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PHYLOGENETIC RELATIONSHIPS OF *LYGODACTYLUS* GECKOS FROM THE GULF OF GUINEA ISLANDS: RAPID RATES OF MITOCHONDRIAL DNA SEQUENCE EVOLUTION?

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Mitochondrial DNA (12S rRNA, 16S rRNA and cytochrome *b*) sequences and nuclear sequences (C-*mos*) were analysed within *Lygodactylus thomensis* from three volcanic islands in the Gulf of Guinea that have never been connected to the continent. Our aim was to assess interrelationships between the three subspecies to test a recent hypothesis suggesting high rates of mitochondrial DNA (mtDNA) sequence evolution in geckos. Our results indicate, based on mtDNA sequence data, that the three subspecies are genetically differentiated at a level more typically observed between species. However, the forms cannot be differentiated using the nuclear marker C-*mos*. These results further substantiate the hypothesis of rapid rates of mtDNA sequence evolution in geckos, although the alternative that C-*mos* is evolving more slowly cannot be discounted. They also suggest that present calibrations for molecular clocks are at the upper limit of divergence over time.

Key words: dwarf gecko, genetic analysis, phylogeny, São Tomé, Príncipe, Annobon

INTRODUCTION

The islands of the Gulf of Guinea are part of a volcanic chain formed during the middle to late Tertiary. Bioko (formerly Fernando Pó) is the largest and closest to Africa, only about 32 km from Cameroon. Smaller and more geographically isolated are São Tomé and Príncipe (1001 km^2 combined), which include a number of small islets, and 160 km southwest of São Tomé, Annobon (17 km^2; Fig. 1). Estimated ages for the origins of Príncipe, São Tomé and Annobon are 31 my, 14 my and 4.8 my respectively (Lee *et al*., 1994). These three islands have never been interconnected, or linked to the continent. This isolation has promoted species divergence and evolution, and they presently harbour several endemic species, including the dwarf gecko, *Lygodactylus thomensis*.

Lygodactylus contains about 60 species, with a centre of distribution in sub-Saharan Africa. Unusually for geckos, dwarf geckos are diurnal. *Lygodactylus thomensis* is the only dwarf gecko known from Príncipe, São Tomé and Annobon. Three subspecies have been recognized, *L. t. thomensis* from São Tomé, *L. t. delicatus* from Príncipe and *L. t. wermuthi* from Annobon.

Although many phylogenetic studies have been performed on the gecko fauna of the more northern Atlantic volcanic islands, such as the Cape Verde archipelago (Carranza *et al*., 2000; Jesus *et al.*, 2001, 2002) and the Canary Islands (Gübitz *et al*., 2005), very little is known about the fauna of the islands of the Gulf of Guinea. A recent phylogenetic study of the geckos *Hemidactylus* indicated that the commonest species, *H. mabouia*, was probably introduced, and also indicated the existence of a genetically distinct lineage (Jesus *et al*., 2005*a*) that may in fact be *H. longicephalus* (Carranza & Arnold, 2006). Like other recent studies on geckos (e.g. Austin *et al.*, 2004; Kasapidis *et al*., 2005; Kronauer *et al*., 2005; Lamb & Bauer, 2000; Harris *et al*., 2004*a*,*b*) this work highlighted extraordinarily high levels of mtDNA sequence divergence within morphologically conservative geckos. However, in the studies where a comparison with nuclear DNA sequence data has been available (Austin *et al*., 2004; Harris *et al*., 2004*b*; Jesus *et al*., 2005*a*) variation within the nuclear markers has been low or non-existent. This led to the speculation that geckos may have a relative fast rate of mtDNA evolution (Jesus *et al*., 2002; Harris *et al*., 2004*a*). Using both mitochondrial and nuclear DNA sequences we aim to (1) examine the levels of variation between forms on the three islands, and compare this with the age of the islands; (2) determine the possible colonization sequence of the islands, and compare colonization rates and patterns to those of other species; and (3) further test the hypothesis of high rates of mtDNA sequence evolution in an additional gecko species.

MATERIALS AND METHODS

The number and geographic locations of the specimens used in this study are given in Table 1 and Fig. 1. Voucher specimens are housed in the collections of the University of Madeira. Total genomic DNA was extracted from small pieces of tail using standard

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TABLE 1. Specimens used in this study. Localities refer to Fig. 1. Codes refer to voucher specimens and to Fig. 2.

Species	Locality	Code
L. t. wermuthi	Annobon	638, 639, 640,641, 642, 643, 645, 646, 649, 650
L. t. thomensis	S. Nicolau, São Tomé	725, 727
L. t. delicatus	Montalegre, Príncipe	699, 700
	Terra Velha, Príncipe	720
L. capensis	Tanzania	TZ32
L. luteopicturatus	Tanzania	TZ1

methods (Sambrook *et al.*, 1989). Primers used in both amplification and sequencing of mitochondrial DNA were 16SL and 16SH, 12Sa and 12S*b*, and Cytochrome *b*1 and 3 from Kocher *et al.* (1989). Amplification conditions were the same as those described by Harris *et al.* (1998). Primers used to amplify a fragment of the nuclear gene C-*mos* were G73 and G74, and were used following the conditions given by Saint *et al.* (1998). C-*mos* sequences have been widely used to infer relationships at many levels within geckos (e.g. Austin *et al.*, 2004; Carranza *et al.*, 2002; Han *et al.*, 2004; Harris *et al.*, 2004*b*). Two outgroup species were also sequenced for all four gene regions, *Lygodactylus luteopicturatus* and *Lygodactylus capensis*. Additionally for the analyses based only on C-*mos*, sequences of *Lygodactylus* sp. and *Lygodactylus bradfieldi* were also included (Austin *et al.*, 2004; Han *et al.*, 2004). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Sequences were aligned using Clustal W (Thompson *et al.*, 1994). Length variation in loop regions of the rRNAs was relatively limited, and all positions were included in the analysis. Mitochondrial DNA sequences were imported into PAUP* 4.0b10 (Swofford, 2003) for phylogenetic analysis. For the phylogenetic analysis of the combined data, we used maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference. We used the approach outlined by Huelsenbeck & Crandall (1997) to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada & Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree employing ML (Felsenstein, 1981) with random sequence addition (10 replicate heuristic search). The MP analysis was also performed with random sequence addition (100 replicate heuristic searches). In both MP and ML support for nodes was estimated using the nonparametric bootstrap technique (Felsenstein, 1985) with 1000 replicates. The Bayesian analysis was implemented using MrBayes (Huelsenbeck & Ronquist, 2001). Two independent replicates were conducted and inspected for consistency to check for local optima. Both analyses were conducted with random starting trees, run for 1 x 10^6 generations, and sampled every 1000 generations using a general-time-reversible model of evolution with a gamma model of among-site rate variation. In both searches, stationarity of the Markov Chain was determined as the point when sampled ln-likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. New sequences from C-*mos* for nine individuals were aligned against the published *Lygodactylus* sequences. There were no indels. Because no characters were homoplastic (consistency index=1) only an MP analysis was performed.

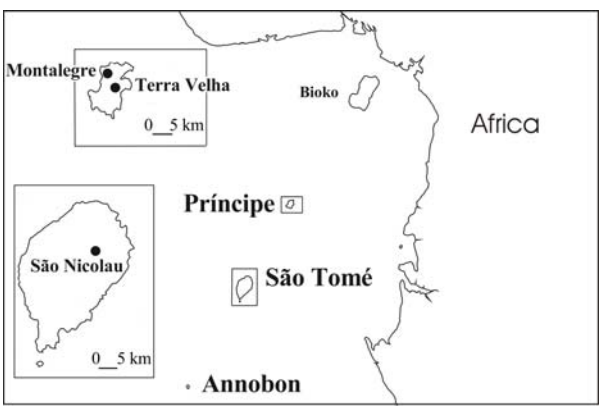

FIG. 1. Map showing the sampling localities of *Lygodactylus* from the Gulf of Guinea. The outgroup samples are both from Tanzania.

RESULTS

For the combined mtDNA gene fragments, 17 individuals were included for a total of 1177 base pairs; ML, MP and Bayesian analyses gave identical estimates of relationships (Fig. 2). The most appropriate model for the combined data was the GTR model with an estimate of invariable sites (0.21) and a discrete approximation of the gamma distribution (0.41). The ML heuristic search using this model found two trees of -Ln 3590. Bayesian analysis produced an identical estimate of relationships to one of these. For MP 176 characters were informative, and the MP search found one tree of 458 steps (Fig. 2). All analyses varied only in relationships between individuals from Annobon. In all analyses all three islands formed monophyletic clades with 100% support. *Lygodactylus thomensis* is a monophyletic group, similarly with 100% support, relative to the included outgroups. Also supported is a sister-taxa relationship between *L. t. delicatus* from Príncipe and *L. t. wermuthi* from Annobon. Average levels of sequence divergence between congeneric rep-

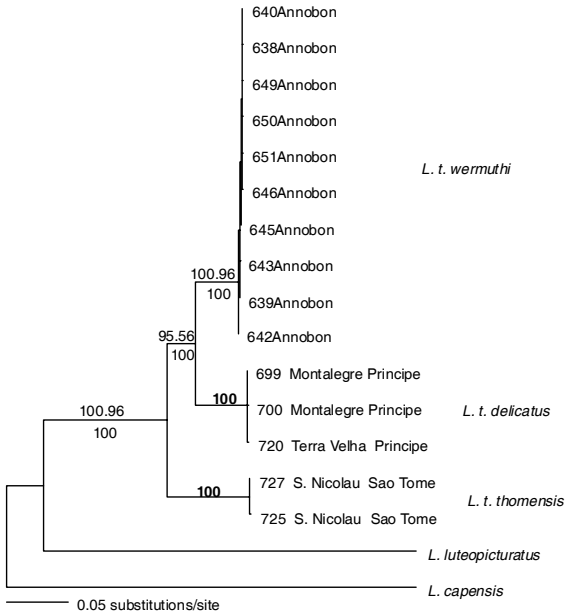

FIG. 2. One of two trees derived from an ML analysis of combined 12S and 16S rRNA fragments using the model described in the text. MP and Bayesian analyses gave identical estimates of relationships. Bootstrap values (>50%) for MP and ML are given above the nodes, and Bayesian probabilities are given below the nodes. When all values were the same, one value is given. The tree was rooted using *Lygodactylus capensis* and *L. luteopicturatus.*

tile species is known to average approximately 12% for cytochrome *b* (Harris, 2002). Sequence divergence for cytochrome *b* between populations from Annobon and Príncipe is approximately 10%, and between Príncipe and São Tomé approximately 15%.

For the C-*mos* nuclear DNA sequences 11 characters were parsimony-informative. A heuristic search found a single tree of 28 steps (Fig. 3). Our analyses of variation of C-*mos* indicate minimal variation within *Lygodactylus thomensis*. Only three haplotypes were found, with two individuals being heterozygous. One haplotype was found in individuals from all three islands. *Lygodactylus thomensis* was clearly differentiated from the other species included in the analysis.

DISCUSSION

Analysis of the mtDNA sequences produced a robust estimate of relationships for populations from the three islands. Presently the species appears to be monophyletic although including additional *Lygodactylus* species in the analysis would be necessary to confirm this. The populations from Annobon and Príncipe are sister taxa. Given the geographical remoteness and younger geological age of Annobon, and that the majority of individuals from Annobon had a derived haplotype for the C-*mos*, it seems very likely that Annobon was colonized from Príncipe. Thus *Lygodactylus* on these islands do not fit a classic "stepping-stones" model of island colonization. Nor do they show the same pattern as *Hemidactylus*, where *H.*

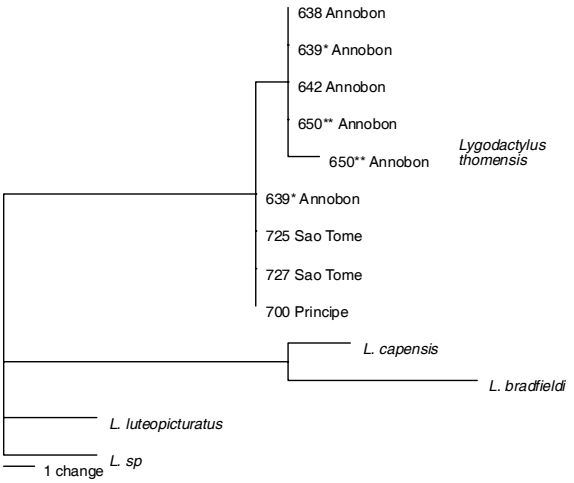

FIG. 3. Single MP tree showing relationships derived from partial sequences of C-*mos*. The * and ** indicates the heterozygous alleles from the same individuals.

newtonii that is endemic to Annobon is sister taxon to a form from São Tomé (Jesus *et al.*, 2005*a*) that is either a new species or may correspond to *H. longicephalus*. They also differ from *Mabuya* skinks, which independently colonized each island (Jesus *et al.*, 2005*b*). These differences in colonization patterns highlight the difficulties in drawing general conclusions regarding how islands are colonized from only a few species – clearly stochastic processes play an important role.

Despite the much greater ages of São Tomé and Príncipe relative to Annobon, the time delay between colonization events is relatively similar. Carranza *et al*. (2000), using 12S rRNA and cytochrome *b* sequences calibrated for *Tarentola* geckos in the Canary Islands, estimated 1.96% sequence divergence per million years. Since *Hemidactylus* have similar size and preferred temperatures this estimate is likely to be appropriate in this group also (Gillooly *et al.*, 2005). Based on our estimate of relationships we cannot determine if São Tomé or Príncipe was the first island colonized. However, the 10% divergence between them for 12S and cytochrome *b* combined sequences suggests that there was an approximately five million year delay between colonization of the first and second islands. The 8% divergence between Príncipe and Annobon would indicate that Annobon was colonized approximately four million years ago, less than one million years after its formation. This is a relatively short delay given the small size and isolation of Annobon – the delay before Madeira was colonized by the lacertid lizard *Lacerta dugesii*, for example, was closer to 10 million years (Brehm *et al.*, 2003). This supports the hypothesis that geckos are relatively rapid reptile colonizers, probably due to the ability of their calcareous-shelled eggs to resist salt water and to be able to be rafted from place to place (Brown & Alcala, 1957). It further suggests that calibration of molecular clocks at a slower rate than that used here would be inappropriate, at least for *Hemidactylus*, as they would predict that Annobon was colonized prior to its formation.

Given the high levels of mtDNA sequence divergence between populations from the different islands we would have expected to see some variation within the C-*mos* sequences. Variation within *Lacerta schreiberi*, for example, is less than half the level seen for mtDNA sequences, but four haplotypes at C-*mos* have been reported (Paulo *et al*., 2002; Godinho *et al*., 2001). Similar situations occur in *Mabuya* from the Cape Verde Archipelago (Brehm *et al*., 2001) and in *Lacerta dugesii* (Brehm *et al.*, 2003; Jesus *et al*., 2005*c*). At the same time almost every study of intraspecific variation within geckos has uncovered extremely high levels of mtDNA sequence variation (e.g. Austin *et al*., 2004; Harris *et al*., 2004*a*,*b*; Kasapidis *et al*., 2005; Kronauer *et al*., 2005; Rocha *et al*., 2005). Such levels are much higher than typically seen in other vertebrates – up to 26.9% for cytochrome *b* in *Thecadactylus rapicauda*, for example (Kronauer *et al*., 2005). Although there are other possible explanations, such as an artefact of taxonomy due to morphological conservativeness of geckos, combined with low levels of variation with C-*mos* the data are consistent with the theory of an elevated rate of mtDNA sequence evolution in geckos. More nuclear markers from diverse groups of geckos will be needed to test this further.

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REFERENCES

Austin, J. J., Arnold, E. N. & Jones, C. G. (2004). Reconstructing an island radiation using ancient and recent DNA: the extinct and living day geckos (*Phelsuma*) of the Mascarene islands. *Molecular Phylogenetics and Evolution* **31**, 109–122.

Brehm, A., Jesus, J., Pinheiro, M. & Harris, D. J. (2001). Relationships of scincid lizards (*Mabuya* spp; Reptilia: Scincidae) from the Cape Verde islands based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **19**, 311–316.

Brehm, A., Jesus, J., Spínola, H., Alves, C., Vicente, L. & Harris, D. J. (2003). Phylogeography of the Madeiran endemic lizard *Lacerta dugesii* inferred from mtDNA sequences. *Molecular Phylogenetics and Evolution* **26**, 222–230.

Brown, W. C. & Alcala, A. C. (1957). Viability of lizard eggs exposed to salt water. *Copeia* **1957**, 39–41.

Carranza, S. & Arnold, E. N. (2006). Systematics, biogeography and evolution of *Hemidactylus* geckos (Reptilia, Gekkonidae) elucidated using mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* **38**, 531–545.

Carranza, S., Arnold, E. N., Mateo, J. A. & López-Jurado, L. F. (2000). Long-distance colonization and radiation in gekkonid lizards, *Tarentola* (Reptilia: Gekkonidae), revealed by mitochondrial DNA sequences. *Proceedings of the Royal Society of London B* **267**, 637–649.

Carranza, S., Arnold, E. N., Mateo, J. A. & Geniez, P. (2002). Relationships and evolution of the North African geckos *Geckonia* and *Tarentola* (Reptilia: Gekkonidae), based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **23**, 244–256.

Godinho, R., Domingues, V., Crespo, E. G. & Ferrand, N. (2001). Extensive intraspecific polymorphism detected by SSCP at the nuclear C-*mos* gene in the the endemic Iberian lizard *Lacerta schreiberi*. *Molecular Ecology* **15**, 731–738.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368–376.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Gillooly, J. F., Allen, A. P., West, G.B. & Brown, J. H. (2005). The rate of DNA evolution: effects of body size and temperature on the molecular clock. *Proceedings of the National Academy of Science USA* **102**, 140–145.

Gübitz, T., Thorpe, R.S. & Malhotra, A. (2005). The dynamics of genetic and morphological variation on volcanic islands. *Proceedings of the Royal Society of London B* **272**, 751–757.

Han, D., Zhou, K. & Bauer, A. M. (2004). Phylogenetic relationships among gekkotan lizards inferred from C-mos nuclear DNA sequences and a new classification of the Gekkota. *Biological Journal of the Linnean Society London* **83**, 353–368.

Harris, D. J. (2002). Reassessment of comparative genetic distance in reptiles from the mitochondrial cytochrome *b* gene. *Herpetological Journal* **12**, 85–86.

Harris, D. J., Arnold, E. N. & Thomas R. H. (1998). Relationships of the lacertid lizards (Reptilia: Lacertidae) estimated from mitochondrial DNA sequences and morphology. *Proceedings of the Royal Society of London B* **265**, 1939–1948.

Harris, D. J., Batista, V., Lymberakis, P. & Carretero, M. A. (2004*a*). Complex estimates of evolutionary relationships in *Tarentola mauritanica* (Reptilia: Gekkonidae) derived from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* **30**, 855–859.

Harris, D. J., Batista, V., Carretero, M. A. & Ferrand, N. (2004*b*). Genetic variation in *Tarentola mauritanica* (Reptilia: Gekkonidae) across the Strait of Gibraltar derived from mitochondrial and nuclear DNA sequences. *Amphibia-Reptilia* **25**, 451-459.

Huelsenbeck, J. P. & Crandall, K. A. (1997). Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics* **28**, 437–466.

Huelsenbeck, J. P. & Ronquist, F. (2001). MR-BAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755.

Jesus, J., Brehm, A. & Harris, D. J. (2001). Relationships of *Hemidactylus* (Reptilia: Gekkonidae) from the Cape Verde islands: what mitochondrial DNA data indicate. *Journal of Herpetology* **35**, 672–675.

Jesus, J., Brehm, A. & Harris, D. J. (2002). Relationships of *Tarentola* (Reptilia: Gekkonidae) from the Cape Verde Islands estimated from DNA sequence data. *Amphibia-Reptilia* **22**, 235–242.

Jesus, J., Brehm, A. & Harris, D. J. (2005*a*). Relationships of scincid lizards (*Mabuya* spp.) from the islands of the Gulf of Guinea based on mtDNA sequence data. *Amphibia-Reptilia* **26**, 467–473.

Jesus, J., Brehm, A. & Harris, D. J. (2005*b*). Phylogenetic relationships of *Hemidactylus* geckos from the Gulf of Guinea islands: patterns of natural colonizations and anthropogenic introductions estimated from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **34**, 480–485.

Jesus, J., Brehm, A. & Harris, D. J. (2005*c*). Is C-mos phylogenetically informative at lower taxonomic levels in reptiles? An assessment of variation within *Lacerta* (*Teira*) *dugesii* (Reptilia; Lacertidae). *Herpetozoa* **18**, 55–59.

Kasapidis, K., Magoulas, A., Mylonas, M. & Zouros, E. (2005). The phylogeography of the gecko *Crytopodios kotschyi* (Reptilia: Gekkonidae) in the Aegean archipelago. *Molecular Phylogenetics and Evolution* **35**, 612–623.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. & Wilson, A. C. (1989). Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science USA* **86**, 6196–6200.

Kronauer, D. J. C., Bergmann, P. J., Mercer, J. M. & Russel, A. P. (2005). A phylogeographically distinct and deep divergence in the widespread Neotropical turnip-tailed gecko, *Thecadactylus rapicauda*. *Molecular Phylogenetics and Evolution* **34**, 431–437.

Lamb, T. & Bauer, A. M. (2000). Relationships of the *Pachydactylus rugosus* group of geckos (Reptilia: Squamata: Gekkonidae). *African Zoology* **35**, 55–67.

Lee, D. C., Halliday, A., Fitton, J. & Poli, G. (1994). Isotopic variations with distances and time in the volcanic islands of the Cameroon line: evidence for a mantle plume origin. *Earth Planet Science Letters* **123**, 119–138.

Paulo, O. S., Jordan, W. C., Bruford, M. W. & Nichols, R. A. (2002). Using nested clade analysis to assess the biology of colonization and the persistence of populations of an Iberian lizard. *Molecular Ecology* **11**, 809–819.

Posada, D., & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Rocha, S., Carretero, M. A. & Harris, D. J. (2005). Diversity and phylogenetic relationships of *Hemidactylus* geckos from the Comoro islands. *Molecular Phylogenetics and Evolution* **35**, 292–299.

Saint, K. M., Austin, C. C., Donnellan, S. C. & Hutchinson, M. N. (1998). C-mos, a nuclear marker useful for squamate phylogenetic analysis. *Molecular Phylogenetics and Evolution* **10**, 259–263.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual (2nd ed.)*. New York: Cold Spring Harbour Press.

Swofford, D. L. (2003). *PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0.b10*. Sunderland, Mass.: Sinauer Associates.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acid Research* **22**, 4673–4680.

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