10-1999

Differentiation of Mexican Species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiformes): Molecular and Morphological Evidence

Virginia León-Règagnon
Universidad Nacional Autónoma de México

Daniel R. Brooks
University of Toronto, dan.brooks@utoronto.ca

Gerardo Perez-Ponce de Leon
Universidad Nacional Autónoma de México, ppdleon@servidor.unam.mx

J. Parasitol., 85(5), 1999 p. 935–946

DIFFERENTIATION OF MEXICAN SPECIES OF *HAEMATOLOECHUS* LOOSS, 1899 (DIGENEA: PLAGIORCHIFORMES): MOLECULAR AND MORPHOLOGICAL EVIDENCE

Virginia León-Règagnon, Daniel R. Brooks*, and Gerardo Pérez-Ponce de León

Laboratorio de Helmintología, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado Postal 70-153, CP 04510 México, D.F., México

ABSTRACT: Molecular evidence is interpreted in the light of morphology to examine the validity of several species of *Haematoloechus* described as Mexican endemics. Internal transcribed spacers 1 and 2 and 28S ribosomal genes were sequenced for 11 isolates. Phylogenetic analysis of separate partitions and combined databases was conducted. Results were analyzed, in the light of morphological evidence. *Haematoloechus macrorchis* is proposed as a junior synonym of *Haematoloechus longiplexus*. *Haematoloechus pulcher* is a sibling species with *Haematoloechus complexus* in Lerma wetlands. In Mexico, *Haematoloechus medioplexus* is distributed along the east coast coinciding with the distribution of *Rana berlandieri*. The sister species of *H. medioplexus* is *Haematoloechus coloradensis*, sharing the distribution of the uterus as a synapomorphic character. *Haematoloechus illimis* is more closely related to *H. medioplexus* and *H. coloradensis* than to *H. complexus*. It can be distinguished by the distribution of the uterus, lobed ovary, and testes.

The members of *Haematoloechus* Looss, 1899 represent 1 of the most common and characteristic groups of digeneans inhabiting anurans. More than 50 species have been described worldwide, all living as adults in anuran lungs. Nine species have been reported from Mexico (Caballero and Sokoloff, 1934; Caballero, 1941, 1942a, 1942b; Bravo, 1943; Martínez, 1969; Guillén-Hernández, 1992; León-Règagnon, 1992; Pulido, 1994), 5 of which have been named as distinct species endemic in the central plateau. The morphological characters used to differentiate these species from those previously described are problematic (Prokopic and Krivanec, 1974; Kennedy, 1980a, 1980b, 1981), and the validity of some of them is doubtful. DNA sequences represent a relatively new and potentially valuable source of data to help solve taxonomic and phylogenetic problems involving parasitic platyhelminths (Blair et al., 1996; McManus and Bowles, 1996). In the present study, we sequenced the ribosomal internal transcribed spacers (ITS1 and ITS2) and the D1 variable region of the 28s gene for 7 nominal species of *Haematoloechus* from Mexico and the U.S.A., following suggestions that these regions would be informative at the scale of closely related species (Luton et al., 1992; Barker et al., 1993). We used a combination of the new molecular data and reassessment of the morphological features of nominal taxa to examine the validity of several of the species of *Haematoloechus* described as Mexican endemics.

MATERIALS AND METHODS

We collected tissue samples during 1996 and 1997 (Table I summarizes collecting localities and hosts). Host and parasite tissues are deposited in the frozen tissue collection of the Zoology Department, Institute of Biology, Universidad Nacional Autónoma de México (UNAM). Worms were allocated to morphospecies in vivo, using morphological characters suggested in the original descriptions (Stafford, 1902; Krull, 1933; Caballero, 1941, 1942b; Bravo, 1943). Voucher specimens were relaxed in hot tap water, fixed with alcohol–formalin–acetic acid or Bouin's fluid, and stored in 70% ethanol before being stained with Mayer's paracarmine, Ehrlich's hematoxylin, or Gomori's trichrome and mounted in Canada balsam as whole mounts for comparison with specimens from the Colección Nacional de Helmintos (CNHE), Instituto de Biología, UNAM, from the U.S. National Parasite Collection (USNPC), Beltsville, Maryland, and from the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum. Samples for molecular work were preserved in absolute ethanol.

Five species previously recorded in Mexico were identified using morphological characters in this study, e.g., *Haematoloechus coloradensis* Cort, 1915, *Haematoloechus complexus* (Seely, 1906) Krull, 1933, *Haematoloechus illimis* Caballero, 1942, *Haematoloechus macrorchis* Caballero, 1941, and *Haematoloechus pulcher* Bravo, 1943. Additionally, 2 specimens were collected from *Rana vaillanti* Brocchi, 1877 in Los Tuxtlas, Veracruz, whose specific identity could not be established using morphological characters due to poor preservation. Sequences of these species and specimens of *Haematoloechus longiplexus* Stafford, 1902 and *Haematoloechus medioplexus* Stafford, 1902 collected in Nebraska, U.S.A. were compared. Worms were dissected to remove host blood from the ceca. When possible, more than 1 sample was sequenced to assess intraspecific variation. Frog tissue was processed for molecular work for comparison and to ensure that the source of the DNA was worms tissues. Standard phenol extraction methods were used to recover DNA from entire worms (a single specimen whenever possible). Laboratory protocols follow Palumbi (1996) and Hillis et al. (1996). Polymerase chain reaction was used for amplifying the DNA sample; parameters and settings follow manufacturer's recommendations and Palumbi (1996). Sequencing used Thermo Sequenase radiolabeled terminator cycle sequencing kits (Amersham Life Science, Inc., Cleveland, Ohio). Protocols follow manufacturer's recommendations with minor modifications. Amplification and sequencing of the 5′ ending of the 28S ribosomal gene (including the D1 variable region) was performed using the primers 28Sy 5′CTA ACC AGG ATT CCC TCA GTA ACG GCG AGT3′ (forward) and 28Sz 5′AGA CTC CTT GGT CCG TGT TTC AAG AC3′ (reverse) (Hillis and Dixon, 1991). The ITS1 and 5.8S ITS2 regions were amplified using the primers BD1 5′GTC GTA ACA AGG TTT CCG TA3′ (forward) and BD2 5′TAT GCT TAA ATT CAG CGG GT3′ (reverse) (Luton et al., 1992). Position of genes in the sequence was obtained from the alignment with the ITS1–5.8S–ITS2 sequence of *Echinostoma revolutum* (Froelich, 1802) Looss, 1899 (Morgan and Blair, 1995) and the sequence of D1 variable domain of the 28S in *Schistosoma* spp. Weinland, 1858 (Barker and Blair, 1996). Sequences are available in GenBank (accession nos. AF133104–AF133114 and AF133186–AF133196).

Clustal W (Thompson et al., 1994) was used with default settings for sequence alignment. The aligned sequences were subsequently edited in ESEE (version 3; Cabot and Beckenbach, 1989). Minor modifications were made by eye to correct the computer-aligned sequences. To evaluate the phylogenetic content of the data sets, we obtained the g1 statistic as suggested by Hillis and Huelsenbeck (1992). These calculations and phylogenetic analyses were performed using PAUP (version 3.1.1; Swofford, 1993) and McClade 3.04 (Maddison and Maddison, 1992). We treated gaps either as missing data or as a fifth base; for both options, we performed exhaustive searches for the independent data sets (ITS1, ITS2, and 28S), and for the combined data set. The 5.8S sequences were only used as a reference for alignment and not used in

Received 2 October 1998; revised 20 March 1999; accepted 20 March 1999.

* Department of Zoology, University of Toronto, Canada. 25 Harbord St. Toronto, Ontario, Canada M5S 3G5.

TABLE I. Host and locality of isolates of *Haematoloechus* spp. collected in Mexico and the U.S.A.

Isolate	Helminth species	Host	Locality
Complexus1	*Haematoloechus complexus*	*Rana montezumae* Baird, 1854	Ciénaga de Lerma, Estado de México
Complexus2	*H. complexus*	*R. montezumae* Baird, 1854	Ciénaga de Lerma, Estado de México
Coloradensis	*Haematoloechus coloradensis*	*Rana montezumae*	Ciénaga de Lerma, Estado de México
Coloradensis*	*H. coloradensis*	*Rana dunni* Zweifel, 1957	Pátzcuaro, Michoacán, México
Illimis	*Haematoloechis illimis*	*R. montezumae*	Ciénaga de Lerma, Estado de México
Longiplexus	*Haematoloechus longiplexus*	*Rana catesbeiana* Shaw, 1802	Genoa, Nebraska, U.S.A.
Macrorchis	*H. longiplexus*	*R. montezumae*	Ciénaga de Lerma, Estado de México
Medioplexus	*Haematoloechus medioplexus*	*Rana pipiens* Schreber, 1782	Holt Creek, Nebraska, U.S.A.
Pulcher1	*H. complexus*	*Ambystoma lermaensis* Taylor, 1940	Ciénaga de Lerma, Estado de México
Pulcher2	*Haematoloechus pulcher*	*A. lermaensis*	Ciénaga de Lerma, Estado de México
Tuxtlas1	*H. medioplexus*	*Rana vaillanti* Brocchi, 1877	Los Tuxtlas, Veracruz, Mexico
Tuxtlas2	*Haematoloechus* sp.	*R. vaillanti*	Los Tuxtlas, Veracruz, Mexico

* This isolate was not included in the analysis because only the 28S gene sequence was obtained, and no variation with respect to the isolate from *R. montezumae* was found.

phylogenetic analyses. Bootstrap resampling was conducted with 1,000 replicates in the branch and bound option.

RESULTS

A total of 1,836 bp—542 bp of the 5′ end of the ITS1 (incomplete), 124 bp of the 5.8S, the entire ITS2 (287 bp), and 883 bp of the 5′ end of the 28S (Fig. 1)—was sequenced and aligned for 7 species of *Haematoloechus* (11 isolates).

The sequenced region of the ITS1 exhibited 16% variability (87 variable sites, not including gaps). In the case of *H. macrorchis* and *H. longiplexus,* there are 3 inserts: the first is 9–15 bp long in position 42, the second is 47 bp long in position 127 and is repeated 3 consecutive times, and the third is 4 bp long in position 277 (Fig. 1). ITS2 shows a higher variability (without considering inserts), with 22.3% of variable sites (64 out of 287); an insert of 17 bp can be seen in *H. complexus, H. pulcher, H. macrorchis,* and *H. longiplexus* close to the 5′ end of the molecule. The sequenced region of the 28S is 15.4% variable (136 variable sites) (Fig. 1). A distance matrix is shown in Table II.

The g1 statistic values are −2.82 for the ITS1 data, −0.65 for the ITS2, −0.79 for the 28s, and −1.39 for the combined data set, showing that the data sets are significantly more structured than random data ($P = 0.01$) (Hillis and Huelsenbeck, 1992).

Tree topology was not affected considering gaps as missing data or as a fifth base. Phylogenetic analyses of each separate data set gave similar topologies for most of the tree, with the exception of pulcher2 and Tuxtlas2, whose position varies when analyzing ITS1 and ITS2, respectively. Consistency indexes and bootstrap values are shown in Figure 2. Following the methodology suggested by Wiens (1998), we combined the 3 data sets that resulted in a single most parsimonious tree with 756 steps and a confidence interval (CI) of 0.84 (430 steps; CI = 0.82 when gaps were treated as missing data). High bootstrap values were obtained for each node and are indicated on the tree (Fig. 3). *Haematoloechus longiplexus* and *H. macrorchis* group together in all cases; the same happens with *H. complexus,* and pulcher1, and with *H. medioplexus,* Tuxtlas1, *H. coloradensis,* and *H. illimis.*

DISCUSSION

Internal transcribed spacers have been used to help reconstruct phylogenetic relationships among closely related helminth groups. ITS1 has been shown to be relatively conservative but has several repeated units that are responsible for its length variation, even among closely related species (Luton et al., 1992; Kane and Rollinson, 1994). Bowles et al. (1995) found divergent paralogues of ITS1 in *Echinococcus* Rudolphi, 1801 (Cestoda), a feature that is very common in plants (Buckler et al., 1997). We did not find divergent paralogues in *Haematoloechus* ITS1, but we did find them in frogs' DNA. Large repeating units are present in the inserts of *H. macrorchis* and *H. longiplexus* sequences, as reported for other genera of digeneans, e.g., *Dolichosaccus* Johnston, 1912 (Luton et al., 1992) and *Schistosoma* (Kane and Rollinson, 1994), although in the species of *Echinostoma* Rudolphi, 1809 no inserts have been found (Morgan and Blair, 1995). The insertion of large sequences in ITS1 seems to be a feature that appears independently in unrelated groups. This feature makes ITS1 only suitable for phylogenetic studies at the species or populations level. ITS2 has been reported to vary from 1.1% in closely related species of *Schistosoma* (Kane and Rollinson, 1994) to 25.87% in distantly related species in the same genus (Bowles et al., 1995). We found a similar amount of variation among *Haematoloechus* spp. (22.3%). The D1 region of the 28S gene has been used in phylogenetic studies at different taxonomic levels, from species of the same genus (Littlewood and Johnston, 1995; Barker and Blair, 1996) to species from different families in a class (Barker et al., 1993). The region we used in this study includes the variable D1, and we found it to be more conservative than the ITS1 and 2 but still variable enough to obtain some phylogenetic information, e.g., >10% (Hillis and Dixon, 1991).

The phylogenetic hypotheses obtained from each data set differ in the position of pulcher2 and Tuxtlas2. Whereas in the ITS1 and ITS2 hypotheses, one or the other were included in the complexus group (complexus1 + complexus2 + pulcher1); in the 28S hypothesis both were included in this group (Fig. 2). The conflicting nodes were strongly supported in each case. There are 2 possible explanations for the difference in the

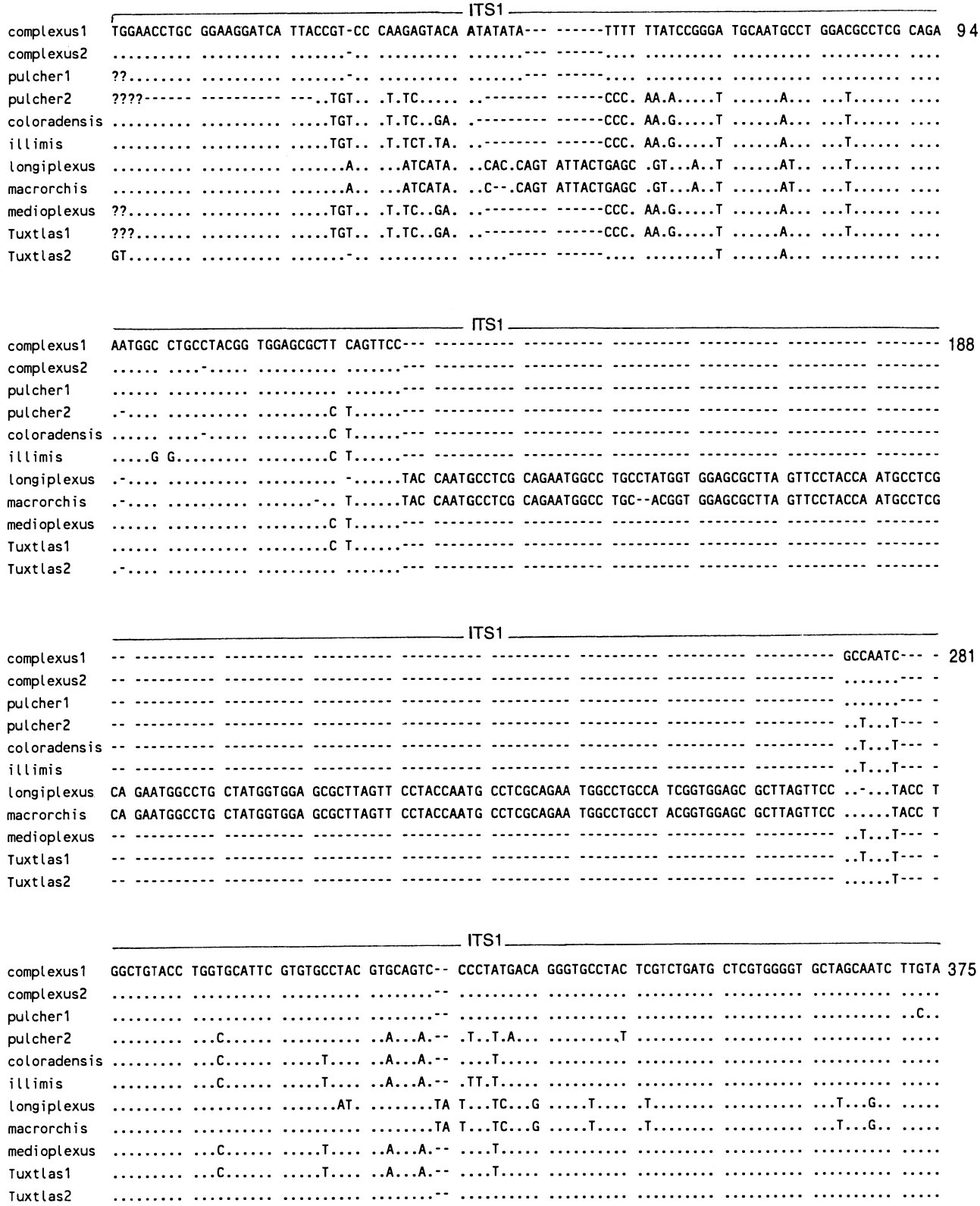

FIGURE 1. Sequences of ITS1 (partial), 5.8S (excluded from analyses), ITS2, and 28S (partial) ribosomal genes of 11 isolates of *Haematoloechus* species from Mexico and U.S.A. For hosts and collecting localities, see Table I. ● = same state as in the top sequence; – = gap; ? = unknown state.

```
                                                           ITS1
complexus1   TTGTC AGTCCACCTT GTGAGCGACG A??TGTGCTG TCGTTCGCGG CAGTGCTAGG CTTAAAGAGT GGTTGATTGC -ACGGCATAG TCACCGCCC 469
complexus2   ..... .......... .......... .......... .......... .......... .......... .......... T.....TAGA .........
pulcher1     ..... .......... .......... .GC....... .......... .......... .......... .......... T.....TAGA .........
pulcher2     ..... .......... ...-.T.... .GTA...... .......... .......... ...G...... .....TGG.. T.....-AGA .........
coloradensis ..... .......... ...-.T.... .GT....... .......... .......... ...G.T.... .....TAG.. T.....-AGA .........
illimis      ..... .......... ...-.T.... .GT....... .......... .......... .....T.... .....TAG.. T.....CAGA ....T....
longiplexus  ...C. .......... ...-.T.... .GT...A... .........T .......... ...G...... .......... T.....TACA ....-....
macrorchis   ...C. .......... ...-.T.... .GT...A... .........T .......... ...G...... .......... T.....TACA ....-....
medioplexus  ..... .......... ...-.T.... .GT....... .........A .......... ...G.T.... .....TAG.. T.....-AGA .........
Tuxtlas1     ..C.. .......... ...-.T.... .GT....... .......... .......... ...GT..... .A...TAG.. T.....-AGA .........
Tuxtlas2     ..... .......... .......... .GC....... .......... .......... .......... .......... T.....TAGA .........

                                            ITS1                                               5.8S
complexus1   T GTTAAATTGT TTACAAAACC -TTTTACACT GTTCAAGTGG TTCAGATCAG CCTCGGTTGG TTTGGATCAT TG   GTACGATG AAGAGCGCC 559
complexus2   . .......... .......... -......... .......... .......... .......... .......... ..   ..-..... .........
pulcher1     . C......... .......... -......... .......... .......... .......... .......... ..   ..-..... .........
pulcher2     . ...---.... .......... G......... .......... ........G. .T....C... .....G.... ..   ..-.-... ........A
coloradensis . ...---.... .......... G......... .......... ........G. .T....C... .....G.... ..   ..-..... .........
illimis      . ...---.... .......... G......... .......... ........G. .T....C... .....G.... ..   .-.GTGGA G........
longiplexus  G T.---G.... .....????? ?????????? ?????????? ?????????? ?????????? ?????????? ??   ..-..... .........
macrorchis   G T..-GT.... .......CTA ?????????? ?????????? ?????????? ?????????? ?????????? ??   ........ .........
medioplexus  . ...---.... ........--. G......... .....-.... ........G. .T....C... .....G.... ..   ..-..... ........A
Tuxtlas1     . ...---.... .......... G...-..... .......... ........G. .T....C... .....G.... ..   ..-..... ........A
Tuxtlas2     . .......... .......... -......... .......... .......... .......... .......... ..   ..-..... ........A

                                                    5.8S
complexus1   C AGAACTGTGT GAATTAATGT G--AACTGCA TACTGCTTTG AACATCGACA TCTTGAACGC ATATTG?GCC ATGGGTTATC ?C??G----- CC 652
complexus2   . .......... .......... .--....... .......... .......... .......... .......... .......... ....------ ..
pulcher1     . .......... .......... .--....... .......... .......... .......... .......... .......... C...-----G ..
pulcher2     G CC........ .......... .--....... .......... .......... .......... ......C.-- .......... C.AT.CCACG ..
coloradensis . .......... .......... .--....... .......... .T........ .......... .C........ ........G. .....CC--- --
illimis      . ...?...... ...??..GTG .TG....... .......... .T........ .......... .C........ ........G. .....CC--- --
longiplexus  . .......... .......... .--....... .......... .......... .......... .C....C... ........G. .....CC--- --
macrorchis   . .......... .......... .--....... .......... .......... .......... .C....C... ........G. .....CC--- --
medioplexus  G CC........ .......... .--....... .......... .T........ .......... .C....C.-- ........G. C.AT.CCACG ..
Tuxtlas1     G CC........ .......... .--....... .......... .T........ .......... .C....C.-- ........G. C.AT.CCACG ..
Tuxtlas2     G CC........ .......... .--....... .......... .T........ .......... .C....C.-- ........G. C.AT.CCACG ..

                                                               ITS2
complexus1   TGTCCGAG GGTCGG   CTTA TAAACTATCA CGACGCCCAA CAAGTCGTGG CTTGGGTCTT GCCAGCTGAC GTGGTTTCCC TATGATGTGT -- 742
complexus2   ........ ......   .... .......... .......... .......... .......... .......... .......... .......... --
pulcher1     ........ ......   .... .......... .......... .......... .......... .......... .......... .......... --
pulcher2     ........ ......   .... .......... .......... .......... .......... ...-...... .......... .......... --
coloradensis ........ ......   .... .......... .......... .......... .......... .......... ...A...... C.CTT.C... GT
illimis      ........ ......   .... .......... .......... .......... .......... .......... ...A...... C.CTT.CG.. GT
longiplexus  ........ ......   .... .......... .......... .......... .......... .......... ...A...... CT.TTCTG.. AT
macrorchis   ........ ......   .... .......... .......... .......... .......... .......... ...A...... CT.TTCTG.. AT
medioplexus  ........ ......   .... .......... .......... .......... .......... .......... ...A...... C.CTT.C... GT
Tuxtlas1     ........ ......   .... .......... .......... .......... .......... .......... ...A...... C.CTT.C... GT
Tuxtlas2     ........ ......   .... .......... .......... .......... .......... .......... ...A...... C.CTT.C... GT
```

FIGURE 1. Continued.

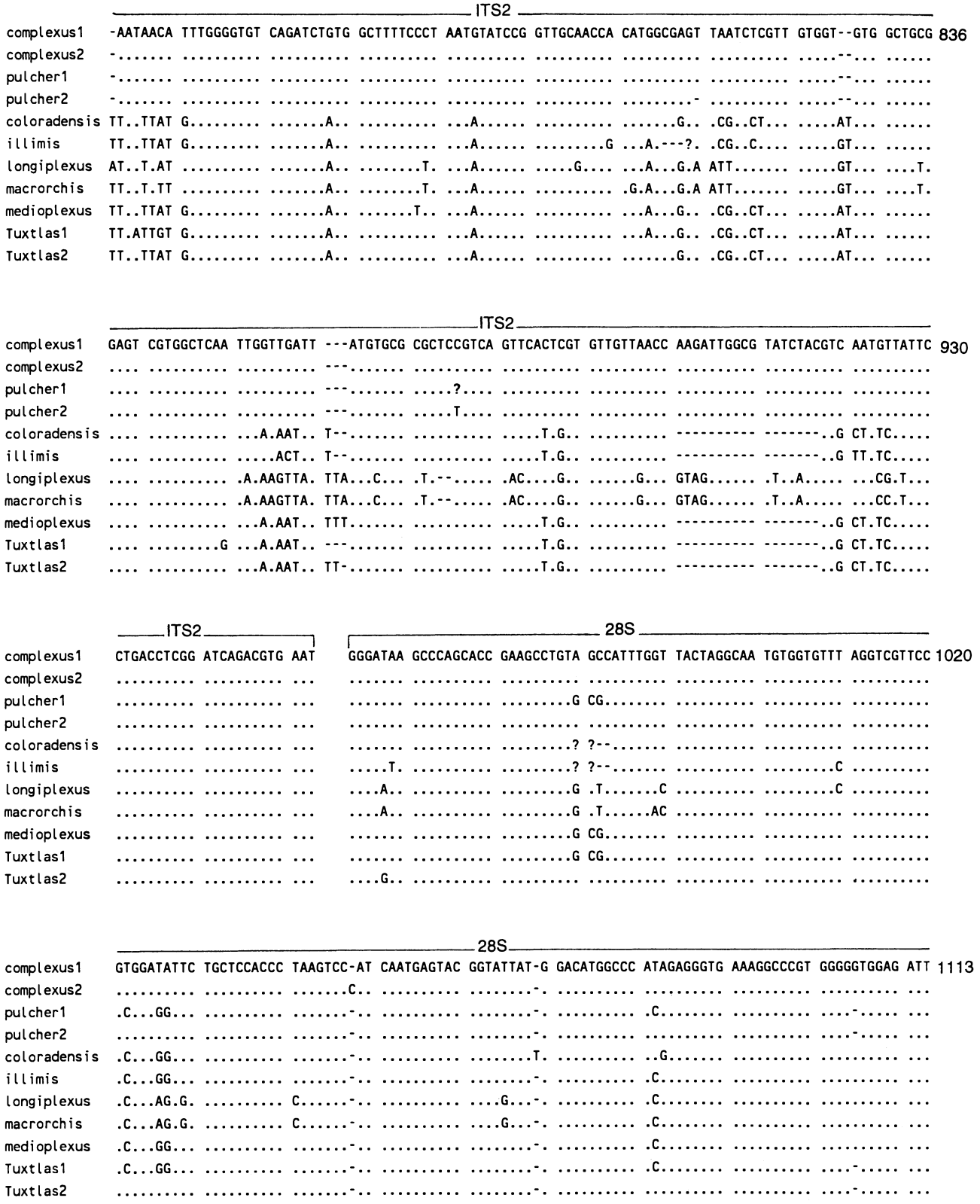

FIGURE 1. Continued.

```
                                                                                28S
complexus1   CGGTAGG ACAGAATATT CTTGGGTAGA CCTTGGAGTC GGGTTGTTTG TGAATGCAGC CCAAAGTGGG TGGT-AAACT CCATCCAAGG CTAAATA 1207
complexus2   ....... .......... .......... .......... .......... .......... .......... ....-..... .......... .......
pulcher1     ....... .......... .......... .........A .......... .......... .......... ....-..... .......... .......
pulcher2     ....... .......... .......... .......... .......... .......... .......... ....-..... .......... .......
coloradensis ....... C.....CT.C .C........ .......... .......... .......... .......... ....-..... .......... .......
illimis      ....... C.....CT.C .C........ .......... .......... A......... .......... ....-..... .......... .......
longiplexus  ....... ....CGCT.C TC........ .......... .......... A......... .......... ....-..... .......... .......
macrorchis   ....... ....CGCT.C TC........ .......... .......... A......... .......... ....-..... .......... .......
medioplexus  ....... C.....CT.C .C........ .......... .......... .......... .......... ...GT..... .......... .......
Tuxtlas1     ....... .........C .......... .......... .......... .......... .......... ....-..... .......... .......
Tuxtlas2     ....... .........C .......... .......... .......... .......... .......... ....-..... .......... .......

                                                                                28S
complexus1   CTT GCACGAGTCC GATAGCGAAC AAGTACCGTG AGGAAAGTTG AAAAGTACTT TGAAGAGAGA GTAAACAGTG CGTGAAACCG CTCAGAGGTA 1300
complexus2   ... .......... .......... .......... .......... .......... .......... .......... .......... ...-......
pulcher1     ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pulcher2     ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
coloradensis ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
illimis      ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
longiplexus  ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
macrorchis   ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
medioplexus  ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Tuxtlas1     ... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Tuxtlas2     ... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                                                                28S
complexus1   AACGGGTGGA GTTGAACTGC AAGCTCTGGG AATTCAACTG GTGAGTGTGG TTTTAGCTTG GTAAAATTGG TGGACATTGG GGTCTGCGTA GTAG 1394
complexus2   .......... .......... .......... .......... .......... .......... .......... .......... .......... ....
pulcher1     .......... .......... .......... .......... .......... .......... .......... .......... .......... ....
pulcher2     .......... .......... .......... .......... .......... .......... .....-.... .......... ..???????? ????
coloradensis .......... .......... .-........ ......-... .......... ...G...... ..C.T..... .....G.C.. .......... .A..
illimis      .......... .......... .......... .......... .......... ...G...... ..C.T..... .....G.C.. .......... ....
longiplexus  .......... .......... ........T. ......G... A......... ...G...... ..C.T..... .......... ......T... ....
macrorchis   .......... .......... ........T. ......G... A......... ...G...... .--CT..... .......... ......T... ....
medioplexus  .......... .......... .......... .......... .......... ...G...... ..C.T..... .....G.C.. .......... .A..
Tuxtlas1     .......... .......... .....T.... .......... .......... ...G...... ..C.T..... .....G.C.. .......... .A..
Tuxtlas2     .......... .......... .....T.... .......... .......... .......... .......... .......... .......... ....

                                                                                28S
complexus1   CAGGTC TTCGCCTTCG GGTGGGGATG CGCGATGCAC TTATCAAGTG TTGTGCTCCT CAGTGGTCAT -ACCGA-CCA ACTCGCTAGT GCACTTTC 1488
complexus2   ...... .......... ........-.. .......... .......... ......G... .......... -.....-... .......... ........
pulcher1     ...... .......... .......... .......... .......... ......G... .......... -.....-... .......... ........
pulcher2     ?????? ?????????? ?????..G.. .......... .......... ......G..G .....-.... AT....-... ......AT.. ........
coloradensis ...... .......... .......... .....?.... .......... ......G... .G..T..--. -C...GG... .......... ........
illimis      ...... .......... .......... .......... .......... ......G... .G..T..--. -C...G-... .......... ........
longiplexus  ...... ..T..-.... .....A.... .......... .......... ......G..C ....T..--. TC...G-... .....TC... ........
macrorchis   ...... ..T....... .....A.... .......... .......... ......G..C ....T..--. TC...G-... .....TC... ........
medioplexus  ...... .......... .......... .......... .......... ......G... .G..T..--. -C...G-... .......... ........
Tuxtlas1     ...... .......... .......... .......... .......... ......G... .G..T..--. -C...G-... .......... ........
Tuxtlas2     ...... .......... .......... .......... .......... ......G... .......... AC....-... .......... ........
```

FIGURE 1. Continued.

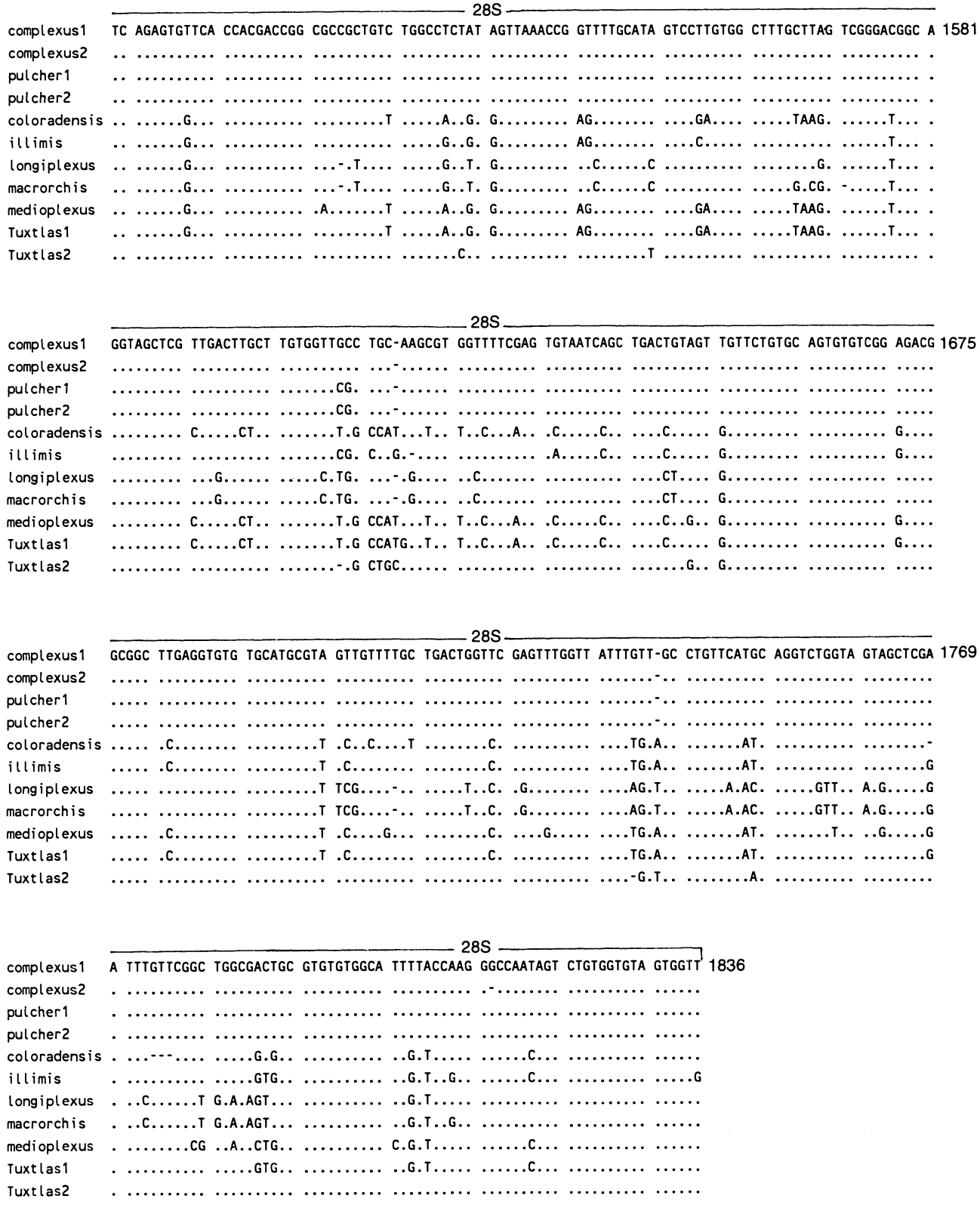

FIGURE 1. Continued.

TABLE II. Pairwise distances between taxa, calculated from the combined data set using PAUP Version 3.1.1 (Swofford, 1993).

Isolates	1	2	3	4	5	6	7	8	9	10	11
1. Complexus1	—	0.007	0.012	0.054	0.106	0.105	0.198	0.199	0.115	0.107	0.056
2. Complexus2		—	0.011	0.053	0.104	0.106	0.197	0.199	0.115	0.106	0.056
3. Pulcher1			—	0.054	0.106	0.103	0.196	0.198	0.112	0.102	0.057
4. Pulcher2				—	0.103	0.103	0.215	0.216	0.103	0.094	0.081
5. Coloradensis					—	0.042	0.203	0.203	0.027	0.025	0.076
6. Illimis						—	0.197	0.197	0.051	0.049	0.083
7. Longiplexus							—	0.017	0.205	0.207	0.201
8. Macrorchis								—	0.205	0.207	0.203
9. Medioplexus									—	0.020	0.073
10. Tuxtlas1										—	0.066
11. Tuxtlas2											—

placement of those isolates. The first is that we are dealing with a case of hybridization (not with F_1 hybrids that would have both parental genomes represented [Rollinson, et al., 1990] but historical hybridization). Nevertheless, if this was the case, the isolates would be strongly associated to 1 or the other parental species, but this is not reflected in the trees (Fig. 2). The second alternative, and the one we think is the best supported by our results, is that the misplacement of these isolates is the result of noise in the data. Combining the 3 data sets allows us to increase the accuracy of the estimated tree by the use of a larger number of characters in the analysis, especially in those parts of the tree unaffected by homoplasy (Kluge, 1989; Kluge and Wolf, 1993; Wiens, 1998). The resulting hypothesis is supported by the morphological evidence. Tuxtlas1 and pulcher2 share with other members of the complexus group a large acetabulum, round testes and ovary, and an unordered array of uterine loops that do not go extracecal.

Specimens identified as *H. macrorchis* and *H. longiplexus* are very similar, differing by only 1.7%. The phylogenetic analysis indicates that, among the taxa used in this study, *H. longiplexus* and *H. macrorchis* are each other's closest relatives. These observations could indicate that the taxa are not distinct species. As noted above, however, ITS2 has been reported to vary as little as 1.1% in closely related species of *Schistosoma* (Kane and Rollinson, 1994). Caballero (1941) differentiated the specimens he described as *H. macrorchis* from *H. longiplexus* by the length of the extracecal uterine loops. In the Mexican specimens, they extend anteriorly halfway between the ovary and the pharynx, whereas in *H. longiplexus* they extend anteriorly to the level of the pharynx. Caballero (1941) also reported specimens of *H. macrorchis* to have a spined tegument, whereas the tegument in *H. longiplexus* was described as aspinose. The presence of spines by itself is a problematic character for differentiating species of this genus. Cort (1915) reported that *H. longiplexus* specimens were aspinose or spinose. Krull (1932, 1933) noted that in *H. longiplexus* and *H. complexus* spines can be lost during the development of the worm or with the fixation techinques, and Brooks (1976) confirmed the presence of tegumental spines on adult specimens of *H. complexus*. Manter (1938) considered *Haematoloechus similiplexus* Stafford, 1902 and *Haematoloechus varioplexus* Stafford, 1902 synonymous because the only distinguishing feature was the presence or absence of tegumental spines.

We examined specimens (CNHE 814, 815, 1555; USNPC 75446, 79466; HWML 20144, 20146, 20147, 20148, 20149, 20150, 21947, 22243, 23255, 34137) and found that in some specimens of *H. longiplexus* the tegument had tiny spines, and in some specimens of *H. macrorchis* the tegument was aspinose. Likewise, the uterine loops in some specimens of *H. longiplexus* reach the pharynx level, whereas in others they reach halfway between the ovary and the pharynx, as in the type specimens of *H. macrorchis*. The information obtained from reexamination of the morphology shows that the characters used originally to distinguish *H. longiplexus* and *H. macrorchis* are variable within samples purported to be one or the other. In conjunction with the low level of molecular difference, the lack of distinguishing morphological traits leads us to propose herein that *H. macrorchis* is a junior synonym of *H. longiplexus*.

The 1.7% variation between samples may indicate that they represent differentiated populations. In addition to the geographic distributions, there are some apparent differences in host species affinities. In the U.S.A. *H. longiplexus* is primarily a parasite of the bullfrog, *Rana catesbeiana* Shaw, 1802, although it has been reported with low prevalences and abundances in other frog species like *Rana blairi* Mecham, Littlejohn, Oldham, Brown, and Brown, 1973 and *Rana pipiens* Schreber, 1782 (Brooks, 1976). In Mexico, *R. catesbeiana* was introduced to the northern states and does not occur farther south than Zacatecas and Tamaulipas (Flores-Villela, 1993). In the present study, we found *H. longiplexus* only in *Rana montezumae* Baird, 1854, a member of the leopard frog clade that includes *R. blairi* and *R. pipiens,* and in very low prevalence (1.2% in this study; Caballero [1941] reported it to be uncommon).

One of the most complex and controversial groups of nominal species of *Haematoloechus* are those inhabiting North American ranid frogs that have no extracecal uterine loops, spherical testes and ovaries, and distinct ventral suckers that are approximately the same size as, or slightly smaller than, the ventral sucker. Included in this group have been *H. complexus* and *H. coloradensis* in the U.S.A. east of the Rocky Mountains, *Haematoloechus confusus* Ingles, 1932, *Haematoloechis kernensis* Ingles, 1932, *Haematoloechus oxyorchis* Ingles, 1932, *Haematoloechus tumidus* Ingles, 1932, and *Haematoloechus buttensis* Ingles, 1936 in the west, and *H. pulcher* and *H. illimis* in Mexico.

Haematoloechus pulcher was differentiated from *H. complexus* by the presence of prominent pharyngeal glands, a rel-

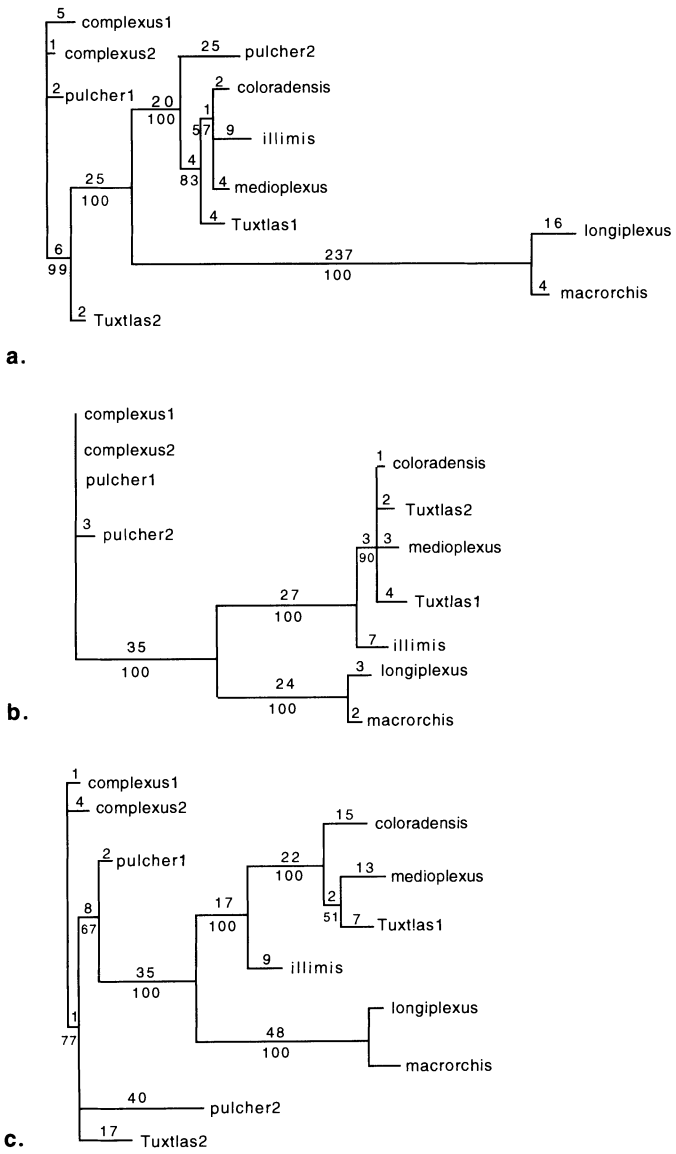

FIGURE 2. Most parsimonious unrooted trees (MPT) from the separate data sets. Values considering gaps as missing data in parentheses. (a) MPT from ITS1 sequences CI = 0.970 (0.962); (b) majority rule consensus of 8 MPTs obtained from ITS2 sequences CI = 0.947 (0.962); (c) majority rule consensus of 6 MPTs obtained from 28s sequences CI = 0.877 (0.868). Bootstrap values (1,000 replicates) shown below the branches; branch length shown above.

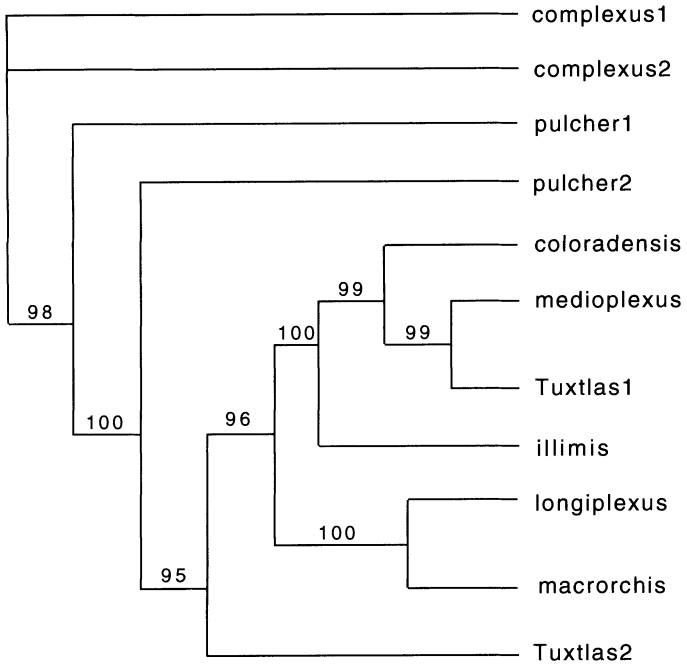

FIGURE 3. Most parsimonious unrooted tree obtained from the combined ITS1, ITS2, and 28s data sets; CI = 0.836. Bootstrap values shown (1,000 replicates).

atively large pharynx, and its host, salamanders of the genus *Ambystoma* Tschudi, 1832 (Bravo, 1943), the latter a circular criterion to use for distinguishing species (Brooks and McLennan, 1993). In the specimens we collected, the pharynx was not clearly larger than in the specimens of *H. complexus* from frogs from the same locality, and the pharyngeal glands, specially in unstained specimens, were no more distinct than those found in other digeneans. Separately sequenced specimens collected from salamanders represented 2 distinct genotypes: pulcher1 differs less (1.2% variation) from *H. complexus* of *R. montezumae* in the same locality than did specimens of *H. longiplexus* from the U.S.A. and Mexico. Specimens of this genotype were likely an infection of *H. complexus* in *Ambystoma lermaensis* Taylor, 1940. Pulcher2, by contrast, showed 5% variation, with *H. complexus,* equivalent to that observed among many clearly differentiated morphospecies. We believe pulcher2, presumably the true *H. pulcher,* is a sibling species with *H. complexus.* If true, we believe that examination of additional material will allow us to discern morphological traits to differentiate them.

Finally, Tuxtlas2 differs from *H. complexus* and *H. pulcher* in 5–8% of its sequence, indicating that it is also a distinct species in the complexus group. Further sampling in the area must be done to clarify the specific identity of this species.

Specimens designated as Tuxtlas1 vary 2.0% from those of *H. medioplexus* in Nebraska. The low level of genetic variation indicates limited geographic differentiation, suggesting that the species should be continuously distributed at least from Nebraska to Los Tuxtlas. *Haematoloechus medioplexus* has been collected in several different host species but most frequently in members of the *R. pipiens,* or leopard frog, clade in central and eastern U.S.A. and Canada. In accordance with the distribution ranges documented by Hillis et al. (1983) and Hillis (1988), published records listing *R. pipiens* as host may have been *R. pipiens, Rana sphenocephala* Cope, 1889, *R. blairi,* or *Rana berlandieri* Baird, 1854. Since the recognition that the leopard frogs represent numerous different species, *H. medioplexus* has been reported in *R. pipiens* and *R. blairi* from Nebraska (Brooks, 1976). We have recently collected *H. medioplexus* in *R. sphenocephala* from Arkansas (D. Brooks, unpubl. obs.). *Haematoloechus medioplexus* has also been reported in *Rana palustris,* another member of the leopard frog clade in Massachusetts and Maine (Rankin, 1945; Bouchard, 1951).

Two members of the leopard frog clade occur in the Veracruz region, where los Tuxtlas is located. *Rana berlandieri* is distributed from Texas southward along the the east coast of Mex-

ico to Veracruz; the northern part of its range overlaps with that of *R. sphenocephala. Rana brownorum* is distributed from Veracruz to Tabasco, Campeche, and Chiapas, Mexico (Frost, 1985).

In Mexico, *H. medioplexus* has been reported at low prevalence in *R. vaillanti* (this study) and *Rana palmipes* from Los Tuxtlas in Veracruz (Guillén-Hernández, 1992), and in *R. montezumae* from the Lerma wetlands and Lake Xochimilco (Caballero, 1941). Material from Lerma was not deposited in the CNHE and is not available for examination. We examined material from Xochimilco (CNHE 1191, 1770) and found that they do not belong to *H. medioplexus.* According to the arrangement of the uterine loops and the lack of acetabulum, they might belong to *Haematoloechus iturbei* Cordero and Vogelsang, 1939 or a closely related form. Further analysis of additional material will allow the identity of these specimens to be determined. Thus, it appears that in Mexico *H. medioplexus* occurs only along the eastern coast. We believe that *R. vaillanti* and *R. palmipes* Spix, 1824 from which *H. medioplexus* has been collected in Los Tuxtlas, are probably not the main hosts for this species of lung fluke in the region. First, both those frog species reach their northernmost extent in eastern Mexico, where *H. medioplexus* reaches it southernmost known distribution. Second, the typical *Haematoloechus* of *R. palmipes* is *H. iturbei* in South America, although this species was misidentified as *H. medioplexus* in Colombia (Uribe-Piedrahita, 1948). We have collected *H. iturbei* in *R. palmipes* from the Area de Conservación Guanacaste in northwestern Costa Rica, and it is clearly distinguishable from *H. medioplexus* by the lack of acetabulum and the unordered disposition of the uterus. We expect *R. berlandieri* to be the main host for *H. medioplexus* in los Tuxtlas as well as throughout its range in the eastern coast of Mexico.

Two species generally considered members of the *H. complexus* group, *H. coloradensis* and *H. illimis,* appear more closely related to *H. medioplexus* than to *H. complexus* in our analysis. They differ 2.7% and 4.2–5.1%, respectively, from *H. medioplexus,* whereas they differ 10.3–10.6% and 10.3–10.5% from *H. complexus. Haematoloechus coloradensis* has generally been considered most similar to *H. complexus* (Kennedy [1981] suggested synonymizing them), from which it has been distinguished by having a relatively larger pharynx with respect to the oral sucker and a spinose tegument. We have already discussed the doubtful validity of the spined tegument as an informative character by itself. We found 2 morphological features, however, that are useful for distinguishing this species. The pharynx in all specimens is relatively larger and generally longer than the oral sucker than that of any members of the *H. complexus* group or of *H. illimis* or *H. medioplexus.* In addition, we discovered that the arrangement of the uterine loops is an informative character. Members of the *H. complexus* group and the *H. medioplexus* group lack longitudinal extracecal uterine loops, the plesiomorphic condition for plagiorchiform digeneans (Brooks et al., 1985). In members of the *H. complexus* group and in *H. illimis,* the postcecal uterine loops are not ordered into a well-differentiated ascending and a descending row, can overlap the ceca, and can occupy the total postcecal space. In *H. coloradensis* and *H. medioplexus,* however, the transverse uterine loops are ordered into a well-differentiated ascending and a descending row and occupy only intercecal space. These are the only 2 species of *Haematoloechus* presently known to exhibit this trait, which we conclude is a morphological synapomorphy linking them, thus corroborating the molecular data. *Haematoloechus medioplexus* further differs from *H. coloradensis* by having an exremely small acetabulum, generally only 25% the width of the oral sucker, and by having extremely dense tegumental spination. These 2 species exhibit some degree of geographic differentiation. *Haematoleochus medioplexus,* as we have indicated, seems to be a species of the lowlands east of the Rocky Mountains down along to the eastern coastal area of Mexico, whereas *H. coloradensis* is a species of the western plateau in the U.S.A. (Colorado, Utah, Idaho, Nebraska) and central plateau in Mexico (Lake Pátzcuaro and Lerma wetlands), although both species occur in Nebraska (Brooks, 1976). Finally, both species inhabit primarily members of the leopard frog clade, with *H. medioplexus* known to occur in *R. palustris, R. pipiens, R. blairi,* and *R. sphenocephala* and presumed to occur in *R. berlandieri,* and *H. coloradensis* known to occur in *R. pipiens, R. blairi, R. montezumae,* and *Rana dunni.*

The sister species of *H. medioplexus* + *H. coloradensis* in this study *is H. illimis,* differing in 4.2–5.1% of its sequence. This species was described from *R. montezumae* in Lerma wetlands more than 50 yr ago (Caballero, 1942b) and never collected again until now. Caballero (1942b) reported it from the lungs, but most of the specimens we collected were found in the eustachian tubes of the frogs, an unusual habitat for *Haematoloechus.* This species, generally considered in the complexus group, differs markedly from other members of the group, together with *H. tumidus,* by having lobed ovary and testes and several short extracecal uterine loops in the posterior half of the body. Caballero (1942b) also mentioned a large metraterm as a distinctive character for *H. illimis.* Molecular data support the exclusion of *H. illimis* from the complexus group and its inclusion in the medioplexus group. Nevertheless, it differs from *H. coloradensis* and *H. medioplexus* in the arrangement of the uterus and in the shape of the ovary and testes. Morphological differences, together with the large amount of molecular variation with respect to *H. coloradensis* and *H. medioplexus,* suggest that it might be more closely related to other groups of species in the genus *Haematoloechus.* Further studies including other North American species might indicate its relation with other members of the genus.

This report has shown the merits and necessity of interpreting molecular data in the light of critical morphological evaluation to document the basic units of evolution and biodiversity: species. Finding substantial agreement between morphological and molecular data gives us hope that a robust phylogenetic hypothesis based on all available evidence (Kluge, 1989; Kluge and Wolf, 1993) can be produced for this fascinating group of digeneans. Given the geographic distribution and host range of *Haematoloechus* species, no doubt this group can become an important model system for historical ecological and parascript studies (Brooks and McLennan, 1991, 1993).

ACKNOWLEDGMENTS

This study was conducted at the Molecular Systematics Laboratory, Department of Zoology, University of Toronto and Royal Ontario Museum (ROM) and financed by Fundación

UNAM, International Council for Canadian Studies Postdoctoral Fellowships and CONACYT proj. J27985N to V.L.-R., NSERC operating grant no. A7696 to D.R.B., and CONACYT proj.2676PN, PAPIIT proj. IN219198 to G.P.-P.L. Special thanks to Jinzhong Fu and Bob Murphy (ROM) and J. P. Laclette and M. García V. (Instituto de Investigaciones Biomédicas, UNAM) for their advice in DNA sequencing, to B. Moore, Skip Sterner, L. García Prieto, A. Jiménez R., B. Mendoza G., G. Pulido F., U. Razo M., C. Rosas V., and A. Sanchez A. for their help in field collections and 3 anonymous reviewers who contributed with valuable suggestions.

LITERATURE CITED

BARKER, S. C., AND D. BLAIR. 1996. Molecular phylogeny of *Schistosoma* species supports traditional groupings within the genus. Journal of Parasitology **82:** 292–298.

———, ———, A. R. GARRETT, AND T. H. CRIBB. 1993. Utility of the D1 domain of nuclear 28s rRNA for phylogenetic inference in the Digenea. Systematic Parasitology **26:** 181–188.

BLAIR, D., A. CAMPOS, M. P. CUMMINGS, AND J. P. LACLETTE. 1996. Evolutionary biology of parasitic platyhelminths: The role of molecular phylogenetics. Parasitology Today **12:** 66–71.

BOUCHARD, J. L. 1951. The platyhelminths parasitizing some northern Maine amphibia. Transactions of the American Microscopical Society **70:** 245–250.

BOWLES, J., D. BLAIR, AND D. P. MCMANUS. 1995. A molecular phylogeny of the genus *Echinococcus.* Parasitology **110:** 317–328.

BRAVO, H., M. 1943. Estudio sistemático de los tremátodos parásitos de los "ajolotes" de México I. Anales del Instituto de Biología Universidad Nacional Autónoma de México **14:** 141–159.

BROOKS, D. R. 1976. Parasites of amphibians of the Great Plains. II. Platyhelminths of amphibians in Nebraska. Bulletin of the University of Nebraska State Museum **10:** 65–92.

———, AND D. A. MCLENNAN. 1991. Phylogeny, ecology and behavior. A research program in comparative biology. University of Chicago Press, Chicago, Illinois, 434 p.

———, AND ———. 1993. Parascript. Parasites and the language of evolution. Smithsonian Institution Press, Washington, D.C., 429 p.

———, R. T. O'GRADY, AND D. R. GLEN. 1985. Phylogenetic analysis of the Digenea (Platyhelminthes: Cercomeria) with comments on their adaptive radiation. Canadian Journal of Zoology **63:** 411–443.

BUCKLER, E. S. I., A. IPPOLITO, AND T. P. HOLTSFORD. 1997. The evolution of ribosomal DNA: Divergent paralogues and phylogenetic implications. Genetics **145:** 821–832.

CABALLERO, Y C. E. 1941. Tremátodos de la Ciénaga de Lerma, Méx. Anales del Instituto de Biología Universidad Nacional Autónoma de México **12:** 623–641.

———. 1942a. Tremátodos de las ranas de la Ciénaga de Lerma, Estado de México. IV. Anales del Instituto de Biología Universidad Nacional Autónoma de México **13:** 635–640.

———. 1942b. Tremátodos de las ranas de la Ciénaga de Lerma, Estado de México. II. Descripción de una nueva especie de *Haematoloechus.* Revista Brasileira de Biologia **2:** 155–158.

———, AND D. SOKOLOFF. 1934. Segunda contribución al conocimiento de la parasitología de *Rana montezumae* con un resumen, descripción de una nueva especie y clave del género *Haematoloechus.* Anales del Instituto de Biología Universidad Nacional Autónoma de México **5:** 5–40.

CABOT, E. L., AND A. T. BECKENBACH. 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. Comparative Applied Biology **5:** 233–234.

CORT, W. W. 1915. North American frog lung flukes. Transactions of the American Microscopical Society **34:** 203–240.

FLORES-VILLELA, O. 1993. Herpetofauna mexicana. Special Publication No. 17, Carnegie Museum of Natural History, Pittsburgh, Ohio, 73 p.

FROST, D. R. 1985. Amphibian species of the world. A taxonomic and geographical reference. Allen Press, Lawrence, Kansas, 732 p.

GUILLÉN-HERNÁNDEZ, S. 1992. Comunidades de helmintos de algunos anuros de Los Tuxtlas, Veracruz. Tesis Maestría Facultad de Ciencias. UNAM, México City, México, 66 p.

HILLIS, D. M. 1988. Systematics of the *Rana pipiens* complex: Puzzle and paradigm. Annual Review of Ecology and Systematics **19:** 39–63.

———, AND T. M. DIXON. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. Quarterly Review of Biology **66:** 411–453.

———, J. S. FROST, AND D. A. WRIGHT. 1983. Phylogeny and biogeography of the *Rana pipiens* complex: A biochemical evaluation. Systematic Zoology **32:** 132–143.

———, AND J. P. HUELSENBECK. 1992. Signal, noise and reliability in molecular phylogenetic analyses. Journal of Heredity **83:** 189–195.

———, B. K. MABLE, AND C. MORITZ. 1996. Nucleic acids IV: Sequencing and cloning. In Molecular systematics, D. M. Hillis, C. Moritz, and B. K. Mable (eds.). Sinauer, Sunderland, Massachusetts. p. 321–383.

KANE, R. A., AND D. ROLLINSON. 1994. Repetitive sequences in the ribosomal DNA internal transcribed spacer of *Schistosoma haematobium, Schistosoma intercalatum* and *Schistosoma mattheei.* Molecular and Biochemical Parasitology **63:** 153–156.

KENNEDY, M. J. 1980a. Host-induced variations in *Haematoloechus buttensis* (Trematoda: Haematoloechidae). Canadian Journal of Zoology **58:** 427–442.

———. 1980b. Geographical variation in some representatives of *Haematoloechus,* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **58:** 1151–1167.

———. 1981. A revision of species of the genus *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **59:** 1836–1846.

KLUGE, A. G. 1989. A concern for evidence and phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). Systematic Zoology **38:** 7–25.

———, AND A. J. WOLF. 1993. Cladistics: What's in a word? Cladistics **9:** 183–199.

KRULL, W. H. 1932. Studies on the life history of *Pneumobites longiplexus* (Stafford). Zoologischer Anzeiger **99:** 231–239.

———. 1933. Studies on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull, n. comb. Parasitenkunde **6:** 192–206.

LITTLEWOOD, D. T., AND D. A. JOHNSTON. 1995. Molecular phylogenetics of the four *Schistosoma* species groups determined with partial 28S ribosomal RNA gene sequences. Parasitology **111:** 167–175.

LEÓN-RÈGAGNON, V. 1992. Fauna helmintológica de algunos vertebrados acuáticos de la Ciénaga de Lerma, Estado de México. Anales del Instituto de Biología Universidad Nacional Autónoma de México **63:** 151–153.

LUTON, K., D. WALKER, AND D. BLAIR. 1992. Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). Molecular and Biochemical Parasitology **56:** 323–328.

MADDISON W. P., AND D. R. MADDISON. 1992. MacClade, Version 3.0. Sinauer Sunderland, Massachusetts.

MANTER, H. W. 1938. A collection of trematodes from Florida amphibia. Transactions of the American Microscopical Society **57:** 26–38.

MARTÍNEZ V., J. M. 1969. Parásitos de algunos anfibios colectados en diferentes áreas de los municipios de Escobedo, Pesquería, Santiago, Nuevo León, México. Tesis Facultad de Ciencias Biológicas. Universidad Autónoma de Nuevo León, Monterrey, México, 51 p.

MCMANUS, D. P., AND J. BOWLES. 1996. Molecular genetic approaches to parasite identification: Their value in diagnostic parasitology and systematics. International Journal for Parasitology **26:** 687–704.

MORGAN, J. A. T., AND D. BLAIR. 1995. Nuclear rDNA ITS sequence variation in the trematode genus *Echinosotoma*: An aid to establishing relationships within the 37-spine group. Parasitology **111:** 609–615.

PALUMBI, S. R. 1996. Nucleic acids II: The polymerase chain reaction. *In* Molecular systematics, D. M. Hillis, C. Moritz, and B. K. Mable (eds.). Sinauer, Sunderland, Massachusetts, p. 205–247

PROKOPIC, J., AND K. KRIVANEC. 1974. Trematodes of the genus *Hae-*

matoloechus Looss, and their variability. Helminthologia **15:** 779–802.

PULIDO F., G. 1994. Helmintos de *Rana dunni* especie endémica del lago de Pátzcuaro, Michoacán, México. Anales del Instituto de Biología Universidad Nacional Autónoma de México **65:** 205–207.

RANKIN, J. S. 1945. An ecological study of the helminth parasites of amphibians and reptiles of western Massachusetts and vicinity. Journal of Parasitology **31:** 142–150.

ROLLINSON, D., T. K. WALKER, R. J. KNOWLES, AND A. J. G. SIMPSON. 1990. Identification of schistosome hybrids and larval parasites using rRNA probes. Systematic Parasitology **15:** 65–73.

STAFFORD, J. 1902. On the american representatives of *Distomum variegatum.* Zoologische Jahrbuecher Abteilung fuer Systematik Oekologie und Geographie der Tiere **16:** 895–912.

SWOFFORD, D. L. 1993. PAUP: Phylogenetic analysis using parsimony, Version 3.1.1. Illinois Natural History Survey, Champaign, Illinois.

THOMPSON, J. D., D. G. HIGGINS, AND T. J. GIBSON. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:** 4673–4680.

URIBE-PIEDRAHITA, C. 1948. Contribuciones al estudio de la parasitología en Colombia II. Caldasia, Bogotá **5:** 211–219.

WEINS, J. J. 1998. Combining data sets with different phylogenetic histories. Systematic Biology **47**: 568–581.