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Short Communication

Extreme difference in rate of mitochondrial and nuclear DNA evolution in a large ectotherm, Galápagos tortoises

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Abstract

We sequenced approximately 4.5 kb of mtDNA from 161 individuals representing 11 named taxa of giant Galápagos tortoises (*Geochelone nigra*) and about 4 kb of non-coding nuclear DNA from fewer individuals of these same 11 taxa. In comparing mtDNA and nucDNA divergences, only silent substitutions (introns, ITS, mtDNA control region, and synonymous substitutions in coding sequences) were considered. mtDNA divergence was about 30 times greater than that for nucDNA. This rate discrepancy for mtDNA and nucDNA is the greatest yet documented and is particularly surprising for large ectothermic animals that are thought to have relatively low rates of mtDNA evolution. This observation may be due to the somewhat unusual reproductive biology and biogeographic history of these organisms. The implication is that the ratio of effective population size of nucDNA/mtDNA is much greater than the usually assumed four. The nearly neutral theory of molecular evolution predicts this would lead to a greater difference between rates of evolution.

Keywords: mtDNA; ITS; Introns; *Geochelone nigra*; Galápagos

1. Introduction

Since the classic demonstration by Brown et al. (1979) that mitochondrial DNA (mtDNA) evolves considerably faster than nuclear DNA (nucDNA), this observation has been repeatedly confirmed in virtually all eukaryotic animals. Quantitatively, the accepted difference, repeated in a number of widely used texts, is that mtDNA evolves 5–10 times faster than nucDNA (e.g., Avise, 1994; Hartl and Clark, 1997; Li, 1997). Here we report a more extreme difference between rates of evolution of mtDNA and nucDNA in a large reptile, the Galápagos giant tortoises, *Geochelone nigra* (*elephantopus*). To our knowledge, this is the largest discrepancy in rates ever documented.

There are 15 described taxa of Galápagos tortoises, of which 11 survive today (MacFarland et al., 1974; Pritchard, 1996). The precise taxonomic status of these taxa is debated, primarily as to whether they should be considered species or subspecies (Zug, 1997). We have been conducting genetic studies of the 11 extant taxa and have shown that many are genetically distinct monophyletic units (Caccone et al., 1999, 2002; Ciofi et al., 2002) that by some species definitions would qualify them as species (Avise and Ball, 1990; Baum, 1992; Cracraft, 1989). Because these taxa are very recently diverged, most of our work has concentrated on mtDNA and microsatellites. The deepest node in the phylogeny is no more than 2.5 mya and possibly as recent as 1.5 mya; the more recent taxa splits are likely less than 0.5 mya (Caccone et al., 2002). In an attempt to confirm the conclusions from the other data sets, we sequenced nucDNA regions that should be the fastest evolving: introns of protein coding genes and the

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internal transcribed spacer (ITS-1)[1] of the nuclear rDNA region. To our surprise, we found virtually no variation for these nucDNA regions.

2. Materials and methods

2.1. Tortoises

The collection of tortoise blood, storage, DNA extractions, etc. has been described previously (Caccone et al., 1999, 2002). We sequenced between one and seven individuals from each taxon for the nucDNA regions described below. As outgroups, we sequenced three mainland South American tortoises: *Geochelone chilensis*, *Geochelone carbonaria*, and *Geochelone denticulata.* On average, 23 tortoises were sequenced for each intron or ITS-1 with a maximum of 37 for the aldolase I intron and a minimum of 11 for the creatine kinase introns.

2.2. Nuclear sequences

A total of 4108 bp of nuclear DNA, largely introns, was sequenced using the EPIC method described by Palumbi (1996). Using the conserved primer sequences in exons described in Palumbi (1996) we PCR amplified actin introns I and II, aldolase introns I and II, and creatine kinase introns 6 and 7; primers described in Duda and Palumbi (1999) were used to amplify calmodulin introns 1 and 2. In addition we sequenced the internal transcribed space of the rDNA region, ITS-1, based on conserved primer regions (Lessa and Applebaum, 1993). All sequencing was performed on an ABI automated sequencer following manufacturer's protocols; both strands were sequenced in all cases. Specific primers for these animals as well as sequencing primers, PCR conditions, and alignments are available from the authors. All nuclear sequences have been deposited in GenBank with Accession Nos. actin, AY101612-37; aldolase 1, AY101638-77; aldolase 2, AY101678-707; calmodulin, AY101708-33; creatine kinase, AY101734-48; ITS-1, AY1011749-63. Accession to the mtDNA sequences can be found in Caccone et al. (2002).

2.3. Data selection

The mtDNA data are from 161 Galápagos tortoises sequenced for a total of 4.5 kb each, including the sequences of the 12S and 16S ribosomal subunit genes, Cytochrome *b*, Nd5, Nd6 genes, and control region (Caccone et al., 2002). In our attempt to compare rates of evolution of mtDNA and nucDNA we excluded the 12S and 16S mtDNA genes from the mtDNA data set. This reduced the mtDNA data set to 3506 bp. For both mtDNA and nucDNA we calculated the number of silent substitutions per site (K_s), with silent including synonymous and noncoding substitutions. For the nuclear DNA, only 257 of the 4108 bp were coding, i.e., the exon priming sites included some coding sequences. We considered only 10 taxa of the 11 analyzed because there is good evidence that the two southern most described taxa on Isabela are not genetically distinct (i.e., there is ongoing gene flow; Caccone et al., 1999, 2002; Ciofi et al., 2002); therefore, we treated these two as a single taxon.

2.4. Analyses

Divergence between taxa was calculated by comparing pairs of individuals from different taxa and average across all individual pairwise comparisons. To compute K_s (here, the number of silent substitutions per site) for each pair of sequences we used the algorithm proposed by Nei and Gojobori (1986), as implemented in DNASP ver 3 (Rozas and Rozas, 1999). Although based on the Jukes and Cantor (1969) distance, Nei and Gojobori's algorithm still performs well, when compared with theoretically more precise maximum-likelihood based algorithms (Castresana, 2002; Smith and Hurst, 1999). The phylogenetic trees presented are distance-based trees: Neighbor-Joining (Saitou and Nei, 1987) based on Tamura and Nei (1993) procedures for estimating distances. Bootstrap analysis was done to determine the strength of the nodes (Felsenstein, 1985). Phylogenetic analyses were performed using PAUP* (Swofford, 2001).

3. Results and discussion

Fig. 1 plots the K_s values for mtDNA and nucDNA. Note that the *Y*-axis is 10 times the *X*-axis, otherwise the line would be virtually flat. Thus the true slope is 1/10 that shown in the figure. The reciprocal of the true slope is about 30. This implies mtDNA is evolving about 30 times faster than nucDNA at what are generally thought to be neutral or nearly neutral sites.

Fig. 2 presents the phylogenetic analysis. The mtDNA tree gives excellent resolution as detailed previously (Caccone et al., 2002) where we also showed that maximum parsimony, maximum likelihood, and Bayesian methods all produce the same, well-supported tree. The nucDNA tree is totally unresolved with virtually no divergence among taxa. The only information in the nucDNA is that it strongly confirms the monophyly of the Galápagos lineage and reasonably supports the previous conclusion that *G. chilensis* is the closest relative on the mainland of South America.

This low variation among taxa is also reflected in low variation among all the sequences. The average

[1] *Abbreviation used:* ITS, internal transcribed space of rDNA.

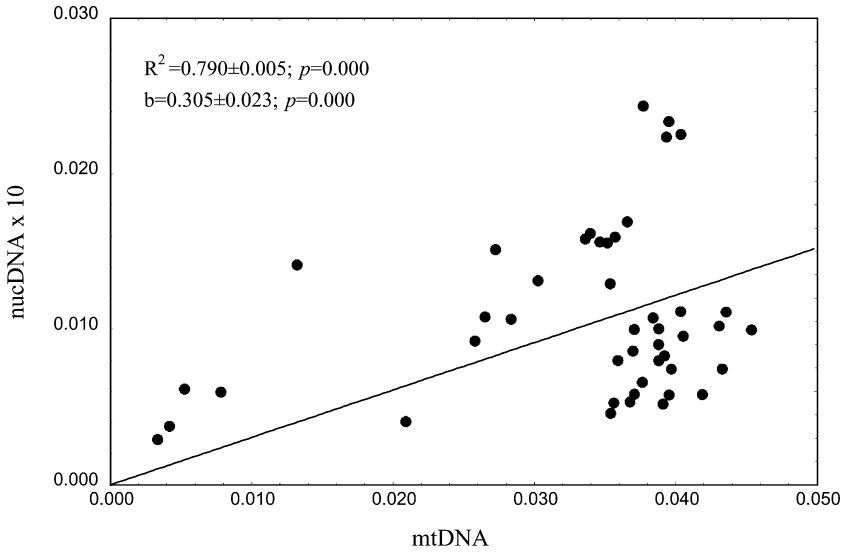

Fig. 1. K_s values (the number of silent substitutions per site, based on Jukes and Cantor, 1969) between all pairs of the 11 named taxa of Galápagos tortoises. Dots indicate the average of all pairwise comparison of individuals from different taxa. Note that the *Y*-axis (nucDNA divergence) is 10 times that of the *X*-axis, and thus the slope is one-tenth that shown.

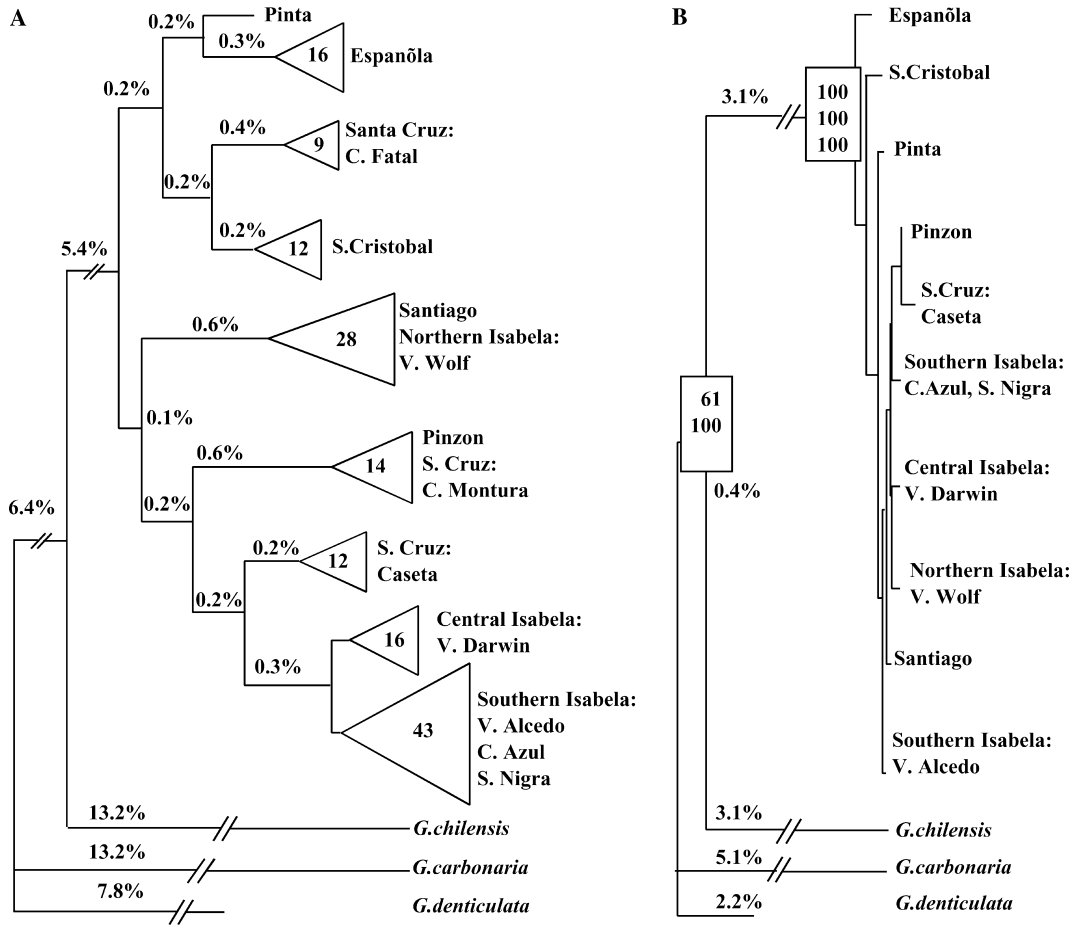

Fig. 2. Comparison of NJ trees for mtDNA (A) and nucDNA (B) drawn to the same scale. Estimated distances are indicated on the branches. Only bootstrap values greater than 50% are shown. Every node labeled in the mtDNA tree has an associated bootstrap value of >98% and an identical topology was obtained for MP, ML, and Bayesian analyses (see Caccone et al., 2002 for details). While the nucDNA tree is an NJ tree, bootstrap values for NJ, MP, and ML are shown (top to bottom) for the only two nodes with values >50%.

pair-wise per site heterozygosity (π, Saitou and Nei, 1987) is 0.011 for mtDNA. The nucDNA introns vary in π from 0 to 0.002; for the ITS π is 0.0005. The overall mean π for the whole nucDNA data set is 0.0003.

In addition to the extreme rate difference between mtDNA and nucDNA, these data also appear to be in conflict with the general pattern of rate differences between ectothermic and endothermic vertebrates (Martin and Palumbi, 1993; Rand, 1994): mtDNA of ectotherms generally evolves slower than endotherms. Also, there is a negative correlation between body size and rate of mtDNA for both ectotherms and endotherms, i.e., vertebrates with larger body size have slower rates of mtDNA evolution. In order to be consistent with the geological history of the Galápagos, we estimated an absolute rate of mtDNA evolution for these tortoises at nearly 1%/my (Caccone et al., 2002). This is among the highest ever recorded for an ectothermic vertebrate and within the range of endotherms (Rand, 1994). Yet these tortoises are the largest extant terrestrial herbivorous ectotherms reaching a size of up to 400 kg (Pritchard, 1996).

The extreme rate difference may be related to the reproductive biology and biogeographic history of these tortoises. Sex is determined by the temperature at which eggs are incubated: warmer temperatures produce females, cooler temperatures males. The Galápagos are notorious for fluctuations in year to year temperatures due largely to ENSO events (''El Niño''). Thus sex ratio may fluctuate widely meaning that the relative effective population sizes of mtDNA and nucDNA may likewise be temporally highly variable. Furthermore, females are known to store sperm from several males for long periods. This means that if a single gravid female founded a new population on a recently emerged island, the number of mtDNA lineages is one whereas the nucDNA copies are $2+2N$, where N is the number of males who mated with the female. Both the widely fluctuating sex ratio and multiple male sperm storage implies that the ratio of nucDNA and mtDNA effective population sizes is even greater than the fourfold assumed for species with 1:1 sex ratios (Birky et al., 1989). This can explain why there is more phylogenetic signal in the mtDNA as lineage sorting and coalescence will occur more rapidly with a lower N_e (Hudson, 1990). But, assuming equilibrium, the larger N_e of nucDNA should lead to greater overall nucleotide diversity under the neutral theory, which is not the case; almost certainly equilibrium predictions do not hold for many of these recent lineages as confirmed by DNA sequence mismatch analysis (Beheregaray et al., 2003).

This observation of a more extreme difference in effective population size of mtDNA compared to nucDNA affecting the rate of substitution is in accord with the nearly neutral theory of molecular evolution (Ohta, 1992, 1995). This theory predicts higher rates of slightly deleterious nucleotide substitutions in smaller populations as well as in populations that have experienced repeated founder events (see also Lynch, 1997). This was first empirically documented in another remote oceanic island system, Hawaiian *Drosophila* (DeSalle and Templeton, 1988). These demographic, biogeographic, and reproductive biology features of Galápagos tortoises, coupled with the well-known higher rate of mtDNA mutation (Rand, 2001), could account for this extreme difference in evolutionary rates.

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References

Avise, J.C., 1994. Molecular Markers, Natural History and Evolution. Chapman & Hall, New York, London.

Avise, J.C., Ball, R.M., 1990. Principles of genealogical concordance in species concepts. Oxford Surveys Evol. Biol. 7, 45–67.

Baum, D., 1992. Phylogenetic species concepts. Trends Ecol. Evol. 7, 1–2.

Beheregaray, L.B., Ciofi, C., Caccone, A., Gibbs, J.P., Powell, J.R., 2003. Genetic divergence, phylogeography, and conservation units of giant tortoises from Santa Cruz and Pinzón, Galápagos Islands. Conserv. Genet. 4, 31–46.

Birky Jr., C.W., Fuerst, P., Maruyama, T., 1989. Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approaches to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. Genetics 121, 613–627.

Brown, W.M., George Jr., M., Wilson, A.C., 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA 76, 1967–1971.

Caccone, A., Gibbs, J.P., Ketmaier, V., Suatoni, E., Powell, J.R., 1999. Origin and evolutionary relationships of giant Galápagos tortoises. Proc. Natl. Acad. Sci. USA 96, 13223–13228.

Caccone, A., Gentile, G., Gibbs, J.P., Fritts, T.H., Snell, H.L., Powell, J.R., 2002. Phylogeography and history of giant Galápagos tortoises. Evolution 56, 2052–2066.

Castresana, J., 2002. Estimation of genetic distances from human and mouse introns. Genome Biol. 3 (research0028.1-research0028.7).

Ciofi, C., Milinkovitch, M., Gibbs, J.P., Caccone, A., Powell, J.R., 2002. Nuclear DNA microsatellite analysis of genetic divergence among and within island populations of giant Galápagos tortoises. Mol. Ecol. 11, 2265–2283.

Cracraft, J., 1989. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns

and processes of differentiation. In: Otte, D., Endler, J.A. (Eds.), Speciation and its Consequences. Sinauer, Sunderland, pp. 28–59.
DeSalle, R., Templeton, A.R., 1988. Founder effects and the rate of mitochondrial DNA evolution in Hawaiian *Drosophila*. Evolution 42, 1076–1084.
Duda, T.F., Palumbi, S., 1999. Developmental shifts and species selection in gastropods. Proc. Natl. Acad. Sci. USA 96, 10272–10277.
Felsenstein, J., 1985. Confidence limits on phylogenetic trees: an approach using the bootstrap. Evolution 39, 783–791.
Hartl, D.L., Clark, A.G., 1997. Principles of Population Genetics, third ed. Sinauer, Sunderland.
Hudson, R.R., 1990. Gene genealogies and the coalescence process. Oxford Surv. Evol. Biol. 7, 1–44.
Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21–132.
Lessa, E.P., Applebaum, G., 1993. Screening techniques for detecting allelic variation in DNA sequences. Mol. Ecol. 2, 119–129.
Li, W.-H., 1997. Molecular Evolution. Sinauer, Sunderland.
Lynch, M., 1997. Mutation accumulation in nuclear, organelle, and prokaryotic transfer RNA genes. Mol. Biol. Evol. 14, 914–925.
MacFarland, C.G., Villa, J., Toro, B., 1974. The Galápagos giant tortoises (*Geochelone elephantopus*) part I: status of the surviving populations. Biol. Conserv. 6, 118–133.
Martin, A.P., Palumbi, S.R., 1993. Body size, metabolic rate, generation time, and the molecular clock. Proc. Natl. Acad. Sci. USA 90, 4087–4091.
Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3, 418–426.
Ohta, T., 1992. The nearly neutral theory of molecular evolution. Annu. Rev. Ecol. Syst. 23, 263–286.
Ohta, T., 1995. Synonymous and nonsynonymous substitutions in mammalian genes and the nearly neutral theory. J. Mol. Evol. 40, 56–63.
Palumbi, S.R., 1996. PCR and molecular systematics. In: Hillis, D., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer Press, Sunderland, pp. 205–248.
Pritchard, P.C.H., 1996. The Galápagos tortoises. Nomenclatural and Survival Status. Chelonian Research Monographs 1, 1–85.
Rand, D.M., 1994. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. Trends Ecol. Evol. 9, 125–131.
Rand, D., 2001. The units of selection on mitochondrial DNA. Annu. Rev. Ecol. Syst. 32, 415–448.
Rozas, J., Rozas, R., 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15, 174–175.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Smith, N.G., Hurst, L.D., 1999. The effect of tandem substitutions on the correlation between synonymous and nonsynonymous rates in rodents. Genetics 153, 1395–1402.
Swofford, D., 2001. PAUP*, Phylogenetic Analysis Using Parsimony (* and other Methods). Version 4.0b. Sinauer Associates, Sunderland.
Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.
Zug, G.R., 1997. Galápagos tortoise nomenclature: still unresolved. Chelonian Conserv. Biol. 2, 618–619.