388996* FJ968141* FJ968142*	6756
11–13	3	*Parachromis friedrichhstalii* (C)	4	Canitzan Lake, Tenosique, Tabasco	17°28′57″	91°25′27″	FJ388975* FJ968126* FJ968127*	FJ388994* FJ968139* FJ968140*	6757
14–16	3	*C. urophthalmus* (C)	5	Las Ilusiones Lake, Tabasco	17°59′46″	92°56′17″	FJ388973* FJ968128* FJ968129*	FJ388992* FJ968143* FJ968144*	
17–19	3	*V. fenestrata* (C)	6	Catemaco Lake, Veracruz	18°25′	95°07′	FJ388967* FJ968112* FJ968113*	FJ388986* FJ968145* FJ968146*	601, 603, 604, 606, 631, 632
20	1	*Amatitlania nigrofasciata* (C)	7	Quebrada Puercos, Santa Rosa, Costa Rica	10°51′	85°34′	FJ388979*	FJ388998*	6757
21–23	3	*Herichthys cyanoguttatus* (C)	8	Axtlan de Terrazas, San Luis Potosí	21°26′1″	98°52′28″	FJ388983* FJ968132* FJ968133*	FJ389002* FJ968147* FJ968148*	6758
24–28	5	*C. urophthalmus* (C)	9	Papaloapan River, Tlacotalpan, Veracruz	18°36′	95°39′	FJ388968* FJ388969* FJ968123* FJ968124* FJ968125*	FJ388987* FJ388988* FJ968149* FJ968150* FJ968151*	6759
29–33	5	*Dormitator maculatus* (E)	10	Alvarado Lagoon, Veracruz	18°45′	95°45′	FJ388966* FJ968108* FJ968109* FJ968110* FJ968111*	FJ388985* FJ968152* FJ968153* FJ968154* FJ968155*	6760
34–38	5	*D. latifrons* (E)	11	Tres Palos Lagoon, Guerrero	16°47′47″	99°44′30″	FJ388972* FJ968114* FJ968115* FJ968116* FJ968117*	FJ388991* FJ968156* FJ968157* FJ968158* FJ968159*	4347–4348
39	1	*D. latifrons* (E)	12	Chamela Estuary, Jalisco	19°31′20″	104°04′53″	FJ388971*	FJ388990*	
40	1	*D. latifrons* (E)	13	Cuitzmala River, Jalisco	19°23′27″	104°58′28″	FJ388970*	FJ388989*	
Neoechinorhynchus roseum 1	1	*Achiurus mazatlanus*		El Caimanero Estuary, Sinaloa	25°36′30″	108°26′25″	FJ388980*	FJ388999*	6762
N. roseum 2	1	*Citharichthys gilbertei*		La Tovara Estuary, Nayarit	21°31′37″	105°14′29″	FJ388981*	FJ389000*	6763
N. schmidti	1	*Trachemys s. venusta*		Pantanos de Centla, Tabasco	18°28′18.9″	92°39′14.9″	FJ388982*	FJ389001*	6764
N. saginatus	1	Nd		Nd	Nd	Nd	FJ388984*	AY829091	

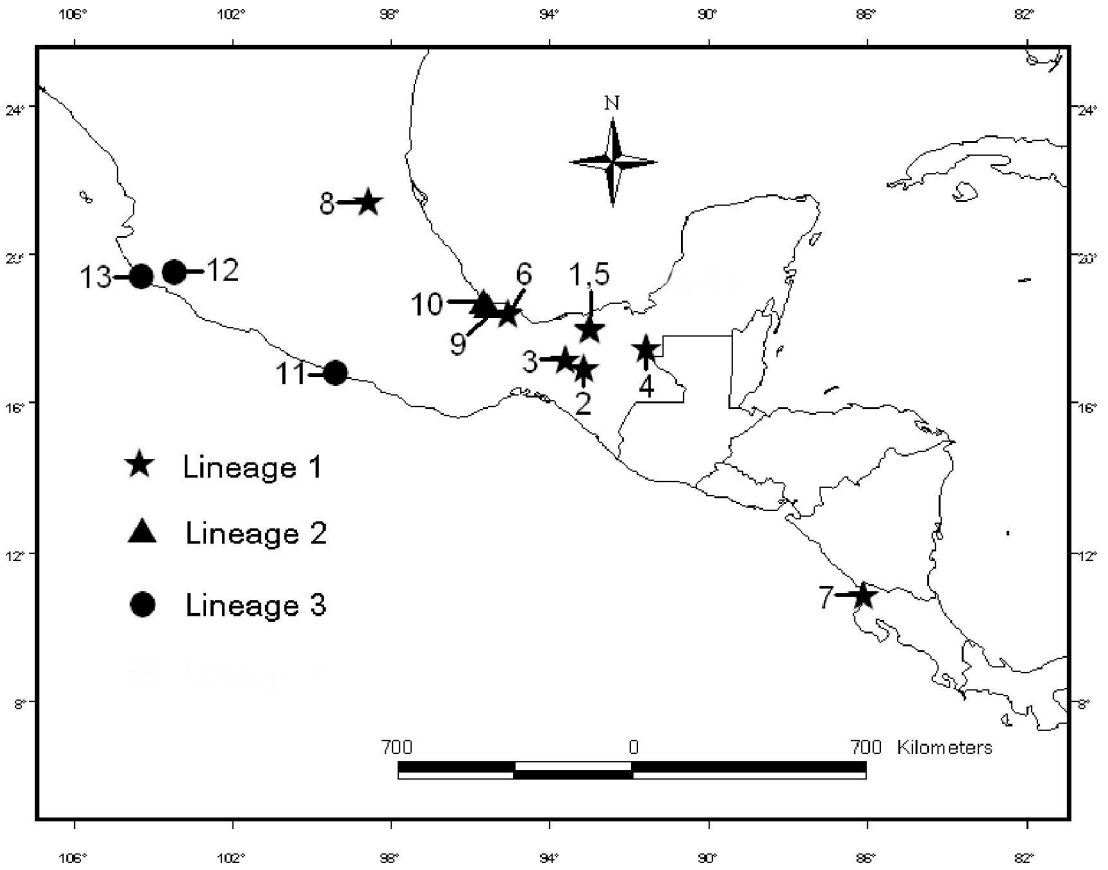

FIGURE 1. Sampling sites of specimens of *Neoechinorhynchus golvani* in Mexico and Costa Rica. Symbols correspond to the lineages uncovered through phylogenetic analyses as shown in Figures 2 and 3. Collection sites are numbered according to Table I.

100 mM Na_2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml Proteinase K. Following digestion, DNA was extracted from the supernatant with the use of the DNAzol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions.

Two regions of nuclear ribosomal DNA (rDNA) were amplified with the use of the polymerase chain reaction (PCR). The ITS1, 5.8S, and ITS2 (ITSs; ~800 bp) were amplified with the use of the forward primer 5′-GTCGTAACAAGGTTTCCGT-3′ and reverse primer 5′-ACCCGCTGAATTTAAGCATA-3′ (Luton et al., 1992). The domains D2 + D3 (~900 bp) of LSU rDNA were amplified using the forward primer 5′-CAAGTACCGTGAGGGAAAGTTGC-3′ and reverse primer 5′-GTCGATAGGACTCCCTTTG-3′ (García-Varela and Nadler, 2005). PCR reactions (25 µl) consisted of 10 µM of each primer, 2.5 µl of 10× buffer, 1.5 µl of 15 mM of $MgCl_2$, and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters for rDNA amplifications included denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 1 min, annealing at 50 C for 1 min, and extension at 72 C for 1 min, followed by a postamplification incubation at 72 C for 10 min.

Each PCR product was purified with the use of Millipore columns (Amicon, Billerica, Massachusetts). Purified products were cloned by ligation into pGEM-T vector (Promega, Madison, Wisconsin) and used to transform competent *Escherichia coli* (JM109). Positive clones were identified by blue/white selection, and clone (insert) size was confirmed by PCR of DNA extracts prepared from bacterial (clone) colonies. Liquid cultures for minipreps were grown in Luria broth containing 50 µg/ml of ampicillin. Plasmids for DNA sequencing were prepared with the use of commercial miniprep kits (Qiaprep, Qiagen, Valencia, California). Plasmids were sequenced for both DNA strands with the use of universal (vector) and internal primers. Sequencing reactions were performed with the use of ABI Big Dye (PE Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry, and reaction products were separated and detected with the use of an ABI 310 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved with the use of Codoncode Aligner version 1.4.5 (Codoncode Corporation, Dedham, Massachusetts). All sequences have been deposited in the Genbank database (accession numbers in Table I).

Alignments and phylogenetic analyses

The ITSs and LSU data sets were aligned separately with the use of the software ProAlign version 0.5 (Loytynoja and Milinkovitch, 2003). For each alignment, a ProAlign guide tree was constructed with the use of corrected (for multiple hits) pairwise distances; this guide tree was used to estimate the hidden Markov model parameters (δ and ε) for progressive multiple alignment. Program (Java) memory and bandwidth were increased as required to complete the alignment. The minimum posterior probability of sites was used as the criterion for detecting and removing unreliably aligned sequences. To reduce the likelihood of excluding correctly aligned sites, the filter threshold was set to 60% minimum posterior probability. For the ITSs sequences, using ProAlign to detect and remove unreliably aligned sequences by their posterior probabilities excluded 644 of 991 sites. For the LSU data set, 161 of 842 sites were excluded based on posterior probability filtering. These (ITSs + LSU) combined data sets included 1,028 characters following removal of unreliably aligned sites. The initial alignment showed many ambiguous positional homology statements, particularly between the ingroups and outgroups. This problem was addressed by removing the outgroups from the ITSs and LSU data sets. For the ITSs data sets, 244 of 771 sites were excluded, and for the LSU data set, 27 of 830 sites were excluded based on a posterior probability filtering with the software ProAlign. A second combined (ITSs + LSU) alignment that contained only in-group taxa (40 samples of *N. golvani*), form a data set of 1, 330 characters. The ITSs and LSU rDNA filtered alignments were analyzed independently and as a combined (ITSs + LSU) data set. Likewise, other combined (ITSs + LSU) data sets that include only 40 samples of *N. golvani* were also analyzed independently. Tree searches were conducted with the optimality criteria

TABLE II. Tree statistics for rDNA data sets. Combined (internal transcribed spacers [ITSs] + large-subunit [LSU]) data sets. Number of informative characters, consistency index, and tree length refer to parsimony inference. Pinv (proportion of invariable sites), Gd (shape of gamma distribution), and −ln likelihood refer to maximum likelihood inference.

Data set	ITSs + LSU	ITSs + LSU (only ingroup)	LSU	ITSs
Total characters	1,028	1,330	681	347
Uninformative characters	39	16	18	21
Constant characters	595	916	406	189
Informative characters	394	398	257	137
Consistency index	0.83	0.93	0.84	0.84
Tree length	740	509	469	269
−ln likelihood	4701.54605	4227.33009	2985.05872	1658.14293
Pinv	0.5000	0	0.5325	0.4368
Gd	Equal	0.6111	Equal	Equal

of maximum parsimony (MP) and maximum likelihood (ML) with the use of PAUP* 4.0b10 software (Swofford, 2002). For ML analyses, the Akaike Information Criterion (AIC) was used to assess the fit of GTR (general time reversible) nucleotide substitution models (Rodríguez et al., 1990) as implemented with the use of Modeltest version 3.0 (Posada and Crandall, 1998). The best ML model for each data set (ITSs and LSU, with and without outgroups) was used for likelihood analysis (Table II). For each data set, the best model with invariable sites (+I), and rate heterogeneity (+G; Yang, 1994) was used, but the estimated parameters varied by data set (Table II). Tree searches were performed with the use of 50 (ML) and 1,000 (MP) random addition heuristic searches with tree–bisection–reconnection (TBR) branch swapping. The support of the clades was assessed by bootstrap resampling, with 10,000 (MP) or 1,000 (ML) bootstrap replicates with the software PAUP* 4.0b10 (Swofford, 2002). Trees were drawn with the use of RETREE and DRAWGRAM from PHYLIP (Felsenstein, 1999). The genetic divergence among population and between congeneric species was estimated with the use of *p* distances with the program *MEGA* version 4 (Tamura et al., 2007). Congeneric species, i.e., *N. roseum*, *N. schmidti*, and *Neoechinorhynchus saginatus* Van Cleave and Bangham, 1949, were chosen as outgroups to conduct ML and MP analyses.

RESULTS

Base composition and genetic divergence

DNA fragments of the ITSs and LSU were amplified, cloned, and sequenced for 40 samples of *N. golvani* and 4 samples representing 3 congeneric species (2 of *N. roseum*, 1 of *N. schmidti*, and 1 of *N. saginatus*), which were used as outgroups. Length of the PCR products among *N. golvani* ranged from 700 to 749 bp and from 813 to 821 bp for ITSs and LSU, respectively, and among congeneric species ranged from 728 to 987 bp and 766 to 818 bp for ITSs and LSU, respectively.

Nucleotide frequencies for the combined (ITSs + LSU) data set that includes outgroups were 0.2656 (A), 0.1769 (C), 0.2802 (G), and 0.2771 (T). The heterogeneity of nucleotide frequencies across taxa was tested with the use of the "basefreq" option implemented in PAUP* ($X^2 = 23.4596$, $P = 1$). This result indicates that rDNA nucleotide frequencies were not significantly heterogeneous across taxa, which is advantageous because MP and ML inference methods perform optimally when nucleotide frequencies are homogeneous (Omilian and Taylor, 2001). Total lengths of the alignments and number of constant and parsimony-informative characters for the ITSs, LSU, and combined (ITSs + LSU) data sets are provided in Table II. The genetic divergence among populations from 3 major clades of *N. golvani* ranged from 9.28 to 19.58% with LSU, from 19.5 to 35.3% with ITSs (Table III), and from 26.9 to 31.6% with LSU and from 40.1 to 45.2% with ITSs among congeners (*N. roseum*, *N. schmidti*, and *N. saginatus*).

TABLE III. Average of genetic divergence of the 3 major clades (lineages) and intraclade detected within *Neoechinorhynchus golvani*. Internal transcribed spacers (ITSs; upper matrix) and large-subunit (LSU; lower matrix). Uncorrected *p* distances are expressed as percentages.

ITSs/LSU	Lineage 1	Lineage 2	Lineage 3	Intraclade ITSs	Intraclade LSU
Lineage 1	—	35.3	34.7	2.1	1.6
Lineage 2	19.49	—	19.5	0.31	0.21
Lineage 3	19.58	9.28	—	0.2	0.1

Combined ITSs + LSU data set

The maximum likelihood tree inferred from the combined (ITSs + LSU) data set that includes 40 samples of *N. golvani* plus 3 congeneric species, yielded 1 tree with −ln of 4701.54605. In this tree, 3 major clades are shown (Fig. 2A). The first clade was composed of 8 populations with 23 specimens of *N. golvani* that exhibit a wide geographic distribution. All these populations are found exclusively in cichlids, including a population from Catemaco, Veracruz (sample nos. 17–19), the type location. Clades 2 and 3 are composed of 10 and 7 specimens from populations distributed along the Gulf of Mexico and Pacific slopes, respectively, and are associated with cichlid and eleotrid fishes (Fig. 2A). The 3 major clades exhibited a relatively high bootstrap support value, ranging from 99 to 100%. Maximum parsimony analysis of the combined ITSs + LSU data set (Fig. 2B) yielded 5 trees with a CI = 0.83 and length of 740 steps (Table II). The strict consensus of 5 trees yielded the same general topology as the ML, with high bootstrap support (100%). The few topological differences between ML and MP trees involved some populations within clades with very short branches as inferred by ML and low bootstrap support (Fig. 2A, B). The second combined (ITSs + LSU) data set included 40 samples of *N. golvani* and 1,330 characters. This alignment was analyzed to explore the effect of including more characters, in this case those that are alignable among the in-group species, but are problematic when the out-group taxa are added due their high divergence. The maximum likelihood tree inferred from this combined data set (ITSs + LSU and no outgroups) yielded 1 tree with a −ln of 4227.33009 (Fig. 3A). This tree also exhibited the same general topologies as ML and MP trees inferred from a combined data set that includes the outgroups (Fig. 2A, B). The nodes of the 3 major clades received high bootstrap support (100%). Maximum parsimony analysis of the combined ITSs + LSU data set without outgroups (Fig. 3B) yielded 12 trees with a CI = 0.93 and length of 509 steps (Table II). The strict consensus of 12 trees yielded the same general topology as the ML tree (Fig. 3A).

LSU data set

Maximum likelihood analysis of the LSU data set (including the outgroups) yielded a tree with a −ln of 2985.05872. This tree had a considerable similarity to the MP tree inferred from the

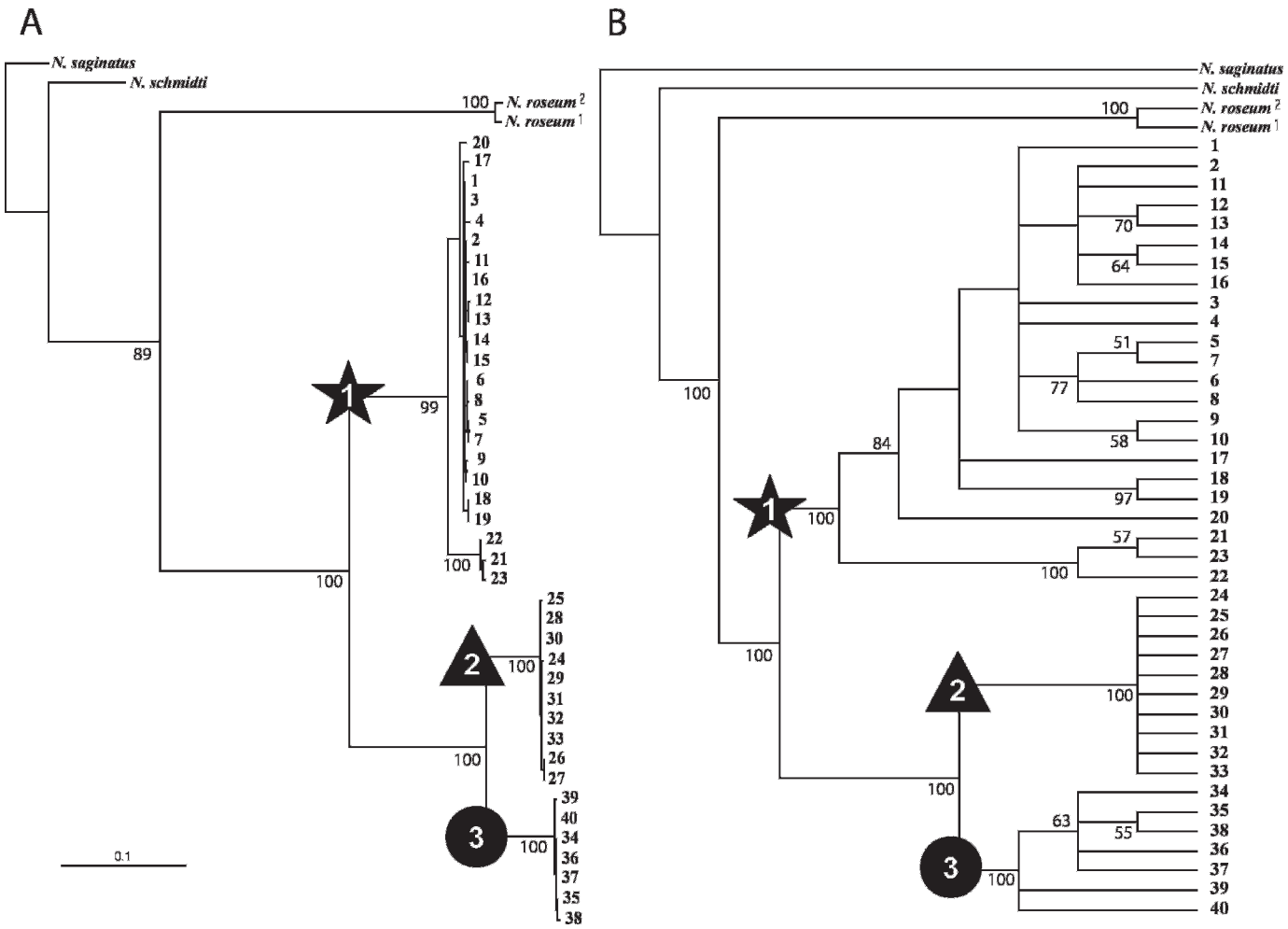

FIGURE 2. Trees recovered from analyses of the combined ITSs + LSU rDNA data set conformed by 40 samples of *Neoechinorhnychus golvani* plus 3 out-group species (1,028 characters). (**A**) Maximum likelihood (ML) tree (−ln likelihood of 4701.54605) obtained from heuristic search with branch lengths scaled to the expected number of substitutions per site. Numbers near internal nodes show ML bootstrap clade frequencies. (**B**) Strict consensus of 5 equally parsimonious trees (740 steps) inferred from heuristic MP analysis. Numbers below internal nodes show maximum parsimony bootstrap clade frequencies. The 3 major lineages discussed in the text are indicated on the trees: Lineages 1 (★), 2 (▲), and 3 (●).

combined data set (with and without outgroups). The 3 major clades received strong bootstrap support (from 87 to 100%). The MP analysis of this data set yielded 150 trees with CI = 0.84 and a length of 469 steps. The strict consensus of 150 trees yielded the same general topology as ML tree inferred from LSU data set alone and the combination of both (ITSs + LSU) data sets with and without outgroups (trees not shown).

ITSs data set

Maximum likelihood analysis of the ITSs data set (including the outgroups) yielded a single tree with a −ln of 1658.14293. This tree produced almost the same topology inferred with ML and MP trees from combined data sets (with and without outgroups) and the LSU data set alone. The MP analysis of this data set yielded 6 trees with CI = 0.84 and a length of 269 steps. The strict consensus of 6 trees revealed the same general topology as the MP trees inferred from LSU data set alone and the combination of both (ITSs + LSU) data sets with and without outgroups (trees not shown).

DISCUSSION

The genetic divergence estimated among populations of *N. golvani* analyzed in the current study ranged from 19.5 to 35.3% by ITSs. This molecular marker was previously used to separate populations of other acanthocephalan species such as *Pomphorhynchus leavis* Müller 1776, and *Leptorhynchoides thecathus* Linton, 1891, which showed a genetic divergence of 20% and between 1 and 8.7%, respectively (Perrot-Minnot, 2004; Steinauer et al., 2007). In the present study, a second nuclear gene (LSU) was used for the first time as molecular marker to determine differences/similarities among populations of acanthocephalans. We hoped that it would also be useful recognizing the presence of cryptic species. The genetic distances estimated for each gene, the reciprocal monophyly of the populations in the phylogenetic trees inferred from each data set, and the combined data set (ITSs + LSU) with and without outgroups, with the use of both ML and MP methods, clearly demonstrate the existence of a complex of cryptic species in *N. golvani*. This complex is composed of at least 3 lineages with evolutionary

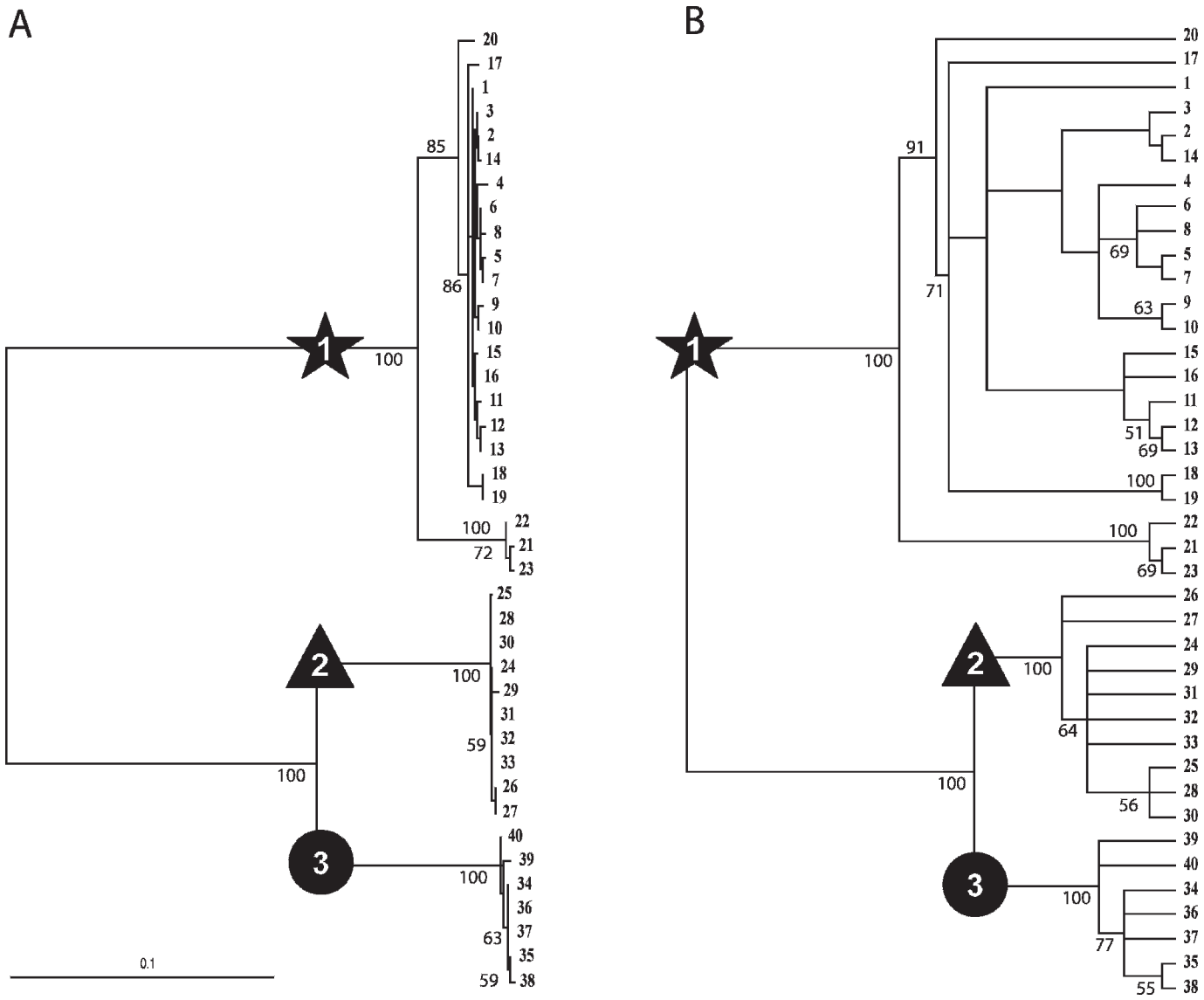

FIGURE 3. Trees recovered from analyses of the combined ITSs + LSU rDNA data set conformed by 40 samples of *N. golvani* (1,330 characters). (**A**) Maximum likelihood (ML) tree (−ln likelihood of 4227.33009) obtained from heuristic search with branch lengths scaled to the expected number of substitutions per site. Numbers near internal nodes show ML bootstrap clade frequencies. (**B**) Strict consensus of 12 equally parsimonious trees (509 steps) inferred from heuristic maximum parsimony (MP) analysis. Numbers below internal nodes show MP bootstrap clade frequencies. The 3 major lineages discussed in the text are indicated on the trees: Lineages 1 (★), 2 (▲), and 3 (●).

independence, particularly when considering that the populations are fragmented.

Lineage 1, which includes *N. golvani* sensu stricto, included 23 specimens (sample nos. 1–23) from freshwater cichlids. This result corroborates the contention that *N. golvani* is a consistent component of the helminth fauna of cichlids (see Pérez-Ponce de León and Choudhury, 2005). The distribution area of lineage 1 (Fig. 1) in Middle America extends from southeastern Mexico to Costa Rica, and it can be explained as a result of the contemporary and historical biogeography of their cichlid hosts (Concheiro-Pérez et al., 2007). According to these authors, Middle American cichlids derive from South American lineages that expanded their distribution range northwards. Ancestors of Middle American cichlids experienced a diversification process while expanding their distribution into southeastern Mexico. The presence of *N. golvani* in cichlids from such a wide distribution range, i.e., from Costa Rica to as far north as the Panuco River Basin in Mexico, indicates an ancient relationship and the fact that parasite distribution is closely tied to the historical biogeography of their hosts.

Lineages 2 and 3 of *N. golvani* are brackish water helminths represented by geographically restricted populations that are distributed in the Gulf of Mexico and the Pacific Ocean slopes, respectively. Moreover, lineage 2 is associated with both eleotrid and cichlid fishes. Meanwhile, the lineage 3 is associated strictly with eleotrid fishes. The genetic divergence between both lineages was 19.5% for ITSs and 9.8% for LSU. Steinauer et al. (2007) found a divergence level of 8.7% for populations of *Leptorhynchoides thecatus* Linton, 1891 with ITSs; their findings were used to postulate the existence of cryptic species for *L. thecatus*. Even

though the present observations may not necessarily represent a genetic yardstick for the group, the values of genetic divergence obtained in our study for clades 2 and 3 are large enough to speculate that both lineages are evolving independently. In addition, these populations have a vicariant distribution (Gulf of Mexico vs. Pacific Ocean slopes) and it seems unlikely that gene flow occurs between them, making it even more likely that they actually represent cryptic species.

Five samples from the Gulf of Mexico slope (lineage 2), were collected from an eleotrid *Dormitator maculatus* Bloch, 1792, (sample nos. 29–33) and another 5 samples were obtained from the cichlid *Cichlasoma urophthalmus* Günther, 1862 (sample nos. 24–28). It is currently known that the Papaloapan River is not considered part of the natural distributional range of *C. urophthalmus* (Miller et al., 2005), although it was recently introduced for aquacultural purposes (Espinosa-Pérez et al., 1993). Therefore, *C. urophthalmus* occurs in sympatry with eleotrids in the same hydrological system. In this scenario, when considering the very low genetic divergence among these individuals, i.e., 0.31% with ITSs and 0.21% with LSU, it can be concluded that they represent the same lineage and that the presence of this lineage in *C. urophthalmus* is due to host sharing or ecological host extension.

The specimens of *N. golvani* belonging to clade 3 were recovered from the eleotrid fish *Dormitator latifrons* Richardson, 1844 from Tres Palos Lagoon, on the Pacific slope of Mexico, (samples 34–38), where it co-occurs with fishes from other families such as Centropomidae, Cichlidae, Gerridae, Gobiidae, and Lutjanidae. Recently, Violante-González et al. (2007) recorded the presence of the acanthocephalan *N. golvani* in several species of brackish water fishes from that locality, including the eleotrid *D. latifrons*. The presence of this species of *Neoechinorhynchus* in fishes other than eleotrids can be explained either as a result of a host-sharing event, or even as an accidental infection, depending on the abundance and mean intensity values. Our prediction is that the acanthocephalans determined as *N. golvani* will correspond to the same lineage we uncovered. Future samplings of fresh specimens from all these hosts to extract DNA are necessary to test this hypothesis. Additionally, a more detailed morphological analysis is needed to look for characters that may corroborate the genetic distinction, probably characters only observed via scanning electron microscopy.

Morphological observations were made and meristic data of some of the diagnostic traits, including number of proboscis hooks as well as the size of anterior, middle, and posterior hooks, and body length and width, were taken from 33 specimens from the sampled populations, representing the 3 different clades. This information was used in a comparative framework, with respect to general morphology and measurements of previous published accounts (Salgado-Maldonado, 1985) in order to look for morphological characters that may indicate the presence of undescribed species. Measurements of our specimens in the current work were compared with 78 specimens from Veracruz and Tabasco, including the type locality. When mean values of individual variables were examined with the use of Student's *t*-tests, no significant statistic difference was found among specimens representing the 3 major clades ($P < 0.005$). Based on these observations, our null hypothesis was that we were dealing with a single species, *N. golvani.* However, the genetic divergence plus the reciprocal monophyly of the 3 major clades in all the phylogenetic analyses leads to detection of 3 cryptic species. These species are not herein described, because further molecular work needs to be conducted by sequencing at least a mitochondrial gene, and an even more detailed morphological examination needs to be undertaken to establish the proper description; that is, more data need to be gathered at this point to establish the species delimitation and description.

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