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Molecules, Morphology, and Mud Turtle Phylogenetics (Family Kinosternidae)

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Previous phylogenetic analyses of morphological and protein characters for the mud and musk turtles of the family Kinosternidae have resolved many of the hypothetical evolutionary relationships among its member species (Seidel et al., 1986; Iverson, 1991). However, several controversial relationships remained unresolved. This is in part due to the incongruence among the phylogenies produced from morphological data alone versus those from only protein data versus those from combined protein and morphological data. Although most of the previously hypothesized species groups were supported by these analyses (Table 1), the monophyly of the *Sternotherus* group (*carinatus*, *depressus*, *minor*, and *odoratus*) and the inclusion of *leucostomum* in the *scorpioides* group (and not with *angustipons* and *dunni*) remained ambiguous. This study was originated in order to obtain mitochondrial DNA sequence data to be submitted to phylogenetic analysis both alone and in combination with the previously published morphological and protein data.

Methods. — Blood samples were obtained from all recognized living species of kinosternid turtles (Kinosterninae and Staurotypinae: Appendix I) except *Kinosternon angustipons*, *K. creaseri*, and *K. oaxacae*, as well as all three subspecies of *Kinosternon subrubrum* (*subrubrum*, *steindachneri*, and *hippocrepis*). DNA isolation and sequencing was done under the supervision of Brian Bowen at the BEECS Genetic Analysis Core at the University of Florida. MtDNA was isolated using standard phenol/chloroform methodology (Hillis and Moritz, 1990). Cytochrome *b* sequences were amplified via the polymerase chain reaction using the universal primers described by Irwin et al. (1991). An 18-base "universal" M13 sequence was added to the 5' end of primers to facilitate automated sequencing. Thermal cycling parameters were: 1 cycle at 94°C (3 min), then 35 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min). Standard precautions (including template-free PCRs as negative controls) were used to test for contamination and to insure the accuracy of the reactions (Innes et al., 1990). Streptavidin-coated magnetic beads (Dynabeads M280 streptavidin, Dynal, Sweden) were used to purify PCR products (Mitchell and Merill, 1989). Single stranded templates were generated by denaturing the magnetically-captured double stranded DNA with fresh 0.15M NaOH and using the released (non-biotinylated) strand as a template for sequencing reactions. Single stranded sequencing reactions were conducted with flourescently labeled M13 primers in a robotic work station (Applied Biosystems model 800); labeled extension products were analyzed with an automated DNA sequencer (Applied Biosystems model 373A).

Both the heavy (forward) and light (reverse) strands were sequenced (at least once) for each individual in order to eliminate ambiguity (except for *dunni* [forward complement sequenced three times] and *leucostomum* [forward complement sequenced twice]). Electrophoretograms generated by the automated sequencer were cross-checked with computer generated sequences to insure accuracy and sequences were then aligned by eye. Any ambiguous base was coded as an unknown.

Aligned sequences (290 bp; Appendix II) were analyzed cladistically using the maximum parsimony program PAUP (version 3.1.1; Swofford, 1993) with the composite outgroup being the Staurotypinae (*Staurotypus salvinii*, *S. triporcatus*, and *Claudius angustatus*; after Bramble et al., 1984, and Hutchison, 1991, among others). Analyses were run with unweighted transversions and transitions as well as with tranversions weighted 4:1 over transitions (the average ratio among the kinosternines); shortest trees were identical under both schemes. Sequence divergence estimates were calculated from the nucleotide sequence differences corrected for "multiple hits" by the two-parameter model of Kimura (1980) using PHYLIP 3.5.5. Shortest trees were generated via the heuristic search mode with random addition of taxa and ten replications. Support for nodes was examined via bootstrap with 1000 replications.

The cladistic analysis was repeated with the DNA data combined with the 27 unordered morphological characters and the 34 protein electromorph characters (as opposed to

Table 1. Prior hypotheses concerning the relationships among various kinosternine turtles.

1) the genus *Kinosternon* (*sensu stricto*) is paraphyletic with respect to *Sternotherus* (Seidel et al., 1986).
2) *carinatus* (Zug, 1966) or *odoratus* (Tinkle, 1958) is the most primitive member of the sternotherine group and of the Kinosternini overall.
3) *carinatus*, *minor*, and *depressus* together are monophyletic (Tinkle, 1958).
4) *minor* and *depressus* are sister taxa (Tinkle, 1958; Iverson, 1977; Seidel and Lucchino, 1981).
5) *baurii* and *subrubrum* are closely related to each other (Lamb and Lovich, 1990; Lovich and Lamb, 1995) and to the sternotherines (based on protein data from Seidel et al., 1986).
6) *herrerai* is the most primitive *Kinosternon* (*sensu stricto*) (e.g., Bramble et al., 1984).
7) *flavescens* is closely related to *subrubrum* and *baurii* (they are ecologically and morphologically similar, and nearly parapatric).
8) *angustipons* and *dunni* are closely related, if not sister taxa (Legler, 1965; Iverson, 1988b).
9) *leucostomum* is most closely related to *angustipons* and *dunni* (Iverson, 1988b, 1991), and not to the *scorpioides* group (but see Iverson, 1991).
10) *sonoriense* and *hirtipes* are sister taxa (Iverson, 1981).
11) the *scorpioides* group (*acutum*, *alamosae*, *chimalhuaca*, *creaseri*, *integrum*, *oaxacae*, and *scorpioides*) is monophyletic (Iverson, 1988b, 1991).
12) *alamosae*, *chimalhuaca*, and *oaxacae* are each derived from *integrum* (Berry, 1978; Berry et al., 1997; Iverson, 1986, 1989).
13) *acutum* and *creaseri* are sister taxa (Iverson, 1988a).

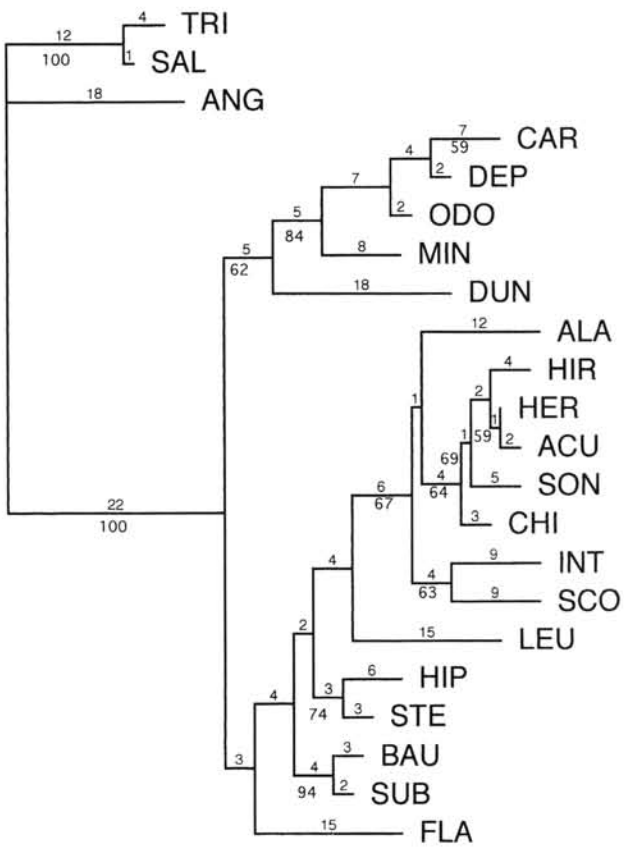

Figure 1. Single most parsimonious tree (PAUP algorithm) of the cladistic relationships among kinosternid species (coded by first three letters of species or subspecies name — see Appendix I) based on the cytochrome *b* mtDNA sequence data coded in Appendix II. Horizontal node lengths are proportional to branch length (number above each branch); numbers below some branches are % support (> 50% only) from 1000 bootstrap replications. The length is 242 steps, and the consistency index is 0.59. The tree was rooted with the composite outgroup of *Claudius* and two species of *Staurotypus*.

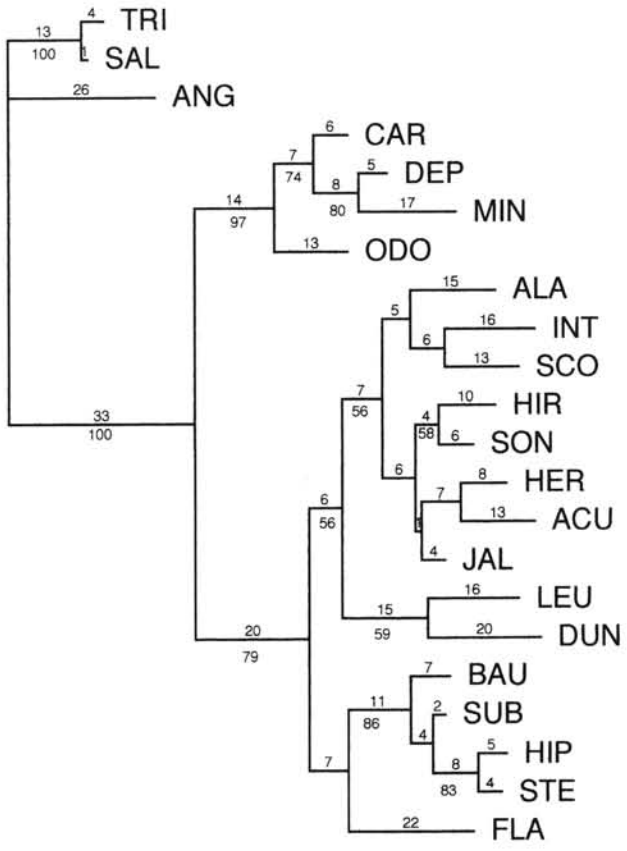

Figure 2. Single most parsimonious tree (PAUP algorithm) of the cladistic relationships among kinosternid species (coded by first three letters of the species or subspecies name — see Appendix I) based on 82 mtDNA characters, 34 protein electromorph products, and 27 morphological characters. Horizontal node lengths are proportional to branch length (number above each branch); numbers below some branches are % support (> 50% only) from 1000 bootstrap replications. The length is 415 steps, and the consistency index is 0.53. The tree was rooted with a composite outgroup of *Claudius* and two species of *Staurotypus*.

locus characters; see Swofford and Olson, 1990; Mabee and Humphries, 1993; and de Queiroz and Lawson, 1994, for logic) reported by Iverson (1991).

Results and Discussion. — Of the 290 cytochrome *b* nucleotides assayed, 121 (42%) were variable among the combined ingroup and outgroup taxa, and 82 of those were phylogenetically informative. Among the ingroup taxa 101 (35%) were variable and 61 of those were informative. Of the 101 variable positions, 92 possessed only two nucleotides; of those 74 (80%) represented transitions (57 C-T; 17 A-G). Sequence divergence estimates ranged from 0.17 to 0.27 for staurotypines (*Staurotypus* and *Claudius*) versus kinosternines, 0.08 to 0.14 between sternotherines and other kinosternine turtles, 0.03 to 0.08 among sternotherine species, and 0.01 to 0.18 among other kinosternine turtles.

The shortest tree generated by the PAUP analysis of the cytochrome *b* nucleotide data (Fig. 1) was completely resolved, and suggested 1) that the *Sternotherus* group is monophyletic; 2) (unexpectedly) that *Sternotherus* is the sister taxon of *dunni*; 3) that members of the *flavescens* group (*flavescens*, *subrubrum*, and *baurii*) are the most primitive members of the *Kinosternon* clade (*sensu stricto*, but excluding *dunni*); and 4) that the *scorpioides* group (Table 1) is monophyletic but includes an unnatural *hirtipes* group (*hirtipes* and *sonoriense*, as well as *herrerai*). However, as indicated by the low bootstrap values, very few of the relationships are resolved with confidence (only two values within the Kinosterninae exceed 74%). In addition, a strict consensus tree of the shortest tree with trees one and two steps longer (total = 295 trees) supported a monophyletic *Sternotherus*, *baurii* as sister taxon to *subrubrum*, and *hippocrepis* as sister taxon to *steindachneri* (each with 100% of the 295 trees), but these clades and all other kinosternine taxa arose from a single unresolved polytomy. Thus, it appears that neither the protein data alone (Seidel et al., 1986; Iverson, 1991) nor the morphologic data alone (Iverson, 1991) nor the cytochrome *b* data alone (this paper) are sufficient to resolve the phylogenetic relationships within the Kinosterninae with confidence. A total evidence analysis was warranted.

The phylogenetic analysis of the mtDNA data combined with the morphological and protein electromorph data sets also generated a single most parsimonious tree (Fig. 2). This tree strongly supported the monophyly of the *Sternotherus* group (based on 14 synapomorphies: 8 mtDNA

[1 unique], 4 electromorph, and 2 morphological), and recognized *Sternotherus* as sister taxon to the genus *Kinosternon* (*sensu stricto*). Within the *Sternotherus* group, *odoratus* was identified as most primitive and sister taxon to the previously recognized *carinatus* group (Table 1), including *carinatus* (most primitive) and the sister taxa *minor* and *depressus* (as hypothesized by Tinkle, 1958).

Within *Kinosternon* two sister clades were identified. The first is the *flavescens* group (Table 1), supported by 7 synapomorphies (3 mtDNA [1 unique], 1 electromorph, and 3 morphological [2 unique]), and in which *flavescens* is basal to the sister taxa *subrubrum* (including *steindachneri* and *hippocrepis*) and *baurii*. Should additional analysis support the monophyly of this *flavescens* group, the genus name *Thyrosternum* Agassiz 1857 (type species, *Thyrosternum pensylvanicum* [= *K. subrubrum*]) is available for this clade; however, that change is not recommended here.

The second clade within *Kinosternon* includes a monophyletic *leucostomum* group (with *dunni*) as sister taxon to the combined *scorpioides* and *hirtipes* groups. Although the latter group was fully resolved in this analysis, minimal branch lengths, low bootstrap values, the lack of mtDNA data for two of its probable members (*creaseri* and *oaxacae*), and biogeographic incompatibilities (e.g., *chimalhuaca* on the Pacific coast, and *herrerai* and *acutum* on the Atlantic) reduce my confidence in the phylogenetic relationships within the *scorpioides* group as depicted in Fig. 2. The analysis of a larger set of characters (particularly including fast-evolving molecular ones) will be necessary before any resolution of the relationships within this latter group can be accepted with confidence.

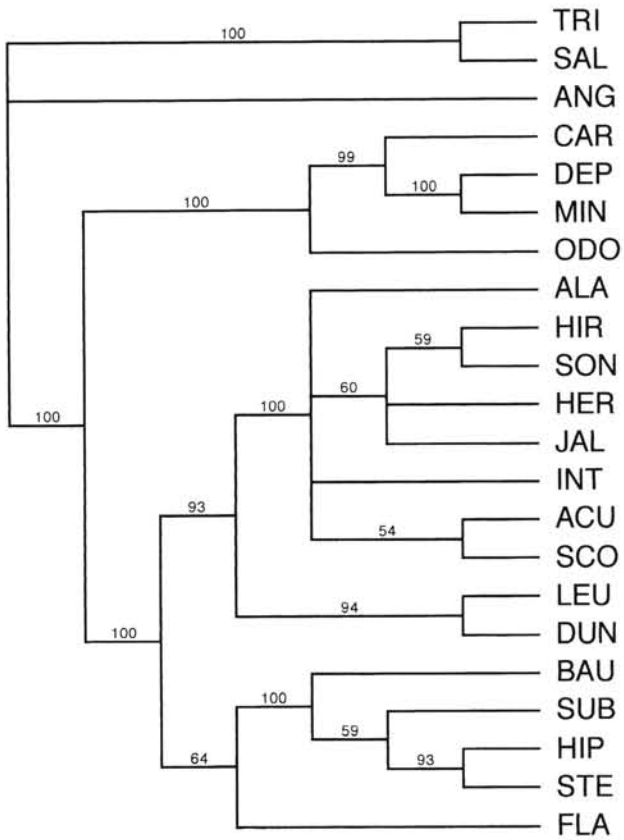

Figure 3. Majority rule (50%) consensus tree of shortest tree (Fig. 2) and 79 trees one or two steps longer (415–417 steps), illustrating the cladistic relationships among kinosternid turtles based on total evidence (see text).

To test the strength of the support for the shortest combined evidence tree, I generated a 50% majority rule tree for the best tree along with the 79 trees that were one or two steps longer (Fig. 3). Each of the major clades evident in the shortest tree are also resolved in this expanded analysis. In conclusion (compare Table 1), the combined analysis supports the monophyly of the genus *Sternotherus* (and hence its resurrection from the synonymy of *Kinosternon*; see Seidel et al., 1986); it indicates that *odoratus* is the sister taxon to all other *Sternotherus*; it recognizes a monophyletic *carinatus* group (with *minor* and *depressus* as sister taxa); it suggests that the central and eastern kinosternids in the USA (*subrubrum* and *baurii*) are monophyletic (with *flavescens* as their sister taxon); and it confirms the monophyly of *dunni* and *leucostomum*, outside of the *scorpioides* group. However, it does not support a primitive position for *herrerai* (e.g., Bramble et al., 1984), and it leaves considerable ambiguity about the relationships within the combined *scorpioides* and *hirtipes* species groups. Further analyses now in progress (J. Parham and J.H. Hutchison, *pers. comm.*; and D. Starkey and S. Davis, *pers. comm.*), involving fossils and additional DNA sequence data, respectively, may resolve the latter relationships.

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APPENDIX I

Three-letter abbreviation codes and sources of blood.

ANG – *Claudius angustatus*: No data; pet trade specimen alive in collection of W.P. McCord.
SAL – *Staurotypus salvinii*: No data; pet trade specimen alive in collection of W.P. McCord.
TRI – *Staurotypus triporcatus*: No data; captive hatched from pet trade specimen in collection of M.A. Ewert.
CAR – *Sternotherus carinatus*: Oklahoma, McCurtain Co., Glover Creek at Hwy 3, NW of Broken Bow; JBI personal collection.
DEP – *Sternotherus depressus*: No data; pet trade specimen alive in collection of W.P. McCord.
MIN – *Sternotherus minor*: Florida, Marion Co., Rainbow Run; UF (University of Florida) 80827.
ODO – *Sternotherus odoratus*: South Carolina, Aiken Co.; personal collection of M. Klemens.
ACU – *Kinosternon acutum*: No data; pet trade specimen alive in collection of W.P. McCord.
ALA – *Kinosternon alamosae*: Mexico, Sonora, Presa La Noria, 1 mi S Alamos Hwy, 1 mi E road to Cerro Prieto Microondas Station; UF 99877-78.
BAU – *Kinosternon baurii*: Florida, Seminole Co., Oviedo; UF 84965.
CHI – *Kinosternon chimalhuaca*: Mexico, Jalisco, 2.3 rd. mi S Hwy 200 Río Purificacion bridge; UF 51791.
DUN – *Kinosternon dunni*: Colombia, no further data; specimen in collection of Olga Victoria Castaño.
FLA – *Kinosternon flavescens*: Nebraska, Garden Co., Gimlet Lake; UF 99881.
HER – *Kinosternon herrerai*: Mexico, Veracruz, Xalapa, Las Animas; MVUZ (Museo Zoologica Universidad Veracruzana) 0455.
HIR – *Kinosternon hirtipes*: Mexico, Chihuahua, Ojo SE of Galeana; UF uncatalogued.
INT – *Kinosternon integrum*: Mexico, Oaxaca, Presa La Noria, 9.0 rd. mi. N Ejutla, Hwy 175; UF 85004.
LEU – *Kinosternon leucostomum*: Costa Rica, Limon Province, 1 km S Puerto Viejo; ASU (Appalachian State University) 17430.
SCO – *Kinosternon scorpioides*: No data; pet trade specimen alive in collection of W.P. McCord.
SON – *Kinosternon sonoriense*: Arizona, Maricopa Co., Tempe, canal along Salt River bottom; specimen alive in collection of P. Rosen.
SUB – *Kinosternon subrubrum subrubrum*: North Carolina, Union Co., Jct SR 1006E and SR 1757N; ASU-WTS 45-92.
HIP – *Kinosternon subrubrum hippocrepis*: Florida, Santa Rosa Co., Goose Pond N of Munson; UF 80820.
STE – *Kinosternon subrubrum steindacheri*: Florida, Lee Co., no further data; UF 84961.

APPENDIX II

Cytochrome *b* sequence data. Abbreviations of taxon names are listed in Appendix I. Ambiguous bases are coded as "9."

TRI TTTTGGATCA CTTCTTGGCA TCTGCTTAAT CCTACAAATT ACTACCGGCA
TCTTCTTAGC TATACACTAC TCATCCGACA TATCCCTAGC ATTCTCAACA
GTAGCCCACA TTACTCGAGA CGTACAATAC GGCTGACTTA TCCGCAACCT
ACACGCCAAC GGCCTTTCAA TATTCTTCAT CTGCATTTAT ATGCACATCG
GACGAGGCAT TTACTACGGC TCATACCTCT ATAAAGAAAC TTGAAACACA
GGAATTCTTC TACTACTATT AACTATAATA ACTGCATTCA

SAL TTTTGGATCA CTTCTTGGCA TCTGCTTAAT CCTACAAATT ACCACCGGCA
TCTTCTTAGC TATACACTAC TCATCCGACA TATCCCTAGC ATTCTCAACA
GTAGCCCACA TTACTCGAGA CGTACAATAC GGCTGACTTA TCCGCAACCT
ACACGCCAAC GG9GCTTCAA TATTCTTCAT CTGCATTTAT ATGCACATCG
GACGAGGCAT TTACTACGGC TCATACCTCT ACAAAGAAAC TTGAAACACA
GGAATTCTAC TACTACTATT AACTATAATA ACTGCATTCA

ANG TTTTGGATCA CTTATTGGCA TTTGCTTAAT CCTACAAATT ACTACCGGTA
TCTTCTTAGC CATACACTAC TCATCTGATA TATCCTTAGC ATTCTCAACA
GTAGCCCACA TCACCCGAGA CGTACAATAC GGTTGACTCA TCCGCAACAT
ACATGCCAAC GGTGCCTCAA TATTCTTCAT CTGCATCTAC ATACACATCG
GACGAGGTGT TTACTACGGT TCATACCTCT ACAAAGAAAC TTGAAACACA
GGAATTTTCT TACTGCTACT AACTATAATA ACTGCATTCA

CAR CTTCGGATCA TTACTCGGCA TCTGCTTAAT CCTGCAAATC ACTACCGGTA
TCTTCCTAGC AATACACTAT TCATCTGACA TATCACTAGC ATTCTCATCA
GTTACCCATA CAATTCGAGA CGTACAATAC GGCTGACTCA TTCGAAACCT
CCATGCCAAC GGCGCTTCTC TATTCTTTAT ATGTATCTAC ATGCACATTG
GACGAGGAAT CTACTACGGC TCATACCTGT ACAAAGAAAC TTGAAACACT
GGAATTATAC TATTACTACT AACCATAGCC ACCGCATTCA

DEP CTTCGGATCA TTACTCGGCA CCTGCTTAAT CCTACAAATC ACCACCGGCA
TCTTCCTAGC AATACACTAT TCATCTGACA TATCACTAGC ATTCTCATCA
GTCACTCATA CAATTCGAGA CGTACAATAC GGCTGACTCA TTCGAAACCT
CCATGCCAAC GGCGCTTCTC TATTCTTTAT ATGCATCTAT ATGCACATTG
GACGAGGAAT CTACTACGGC TCATACCTGT ACAAAGAAAC TTGAAACACT
GGAATTATAC TACTACTACT AACCATAGCC ACCGCATTCA

MIN CTTCGGATCA TTACTCGGCA CCTGTTTAAT CCTACAAATC ATTACTGGTA
TCTTCCTAGC AATACACTAC TCATCTGACA TATCATTAGC ATTCTCATCA
GTCACCCATA CAATTCGAGA CGTACAATAC GGCTGACTTA TCCGAAACCT
CCATGCCAAC GGCGCTTCCC TATTTTTTAT GTGTATCTAC ATGCATATCG
GACGAGGAAT CTACTACGGC TCATACCTAT ACAAAGAAAC TTGAAACACC
GGAATTATAC TACTACTACT AACTATAGCT ACCGCATTCA

ODO CTTCGGATCA TTACTCGGCA CCTGCTTAAT CCTACAAATC ACCACCGGCA
TCTTCCTAGC AATACACTAT TCATCTGACA TATCACTAGC ATTCTCATCA
GTCACCCATA CAATTCGAGA CGTACAATAT GGCTGACTTA TCCGAAACCT
CCATGCCAAT GGCGCTTCCC TATTCTTTAT ATGTATCTAT ATGCACATTG
GACGAGGAAT CTACTACGGC TCATACCTCT ACAAAGAAAC TTGAAACACT
GGAATTATAC TACTACTACT AACTATAGCC ACCGCATTCA

ALA CTTCGGATCA TTACTTGGCA CCTGCTTAAT ACTACAAATT ATTACCGGCA
TCTTCCTAGC AATACACTAT TCATCTGACA TATCACTAGC ATTCCAATCA
ATCACCCATA CAATTCGAGA CGTACAATAC GGCTGACTCA TCCGAAACCT
CCACGCCAAT GGCGCCTCCC TATTTTTTAT ATGTATCTAC ATTCATATTG
GACGAGGAAT CTACTACGGC TCATACCTCT ACAAAGAAAC TTGAAATACC
GGAATTATAT TACTACTACT AACCATAGCT ACTGCATTTA

BAU CTTTGGATCA CTACTTGGCA CCTGCTTAAT CCTACAAATT ACTACCGGTA
TCTTCCTAGC AATACACTAT TCATCTGATA TATCATTAGC ATTCTCATCA
ATTACTCATA CAATTCGAGA CGTACAATAC GGCTGACTTA TCCGAAACCT
CCATGCCAAC GGTGCTTCCC TATTCTTCAT ATGTATCTAC ATACACATCG
GACGAGGAAT CTACTACAAC TCATACCTTT ACAAAGAAAC TTGAAACACC
GGAGTTATAC TACTACTACT AACCATAGCT ACTGCATTCA

FLA CTTTGGATCA AAACTCGGCA CCTGCTTAGT CCTACAAACT ACTACCGGCA
TCTTCGTAGC AATACACTAC TCATCTGACA TATCATTAGC ATTCTCATCA
GTCACCCATA CAATTCGAGA CGTACAATAC GGTTGACTTA TCCGAAACCT
CCACGCCAAT GGGGCTTCCT TATTTTTCAT GTGCATCTAC ATACACATCG
GACGAGGAAT CTACTACGGC TCATACCTCC GCAAAGAAAC TTGAAACACT
GGAGTTATAC TACTACTACT AACCATAGCC ACTGCATTCA

HIR CTTCGGATCA TTACTTGGCA CCTGCCTAAT CCTACAAATT ATTACCGGCA
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