Molecular systematics and historical biogeography of tree boas (Corallus spp.)

Timothy J. Colston a,b,* , Felipe G. Grazziotin c , Donald B. Shepard d , Laurie J. Vitt a,b , Guarino R. Colli e , Robert W. Henderson f , S. Blair Hedges g , Sandro Bonatto h , Hussam Zaher c , Brice P. Noonan i , Frank T. Burbrink j

a Zoology Department, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019, USA
b Sam Noble Oklahoma Museum of Natural History and Zoology Department, University of Oklahoma, 2401 Chautauqua Avenue, Norman, OK 73072, USA
c Museu de Zoologia, Universidade de São Paulo, Caixa Postal 42.494, 04218-970, São Paulo, SP, Brazil
d Bell Museum of Natural History and Department of Fisheries, Wildlife, and Conservation Biology, University of Minnesota, Saint Paul, MN 55108, USA
e Departamento de Zoologia, Universidade de Brasília 70910-900 Brasília, DF, Brazil
f Milwaukee Public Museum, 800 W. Wells St. Milwaukee, WI 53233, USA
g Milwaukee Public Museum, 800 W. Wells St. Milwaukee, WI 53233, USA
h Department of Biology, University of Mississippi, Box 1848, University, MS 38677, USA
i Department of Biology, University of Mississippi, Box 1848, University, MS 38677, USA
j Department of Biology, 6S-143, The College of Staten Island, 2800 Victory Blvd, Staten Island, NY 10314, USA

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ABSTRACT

Inferring the evolutionary and biogeographic history of taxa occurring in a particular region is one way to determine the processes by which the biodiversity of that region originated. Tree boas of the genus Corallus are an ancient clade and occur throughout Central and South America and the Lesser Antilles, making it an excellent group for investigating Neotropical biogeography. Using sequenced portions of two mitochondrial and three nuclear loci for individuals of all recognized species of Corallus, we infer phylogenetic relationships, present the first molecular analysis of the phylogenetic placement of the enigmatic C. cropanii, develop a time-calibrated phylogeny, and explore the biogeographic history of the genus. We found that Corallus diversified within mainland South America, via over-water dispersals to the Lesser Antilles and Central America, and via the traditionally recognized Panamanian land bridge. Divergence time estimates reject the South American Caribbean-Track as a general biogeographic model for Corallus and implicate a role for events during the Oligocene and Miocene in diversification such as marine incursions and the uplift of the Andes. Our findings also suggest that recognition of the island endemic species, C. grenadensis and C. cookii, is questionable as they are nested within the widely distributed species, C. hortulanus. Our results highlight the importance of using widespread taxa when forming and testing biogeographic hypotheses in complex regions and further illustrate the difficulty of forming broadly applicable hypotheses regarding patterns of diversification in the Neotropical region.

* Corresponding author. Address: Department of Biology, University of Mississippi, Box 1848 University, MS 38677, USA.
E-mail addresses: tim@maddreptiles.com (T.J. Colston), fgrazziotin@gmail.com (F.G. Grazziotin), dshepard@umn.edu (D.B. Shepard), vitt@ou.edu (L.J. Vitt), grcollipush.br (G.R. Collin), henderson@mpm.edu (R.W. Henderson), sbh1@psu.edu (S. Blair Hedges), slbonatto@upcsr.br (S. Bonatto), hussam.zaher@gmail.com (H. Zaher), hnoonan@olemiss.edu (B.P. Noonan), burbrink@csi.cuny.edu (F.T. Burbrink).

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1. Introduction

Inferring the evolutionary history of a group in a particular region is one way to elucidate the processes by which the fauna of that region originated (Crisp et al., 2011; Riddle, 2009). In particular, comparing the evolutionary histories of multiple codistributed lineages allows one to formulate general biogeographic hypotheses that can be further tested and refined with additional taxa (Castoe et al., 2009; Daza et al., 2009). Extreme geological and environmental complexity as well as areas of high richness, high endemism, and multiple major biogeographic provinces (Udvardy, 1975) make the Neotropical region particularly interesting to biogeographers (Beheregaray, 2008; Graham, 1997). However, complex interactions among multiple factors have also precluded a consensus regarding the ecological and historical processes that have generated observed patterns of diversity. Although several broad biogeographic hypotheses have been proposed, the evolutionary history of most Neotropical taxa remains poorly understood and thus, the generality of these hypotheses remains untested in many clades (Beheregaray, 2008).

Multiple historical events have played a role in biological diversification in South America, but four major ones are of primary importance and have received the most attention (Rull, 2011).
First, marine incursions from the Pacific and Caribbean covered large tracts of Amazonia with huge lakes and swamps during the Tertiary, isolating lineages in upland areas such as the Brazilian and Guianan shields (Haffer, 2008; Webb, 1995). Second, the Andean uplift during the Miocene created a major continental barrier to dispersal while also providing new montane habitat and opportunities for colonization and diversification (Haffer, 2008; Rull, 2011). Third, contraction of forests during Pleistocene climatic fluctuations is thought to have isolated populations of forest-adapted taxa in refugia, which subsequently led to speciation (Haffer, 1969, 2008; Vanzolini, 1970). Finally, closure of the Panamanian Isthmus provided a dispersal route between North and South America which has been coined the "Great American Biotic Interchange-GABI" (Lessa et al., 1997; Simpson, 1940). The GABI was first recognized by Wallace (1876) and has since been identified as important in structuring biogeographic patterns in multiple taxa including birds, mammals, and invertebrates (Johnson and Weckstein, 2011; Lessa et al., 1997; Zeh et al., 2003); however divergences in many squamate taxa that span both sides of the Panamanian Isthmus predate its formation (Castoe et al., 2009; Daza et al., 2009, 2010). Among these events, the formation of Pleistocene refugia has received the most attention and debate, whereas the closure of the Panamanian Isthmus is the only event for which the timing is precisely dated (Rull, 2011).

The genus Corallus is a member of the subfamily Boinae, a group with clear Gondwanan origins (Noonan and Chippindale, 2006). Currently, eight species are recognized based on morphology or in some cases morphology and molecular data: C. annulatus (sensu lato, but see taxon sampling in Materials and Methods), C. batesii, C. caninus, C. cookii, C. cropanii, C. grenadensis, C. hortulanus, and C. ruschenbergerii (Henderson et al., 2009). Four species are endemic to South America, including the rare and enigmatic C. cropanii, which is only known from four specimens (three existing) and whose taxonomic placement has been of great interest (Kluge, 1991; Machado-Filho et al., 2011). Two species are distributed in both Central and South America (C. annulatus and C. ruschenbergerii), and two species are endemic to the Lesser Antilles (C. cookii and C. grenadensis) (Henderson, 2002; Henderson et al., 2009). Phylogenetic relationships have been explored using morphology (Kluge, 1991; Henderson, 1997, 2002; Henderson and Hedges, 1995; Henderson et al., 2009) and molecular phylogeographic analyses have been conducted on individual species (Vidal et al., 2005). However, a time-calibrated phylogeny for all members of the genus, which could be used to test both spatial and temporal components of biogeographic hypotheses, is not presently available.

Here, we use DNA sequence data from two mitochondrial and three nuclear genes of all species of Corallus to infer their phylogenetic relationships. We then reconstruct the biogeographic history of the group by integrating divergence times of species, as estimated by relaxed-clock divergence dating methods, with ancestral area analyses that account for dispersal and extinction to infer the biogeographic history of the group. Finally, we use our results to evaluate the potential roles of major Neotropical biogeographic events (e.g., marine incursions, Andean uplift, Pleistocene refugia, and Panamanian Isthmus) in the diversification of Corallus.

2. Materials and methods

2.1. Taxon sampling

We used 20 specimens of Corallus including all species from 18 localities across the distribution of the genus (Fig. 1, S1). We did not have access to tissue samples from specimens positively identified as C. batesii and available sequence data were restricted to cytochrome-b (cyt-b) from two individuals. Our Ecuadorian sample of C. annulatus could not be definitively classified as either C. annulatus or C. blombergi, and as there is no question whether these species are sister and we are not addressing the validity of C. blombergi, we treat C. annulatus herein as including both C. annulatus and C. blombergi. Tissue samples were collected by us, obtained through museum loans, or donated by private individuals from personal tissue collections. For phylogenetic analyses, we included sequences from GenBank for additional boid taxa and Cylindrophis for use as outgroups. These outgroup taxa were also included in divergence dating analysis in order to provide external nodes on which we could place prior age information based on the fossil record. A detailed list of specimens, including voucher numbers and GenBank accession numbers, is provided in Supplementary Information (S1).

2.2. Laboratory methods

We extracted total genomic DNA from liver tissue, muscle tissue, or shed skins either stored frozen or in ethanol using Qiagen DNeasy kits (tissue protocol). We amplified the complete

Fig. 1. (A) Map of Central and South America showing approximate distributions and sampling localities of Corallus species used in this study. (B) Phylogenetic estimate of relationships of the genus Corallus based on Bayesian Inference in the program Mr. Bayes. Tree topology was identical in both BI and ML inference. Node numbers indicate Bayesian posterior probability and ML bootstrap values (PP/ML). Nodes labeled with an asterisk (*) had a posterior probability of 1 and a bootstrap value of 100%. Vertical colored bars correspond to colors in distribution map whereas symbols to the right of the bars correspond to sample localities on the map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
cytochrome-\(b\) (\(cyt-b\)) mitochondrial gene and a portion of the 12S ribosomal RNA (\(rRNA\)) gene using Polymerase Chain Reaction (\(PCR\)) with GoTaq Green Master Mix (Promega Corp) following manufacturer’s specifications. Additionally, we amplified and sequenced portions of three nuclear protein-coding loci: brain ribosomal RNA (\(rRNA\)) gene using Polymerase Chain Reaction (\(PCR\)) with GoTaq Green Master Mix (Promega Corp) following manufacturer’s specifications. We cleaned PCR products using ExoSap-IT (USB Corp.) and used purified double-stranded products sequenced portions of three nuclear protein-coding loci: brain ribosomal RNA (\(rRNA\)) gene using Polymerase Chain Reaction (\(PCR\)).

### Table 1

<table>
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<tr>
<th>Primers</th>
<th>Primer sequence</th>
<th>Source</th>
<th>Fragment length (aligned)</th>
<th>Informative sites</th>
<th>Selected model</th>
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<td>Noonan and Chippindale (2006)</td>
<td>713</td>
<td>30</td>
<td>HKY + GAMMA</td>
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<tr>
<td>BDNF-R</td>
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<tr>
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<td>11</td>
<td>TrN93 + GAMMA</td>
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<tr>
<td>NT3F</td>
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<td>c-mos</td>
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<td>HKY + GAMMA</td>
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<td>L14010</td>
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<td>H16064</td>
<td>TAACTGATTTTCCTTCACTACA (SEQ)</td>
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<td>Corcyt-bF</td>
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<tr>
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<td>439</td>
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</table>

2.3. Phylogenetic inference

We conducted phylogenetic analyses using Bayesian Inference (\(BI\)) and Maximum Likelihood (\(ML\)) methods with different substitution models applied to each gene and codon position (Brandley et al., 2005; Castoe and Parkinson, 2006). The best substitution model for each partition was determined using the Akaike Information Criterion (\(AIC\)) in the program FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html), a web implementation of ModelTest (Posada and Crandall, 1998).

For Bayesian Inference, we used MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) with two independent runs of four chains (3 hot and 1 cold) for 20 \(\times\) 10\(^6\) generations sampling every 1000. We used default priors for all parameters and assessed convergence of the MCMC chains and independent runs by viewing trace plots of the tree –lnL values in the program Tracer v1.5 (Rambaut and Drummond, 2007). Additionally we used the program AWTY (Nylander et al., 2008) to view changes in the posterior probability of splits over the course of each MCMC run. We discarded trees sampled prior to stationarity as burn in and combined post-burn in trees from the two runs.

We conducted maximum likelihood analysis using the program RAxML 7.2.5 (Stamatakis et al., 2008) via the CIPRES portal v2.2 (Miller et al., 2010). The tree was inferred using the GTR(+\(\Gamma\)) model, partitioned by gene and codon position, and node support was obtained from 1000 non-parametric bootstrap replicates under the partitioned rapid bootstrapping GTRCAT model.

We compared trees from \(BI\) and \(ML\) analyses to infer the most credible relationships and considered nodes well supported when both the Bayesian posterior probability was \(\geq 0.95\) and the non-parametric bootstrap value \(\geq 70\%.\) Including the \(cyt-b\) sequences from the two individuals identified as \(C.\) batesii in phylogenetic analyses produced questionable topologies therefore we ran comparative analyses both with and without these data.

### Table 2

| Fossil material and lognormal prior settings used to constrain minimum ages of nodes in Fig. 2. |
|---|---|---|---|---|---|
| Taxon | Group | Period (Age) | Mean (SD) | Offset | Node |
| Corallus | Boinae | Middle Paleocene (61-58MY) | 1 | 58 | C |
| | | | | | (Pascual and Ortiz-Jaureguyizar [1991]; Medeiros and Bergqvist [1989]; Rage [2001]) |
| Titanoboa | Boinae | Middle Paleocene (60 MY) | 1 | 60 | B |
| | | | | | (Head et al. [2009]) |
| Morelia | Boidae | Miocene (25-22 MY) | 1 | 22 | A |
| | | | | | Scanlon [2001]) |
Corallus included the sister group of Boa constrictor, which we rooted the tree with the outgroup taxa pruned for ancestral area reconstruction analyses. Branch colors reflect biogeographic ranges for terminal tips and pie charts reflect the area with highest probability from Lagrange and DIVA analyses. Biogeographic regions are SA = South America, C = Central America, and LA = Lesser Antilles. (B) Chronogram for Corallus with outgroup taxa pruned for ancestral area reconstruction analyses. Branch colors reflect biogeographic ranges for terminal tips and pie charts correspond to fossil calibration points described in Table 2 and divergence estimates reported in Table 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We conducted two independent searches of $30 \times 10^6$ generations on the five-gene dataset (cyt-b + cyt-c + cyt-d + cyt-e) using every 1000 generations under the same models and partitioning strategy used in BI. We combined output log files from the two runs using the program LogCombiner v1.6.2 (Drummond and Rambaut, 2007) and discarded the first 7.5 million generations as burn in. We observed that all ESS values based on post-burnin samples were $>200$ for all estimated parameters in the program Tracer v1.5 (Rambaut and Drummond, 2007). We used Tree Annotator (Drummond and Rambaut, 2007) to combine post-burn in trees from the two runs and present the mean and 95% highest posterior density (HPD) of node ages.

2.5. Biogeographic analyses

We inferred the historical biogeography of Corallus using the ingroup topology and where appropriate the node ages of the BEAST chronogram (Fig. 2). We categorized species as having one of four distributions: (1) South American (SA) only = C. caninus, C. cropanii, C. hortulanus; (2) Central American (CA) and SA = C. annulatus, C. ruschenbergeri; (3) Lesser Antilles only = C. cookii, C. grenadensis, or (4) CA, SA, and Lesser Antilles (for outgroup ranges).

We reconstructed ancestral areas for each node in the phylogeny of Corallus using the program DIVA 1.2 (Ronquist, 2001). DIVA is a parsimony method that places ancestral areas onto internal nodes of a phylogeny using a three-dimensional step matrix. Ancestral areas are optimized by minimizing the allowed number of duplication and extinction events required to explain the current distributions of descendant lineages. Because optimizations can become less reliable as the root node is approached (Ronquist, 2003, 1997) we rooted the tree with the outgroup Boa constrictor, included the sister group of Corallus (Eunectes + Epicrates), and assumed the ancestral area of Boa constrictor encompassed all possible ancestral areas for Corallus (as does Eunectes + Epicrates). Additionally, we used a likelihood method of geographic range reconstruction implemented in the program Lagrange v2.9.1 (Ree and Smith, 2008). This maximum likelihood approach is based on a stochastic model of range evolution that incorporates rates of dispersal, extinction and cladogenesis. Instantaneous transition rates between ranges along phylogenetic branches were specified and these rates were used to estimate the likelihood of ancestral range inheritance at branching events using the program's default settings.

3. Results

3.1. Phylogenetic relationships

FindModel identified the best model for each locus (Table 1). Results from the BI and ML analyses were congruent and support was high for the monophyly of Corallus. The cyt-b sequences from the two individuals identified as Corallus batesii produced questionable topologies with low support (e.g., placing Corallus batesii sister to Eunectes rather than C. caninus). A NCBI blast-n search of one sequence identified the individual as Eunectes notaeus with a 85.9% match (e-score 2.0e−177). Because neither specimen was available for examination by the primary author and the sister relationship of Corallus batesii and Corallus caninus is not in question we chose to exclude these two sequences from further analyses. We note however that the species status of Corallus batesii and divergence times from Corallus caninus warrant further investigation.

In both BI and ML analyses, we found strong support for the sister relationship of C. caninus and all other species of Corallus. The enigmatic C. cropanii was strongly supported as sister to all
The one notable exception between molecular and morphological phylogenies is the nesting of *C. grenadensis* and *C. cookii* within *C. hortulanus* in our molecular phylogeny. This relationship was recovered using mitochondrial, nuclear, and the combined datasets, indicating that either *C. grenadensis* and *C. cookii* are not valid species or that *C. hortulanus* is composed of several cryptic species, some of which are more closely related to *C. grenadensis* and *C. cookii* than they are to each other. Mitochondrial DNA sequence divergence among these three taxa is minimal (<2% uncorrected sequence divergence), questioning the validity of the taxonomic status of *C. grenadensis* and *C. cookii*. However, additional studies of these taxa that include better sampling and additional loci, and explicit tests of alternative species hypotheses using coalescent methods for species delimitation (e.g. Yang and Rannala, 2010; Fujita et al., 2012) are needed to properly address their status.

### 4.2. Historical biogeography

Species distributions in *Corallus* largely follow major Neotropical biogeographic provinces. The Amazon Basin, Guiana Shield, Brazilian Atlantic Forest, and Lesser Antilles all contain endemic *Corallus* lineages, and a few widespread taxa span multiple regions. Interestingly, the two Central American species are co-distributed in northern South America as well. These patterns reveal that multiple dispersal and vicariant events have shaped diversification in *Corallus*, highlighting the complexity of processes driving species diversification in the biodiverse Neotropical region.

The South American–Caribbean track (SAC) hypothesis, which describes the biogeography of faunal exchange between North and South America, has been posited for multiple squamate clades (Savage, 2002), although this hypothesis has yet to be explicitly tested. The SAC predicts that *Corallus* initially diversified within mainland South America during the Eocene, dispersed to the Lesser Antilles via the Aves Ridge and subsequent stepping stone islands in the Late Tertiary (and should thus be relatively older than the lower Central American clades of South American origin), and then dispersed to Central America in the Middle to Late Pliocene (~3 Ma) with the closure of the Panamanian Isthmus (Savage, 1982; see Savage, 2002 for further review). Consistent with this, we found that *Corallus* initially diversified within South America with speciation events giving rise to *C. annulatus*, *C. caninus*, *C. cropanii*, *C. hortulanus*, and *C. ruschenbergeri* dating to the Oligocene and Miocene. The times of these speciation events suggest that factors such as marine incursions and/or the uplift of the Andes may have been important drivers of diversification (Haffer, 2008; Webb, 1995). However, inconsistent with the SAC model, we found that *C. annulatus* arrived in Central America during the Late Miocene, prior to the formation of the Panamanian land bridge. The closure of the Panamanian Isthmus in the Pliocene (~3 Ma) was pivotal for faunal exchange between North and South America in groups such as mammals, but earlier events may have also been important in taxa such as squamates (Savage, 2002). For example, the Proto-Antilles in the Early Paleocene may have provided a dispersal route between continents or dispersal via rafting or island hopping may have been possible in the Miocene before final closure of the Panamanian Isthmus as lower CA formed off the coast of northern SA (Savage, 2002). In contrast, *C. ruschenbergeri* appears to have dispersed from SA to CA during the Pleistocene via the traditionally recognized Panamanian land bridge, although the 95% HPD (Table 3) of our results allow for the possibility that *C. ruschenbergeri* may have arrived in CA prior to the closure of the Panamanian Isthmus.

Our results support previous studies (Henderson and Hedges, 1995) suggesting over-water dispersal of *C. grenadensis* and *C. cookii* from a northern, Guianan Shield ancestor. However,
inconsistent with the SAC model, divergence estimates place the age of the MRCA of these two species and *C. hortulanus* from Guyana at approximately ~2 Ma (Early Pleistocene), well after the Late Tertiary. There is no evidence for a land connection between the Guyana Shield and the Lesser Antilles at any time and ancestral area reconstructions place the distribution of the common ancestor of *C. grenadensis* and *c. cookii* in the Lesser Antilles during the Pleistocene, thus indicating over-water dispersal by these species from South America. This result is likely due to the two species being nested within *C. hortulanus*, which occurs only in South America.

The biogeography of *Corallus* shows a similar pattern to other codistributed Neotropical squamate and amphibian taxa. Phylogetic and biogeographic evidence has repeatedly revealed multiple dispersal events between Central and South America prior to the formation of the Panamanian land bridge and subsequent dispersal events back to South America after its closure (Daza et al., 2009; Koepfli et al., 2007). For example, evidence for colonization of Central America prior to the closure of the Panamanian Isthmus has been found in frog taxa with clear South American origins (Heinicke et al., 2007; Santos et al., 2009), and over-water dispersal to the Antilles rather than via the Proto-Antilles or Aves Ridge has been found in frogs and lizards (Censky et al., 1988; Heinicke et al., 2007; Hower and Hedges, 2003). The uplift of the Andes in the Miocene has been shown to have been both a dispersal barrier and provided areas of colonization of montane habitat in anurans (Garda and Cannatella, 2006; Noonan and Wray, 2006). The geography and timing of these incursions corresponds with the patterns we recovered in some *Corallus* lineages (e.g. *C. caninus*).

4.3. Conclusion

Our study indicated that *Corallus* diversified multiple times within mainland South America (*C. annulatus, C. caninus, C. cropa-nii, C. hortulanus, C. ruschenbergerii*), dispersed over water once to Central America (*C. annulatus*) and once to the Lesser Antilles (*C. cookii, C. grenadensis*), and dispersed once to Central America via the traditionally recognized Panamanian land bridge (*C. ruschen-bergerii*). The taxonomic validity of *C. grenadensis* and *c. cookii* are questionable and additional study of these taxa with respect to *C. hortulanus* is needed. We find incomplete support for the South American–Caribbean Track as a general biogeographic model for *Corallus*, and instead highlight the importance of over-water dispersal. The timing of speciation events within South America suggest possible roles for marine incursions, and the uplift of the Andes, but all South American species arose prior to the Pleistocene rejecting a role for refugia in speciation. The biogeographic history of *Corallus* further illustrates the difficulty of forming broadly applicable hypotheses regarding patterns of diversification in the Neotropical region.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.11.027.

References


