The Royal Society

Phylogeography of *Bufo marinus* from its natural and introduced ranges

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The marine toad, *Bufo marinus*, has a broad natural distribution extending from the south-west of the USA to southern Peru and the central Amazon. It was introduced to several localities in the Caribbean and Pacific Oceans to control sugar cane pests. We sequenced 468 bp of mitochondrial DNA (mtDNA) containing the *ND3* gene, and flanking *tRNA* genes from toads spanning the broad natural and introduced ranges. Consistent with the known history of introductions and expected effects of serial bottlenecks, mtDNA within introduced populations in Hawaii and Australia was uniform and most closely related to samples from eastern Venezuela and French Guiana. However, mtDNA nucleotide diversity in the geographic region spanning the source areas is also relatively low (0.18–0.46%) and the absence of variation in the introduced populations precludes quantitative assessment of the reduction in genetic diversity. Unexpectedly, there was a large phylogeographic break (5.4% sequence divergence) within the natural range separating populations east and west of the Venezuelan Andes. We hypothesize that the two major lineages of *B. marinus* were isolated by the uplift of the eastern Andean cordillera which was completed approximately 2.7 Ma. Another species of the *marinus* group, *B. paracnemis*, had mtDNA paraphyletic, with *marinus*, being nested within the eastern lineage. Thus, at least one speciation event within the *marinus* group postdates the split within *marinus*. These findings suggest that the taxonomy of *B. marinus* should be re-evaluated and that the search for pathogens to control Australian populations should be conducted in populations from both lineages in the natural range.

Keywords: marine toad; bottleneck; mtDNA; taxonomy; biological control

1. INTRODUCTION

The marine toad, *Bufo marinus* is of particular interest for three reasons. First, its natural geographic range is unusually broad for a neotropical amphibian, extending from south-east Texas (USA) to south-eastern Peru and central Amazonia (Zug & Zug 1979; figure 1*a*). Second, the species was introduced to islands across the Caribbean and Pacific Oceans in order to control cane pests, but is now itself perceived as a major pest that potentially threatens native wildlife (Covacevich & Archer 1975; Freeland 1987). The range of the species continues to expand in Australia (Easteal 1988; Lampo & De Leo 1998; figure 1*b*) and there is now a major effort to identify parasites and pathogens from toads in the natural range, and from Venezuela in particular, that might be used to control toad populations (Lampo & Bayliss 1996; Zupanovic *et al*. 1998). Third, because of the well-documented history of translocations and subsequent range expansions (Easteal 1981), toads from the introduced range constitute a natural experiment for testing population genetics theory (Easteal 1988, and references therein).

Given its widespread natural distribution, it is conceivable that *B. marinus* consists of multiple lineages or species against which biocontrol agents will act differently. Notably, Taylor & Smith (1945) suggested that the name *B. horribilis* be ascribed to Mexican populations, but Duellman (1961) synonymized this species with *B. marinus* on the grounds that there had been no comprehensive systematic study. Several other currently recognized species within the *marinus* group have at one time or another also been treated as subspecies of *B. marinus* and these are currently discriminated on the basis of morphological differences, mostly among allopatric distributions (reviewed by Cei 1972; Easteal 1986).

The history of translocations leading to the Australian populations is known in considerable detail (Easteal 1981), although there is less information about the early stages. The population on Barbados was founded before 1844 from both Guyana and French Guiana. This stock was then used to found the Puerto Rican population in the 1920s, which in turn was used to establish the Hawaiian population in 1932. Australian toads descend from 101 individuals sampled from Oahu in 1935. The founder events associated with these translocations were predicted to result in genetic divergence through drift, and a comparison of variable protein loci among populations from Hawaii and Australia confirmed this (Easteal 1985, 1988). There is, however, no information on the genetic relationships of the Hawaiian and Australian populations to those in the Americas. Similarly, there have been no investigations of genetic variation among populations within the natural distribution, so it is not

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Received 19 December 1997 *Accepted* 19 January 1998

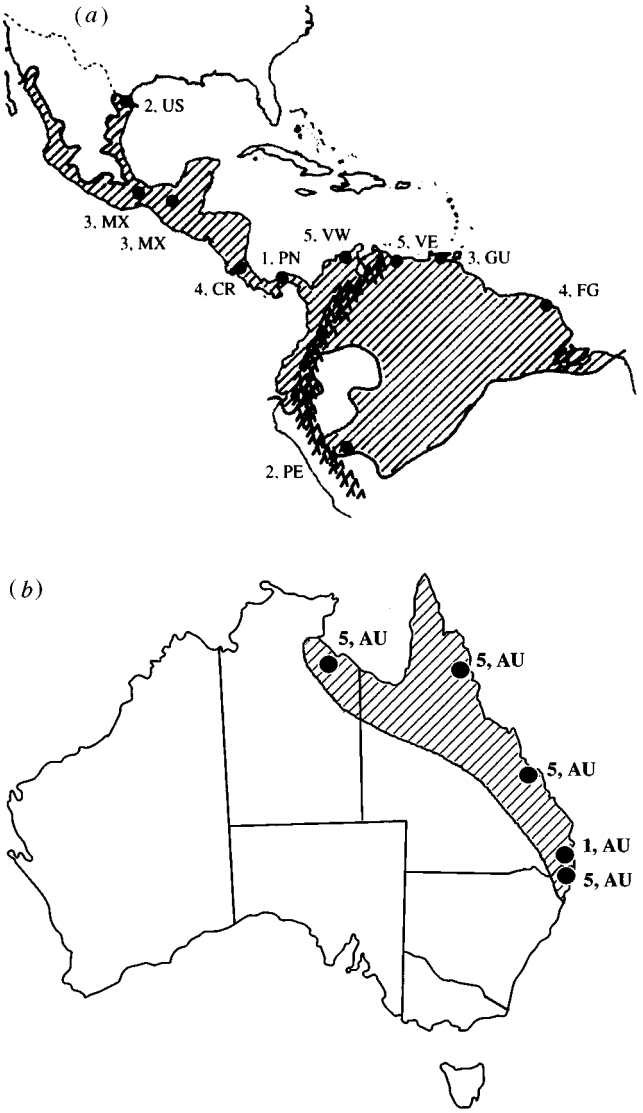

Figure 1. (*a*) Natural range of *Bufo marinus* in central and south America (after Zug & Zug 1979). (*b*) Range of the introduced population in Australia (after Easteal 1986; Zupanovic *et al*. 1998). The locations sampled (codes as in table 1) and the number of individuals analysed by DNA sequencing are indicated.

known whether the Venezuelan populations are genetically similar to those from the adjacent source areas or even whether they are a single taxon.

The principal aim of the present study was to survey the natural range for the presence of cryptic evolutionary lineages and, secondarily, to compare populations from the introduced and natural ranges in the context of the documented history of translocations (Easteal 1981). Phylogeographic analyses of mtDNA have been particularly effective at identifying cryptic evolutionary lineages and/or species in many animal taxa (e.g. Avise 1994), including amphibians (e.g. McGuigan *et al*. 1998) where morphology is relatively conservative. Identified discontinuities can then be used to develop hypotheses about biogeographic events, using additional information about landscape history and, if possible, corroborating evidence from other genes. One danger of this 'gene tree' approach is that mtDNA might be under selection which can alter the level and distribution of sequence divergence independent of population history (Ballard & Kreitman 1995). Following the approach adopted in other recent studies of the same gene (Nachman *et al*. 1994, 1996), we therefore tested for the signature of selection by comparing the proportions of silent and replacement substitutions within- versus across major lineages (McDonald & Krietman 1991).

2. METHODS

(a) *Sampling*

Sampling was designed to balance the need for geographic coverage of the natural and introduced ranges with adequate sampling of individuals from each location and nucleotides sequenced per individual (table 1). For the introduced populations, we sampled 21 individuals from Australia representing the approximate geographic limits of the current range (figure 1*b*), 10 individuals from Hawaii and 1 from Indonesia. For the natural range (figure 1*a*), our samples ($N=32$) span most of the distribution (although we were unable to obtain samples from the Amazon basin), with a focus on the Venezuelan sites being examined for biological control and toads from original source areas (e.g. French Guiana). As outgroups, we examined material from three species, *B. paracnemis* (another member of the *marinus* group), *B. granulosis* and *B. beebei*.

(b) *mtDNA sequencing and analysis*

Purified mtDNA was extracted from heart tissue of a Brisbane toad by ultracentrifugation (Dowling *et al*. 1996). This DNA was used for the initial PCR and sequencing to help confirm that the primers amplified an mtDNA gene fragment rather than a nuclear copy (Zhang & Hewitt 1996). For routine PCR, DNA was extracted using a Chelex/Pro K method from very small amounts of liver tissue. Primers *ND3.1* (5′-GGCTCATATTTTCTTAGTATAAA-3′) and *ND3.2* (5′-AGTAATCAGGACTCGTTTGTT-3′) were designed from *marinus* sequences that had been amplified and sequenced with primers designed for conserved regions of the *COIII*, *ND3*, and *ND4* mtDNA genes (R. Slade and C. Moritz, unpublished data). For *marinus*, *beebei*, and *granulosus*, the 25 mL PCR reaction contained 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 0.01% Nonidet P-40, 0.01% Tween 20, 200 mM dNTPs, 3 mM $MgCl_2$, 0.4 mM of primers *ND3.1* and *ND3.2*, 0.5 units of Taq DNA polymerase, and DNA template. For *paracnemis* the PCR reaction contained the same components except that the $MgCl_2$ was at 1.5 mM, and the primers were at 0.25 mM. For *marinus*, the cycling parameters were 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min for 35 cycles, while for the other species the cycling parameters were the same except that the annealing temperature was 48 °C. The resulting fragment (*ca*. 500 bp) was sequenced using standard cycle sequencing protocols and *ND3.1*, *ND3.2*, and internal primers *ND3U2* (5′-TAATCCA GACTCAGAAAAAC-3′) and *ND3L2* (5′-AATCGCATAGAATATGGTAG-3′) giving a sequence of 468 bp in *B. marinus*.

Sequences were aligned initially with Clustal V (Higgins & Sharp 1988), and thereafter manually. The distances between sequences was corrected using the Kimura two-parameter model (Kimura 1980). Phylogenies were reconstructed using the neighbour-joining method (Saitou & Nei 1987). The protein translations, distances, and trees were constructed using MEGA (Kumar *et al*. 1993). The nucleotide diversities and divergences within and between populations were generated in REAP

Table 1. *Samples analysed for mtDNA sequence variation*

species	location (ID[a])	N	specimen ID[b]
B. marinus			
introduced	Borroloola, NT, Australia (AU)	5	—
	Mossman, Qld, Australia (AU)	5	—
	Rockhampton, Qld, Australia (AU)	5	—
	Lennox Head, NSW, Australia (AU)	5	—
	Brisbane, Qld, Australia (AU)	1	—
	Manoa, Oahu, Hawaii (HW)	9	—
	Coconut Island, Hawaii (HW)	1	—
	Indonesia	1	—
natural	Lake Maracaibo, Venezuela (VW)	5	—
	Higuerote, Venezuela (VE)	5	—
	Guiria, Venezuela (GU)	3	—
	Cayenne, French Guiana (FG)	4	—
	Madre de Dios, Atalaya, Peru (PE)	2	USNM 206333, 206332
	Mexico (MX)	6	MVZ 12191, 12192, 12193, 13558, 13559, 13560
	Costa Rica (CR)	4	MVZ AG34, AG35; ASDM 88011, 88012
	Panama (PN)	1	USNM 051864
	Texas, USA (US)	2	—
B. paracnemis	Uruguay	6	—
B. granulosis	Brazil	2	USNM 051337, 051338
B. beebei	Trinidad	2	USNM 286991, 286990

[a]IDs in parentheses indicate abbreviations used in figure 2.
[b] Australian specimens not assigned museum numbers are currently housed in the Department of Zoology, University of Queensland, and will be lodged with the Queensland Museum on completion of the study. Others were sampled non-destructively by colleagues. Abbreviations: USNM, Smithsonian Museum of Natural History; MVZ, Museum of Vertebrate Zoology, UC Berkeley; ASDM, Arizona Sonora Desert Museum.

(McElroy *et al*. 1992), and AMOVA (Excoffier *et al*. 1992) was used to estimate the distibution of variation within and among populations and regions. Departures from neutrality were tested against the null hypothesis that the ratio of silent to replacement substitutions is the same within as it is between major clades (McDonald & Kreitman 1991).

3. RESULTS

(a) *mtDNA sequence diversity within and between populations of* B. marinus

The amplified sequence of 468 bp (plus 42 bp of primers) comprised most of $tRNA^{Gly}$, all of the *ND3* gene, and all of $tRNA^{Arg}$ (figure 2). There were two characteristics of the *ND3* sequence that should be noted. First, in the *ND3* sequence of the two samples from Peru (PE) the start codon was shifted down one amino acid due to an insertion of a single adenine base at nucleotide position 80 (figure 2). Second, the position of the stop codon in all of the sequences is unclear. The stop codon is expected to be at nucleotide positions 410–412 where it abuts the beginning of the $tRNA^{Arg}$ sequence. However, in all the *Bufo* sequences, this position is the triplet 'TGA' which, in the mtDNA genetic code assumed for amphibians (Roe *et al*. 1985), codes for tryptophan. In this assumed amphibian code, the first stop codon in the *Bufo ND3* sequence, 'TAA', is near the end of the $tRNA^{Arg}$ sequence at positions 479–481. Either there is extensive overlap of the *ND3* gene and $tRNA^{Arg}$, resulting in an unusually large *ND3* product of 134–135 codons (cf. 114–115 in mammals; Brown 1985), or a stop codon is created near the usual place by processing and polyadenylation (Roe *et al*. 1985), or the assumed genetic code does not apply to all amphibian species.

From the 64 samples analysed, there were 15 different haplotypes in the 468 bp of mtDNA sequence. Within the *ND3* reading frame, there were 50 (12.4%) variable nucleotide sites, but only three (2.2%) variable amino acid residues, reflecting the general conservatism of amino acid sequences in amphibian mtDNA (Adachi *et al*. 1993; Graybeal 1993). Fourteen of the 15 variants occurred within the natural range, whereas only one haplotype was found in the 32 samples from the introduced populations of Australia, Hawaii, and Indonesia. Sequence divergence among haplotypes of *B. marinus* varied from 0.21 to 9.57%. Nucleotide diversity, the average pairwise sequence difference among individuals across the natural range was substantial at 4.79%, and compares with zero for that observed across the introduced range (Hawaii and Australia).

On a smaller scale, several regions from the natural range contained enough samples ($n \geqslant 4$) to gain some idea of the level of mtDNA diversity within populations (table 2). The highest within-population diversity was 0.47% (Costa Rica), an order of magnitude less than the total diversity across the natural range. Thus, most of the diversity within *marinus* occurs between populations (table 2). This conclusion is supported by the AMOVA analysis of the five populations from the natural range with adequate sample sizes (i.e. French Guiana, eastern Venezuela (Higuerote and Guiria), western Venezuela, Mexico and Costa Rica) in which 94% of the genetic variation is distributed among populations and 6% is distributed within populations. The sequence variation appeared to be strongly partitioned to either side of the Venezuelan Andes (table 2). If the five populations are placed into two regions representing those east of the Andes (French Guiana, eastern Venezuela) and

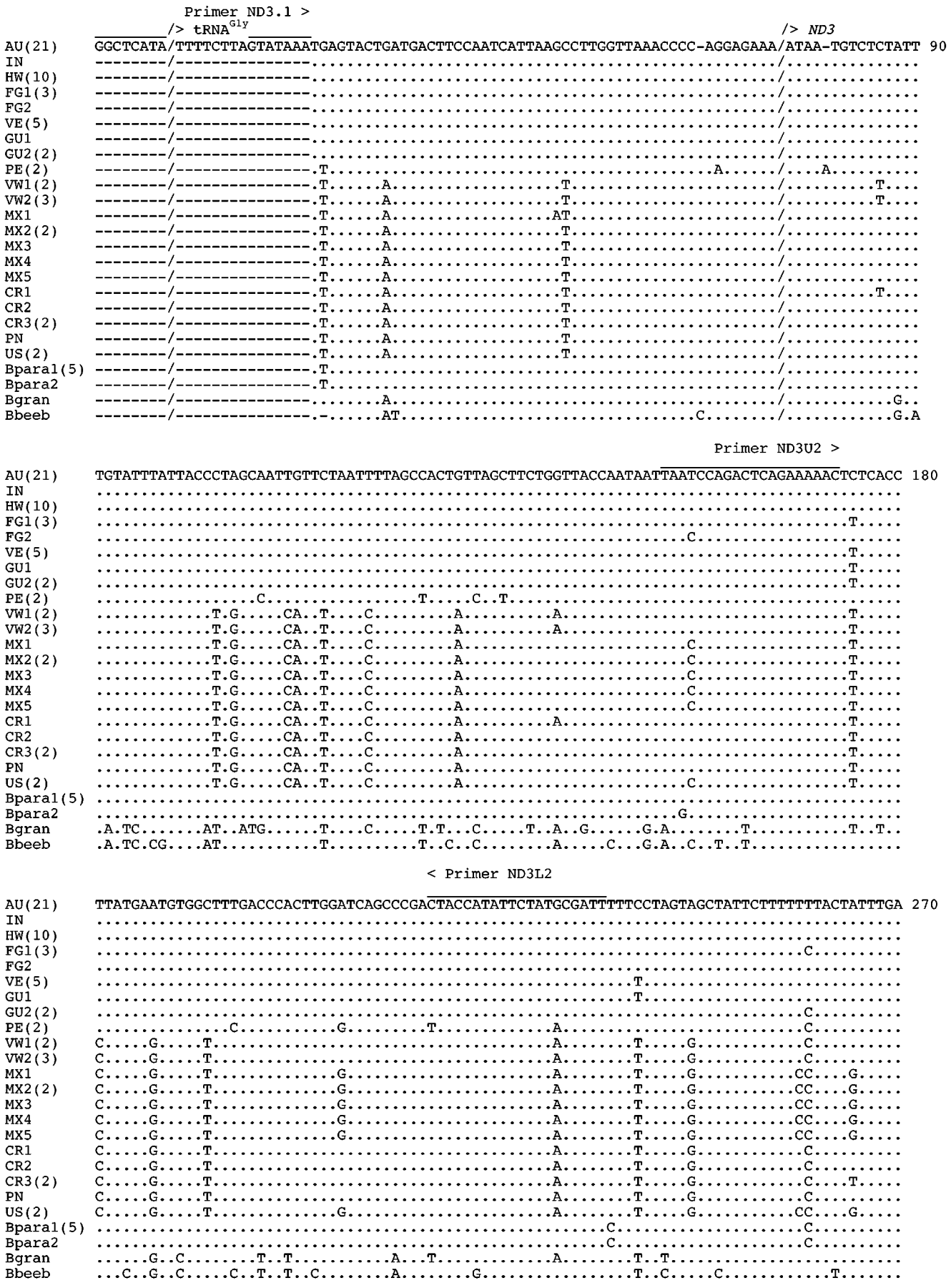

Figure 2. MtDNA sequences of *Bufo marinus* from several localities (codes as in table 1), with homologous sequences from *B. paracnemis*, *B. granulosis*, and *B. beebei*. Numbers in parentheses are the number of individuals with that haplotype. This alignment is available from the EMBL FTP server at `ftp://ftp.ebi.ac.uk/pub/databases/embl/align/`, and has the accession number DS33151.

```
AU(21)    TTTAGAAATTGCCCTTCTCCTCCCCACCCCCTGGGCAGCGCAACTTCCTCACCCCACATTATCAATTTTTTGAGCATCAATTATTCTTAT 360
IN        ..........................................................................................
HW(10)    ..........................................................................................
FG1(3)    .....................T....................................................................
FG2       ...............C.....T....................................................................
VE(5)     .....................T....................................................................
GU1       .....................T....................................................................
GU2(2)    .....................T....................................................................
PE(2)     C...........T.................T..A........................................................
VW1(2)    C...........T........T.....T..T...........G........T......C....T...............G..........
VW2(3)    C...........T........T.....T..T...........G........T......C....T...............G..........
MX1       C...........T........T.....T.................A.....T......C....T..........................
MX2(2)    C...........T........T.....T.................A..C..T......C....T..........................
MX3       C...........T..............T.................A.....T......C....T..........................
MX4       C...........T........T.....T.................A.....T......C....T..........................
MX5       C...........T...........T..T.................A.....T......C....T..........................
CR1       C...........T........T.....T..T...........G........T......C....T...............G..........
CR2       C...........T........T.....T..T....................T......C....T...............G..........
CR3(2)    C...........T........T.....T..T....................T......C....T...............G..........
PN        C...........T........T.....T..T....................T......C....T...............G..........
US(2)     C...........T........T.....T.................A.....T......C....T..........................
Bpara1(5) C........C..T........T.....................................C...............................
Bpara2    C........C..T........T.....................................C...............................
Bgran     CC...............C..G..T..A.....A..A..T..A.....A..AT....G..CC....T..C.......................
Bbeeb     C..............C..T..T..T...........T..A.....G..A..T..A..C.....C..C.......................

                                                                 ND3? </> tRNA^Arg
AU(21)    TCTTTTAACTTTAGGTTTATTTACGAATGACTTCAAGGAGGCCTAGAATGA/GCTGAATGAGGAGTTAGTCTAAAAAAGACAGCTGATTT 45
IN        .................................................../......................................
HW(10)    .................................................../......................................
FG1(3)    .................................................../......................................
FG2       .................................................../......................................
VE(5)     .................................................../......................................
GU1       .................................................../......................................
GU2(2)    .................................................../......................................
PE(2)     ....C.............C.....T........................../......................................
VW1(2)    ...C..G...........C.............C.....C...T......../......................................
VW2(3)    ...C..G...........C.....T.......C.....C...T......../......................................
MX1       ...C..G...........C.............C.....C...T......../......................................
MX2(2)    ...C..G...........C.............C.....C...T......../......................................
MX3       ...C..G...........C.............C.....C...T......../......................................
MX4       ...C..G...........C.............C.....C...T......../......................................
MX5       ...C..G...........C.............C.....C...T......../......................................
CR1       ...C..G...........C.............C.....C...T......../......................................
CR2       ...C..G...........C.............C.....C...T......../......................................
CR3(2)    ...C..G...........C.............C.....C...T......../......................................
PN        ...C..G...........C.............C.....C...T......../......................................
US(2)     ...C..G...........C.....T.......C.....C...T......../......................................
Bpara1(5) .................................................../......................................
Bpara2    .................................................../......................................
Bgran     ......G........C.......................C..T.....G.../......................................
Bbeeb     ....C..........C................C........T......../......................................

                                                               < Primer ND3.2
                                                ND3? </tRNA^Arg</
AU(21)    CGGCTCAGCAAATTATGGTTTAACCCCATAA/CACCTTT/ATGTTAACAAACGAGTCCTGATTACT 514
IN        .............................../......./.....---------------------
HW(10)    .............................../......./.....---------------------
FG1(3)    .............................../......./.....---------------------
FG2       .............................../......./.....---------------------
VE(5)     .............................../......./.....---------------------
GU1       .............................../......./.....---------------------
GU2(2)    .............................../......./.....---------------------
PE(2)     .............................../......./.....---------------------
VW1(2)    .............................../.....C./.....---------------------
VW2(3)    .............................../.....C./.....---------------------
MX1       .............................../.....C./.....---------------------
MX2(2)    .............................../.....C./.....---------------------
MX3       .............................../.....C./.....---------------------
MX4       .............................../.....C./.....---------------------
MX5       .............................../.....C./.....---------------------
CR1       .............................../.....C./.....---------------------
CR2       .............................../.....C./.....---------------------
CR3(2)    .............................../.....C./.....---------------------
PN        .............................../.....C./.....---------------------
US(2)     .............................../.....C./.....---------------------
Bpara1(5) ..........................G..../......./.....---------------------
Bpara2    ..........................G..../......./.....---------------------
Bgran     .............................../......./.....---------------------
Bbeeb     ..A..........C...............G./.....C./.....---------------------
```

Figure 2. (*Cont.*)

Table 2. *Per cent diversities and divergences within and between* Bufo marinus *populations*[a]

	Australia (21)	Hawaii (10)	French Guiana (4)	eastern Venezuela (8)	western Venezuela (5)	Mexico (6)	Costa Rica (4)
Australia	*0*	0.00	0.65	0.65	8.70	8.61	8.10
Hawaii	0.00	*0*	0.65	0.65	8.70	8.61	8.10
French Guiana	0.44	0.44	*0.43*	0.46	8.20	8.16	7.61
eastern Venezuela	0.56	0.56	0.15	*0.18*	7.95	8.03	7.36
western Venezuela	8.64	8.64	7.92	7.79	*0.13*	2.54	0.72
Mexico	8.43	8.43	7.75	7.75	2.29	*0.37*	1.90
Costa Rica	7.87	7.87	7.16	7.04	0.43	1.48	*0.47*

[a]Within population diversities in italics on diagonal, between population diversities and divergences above and below diagonal respectively. Sample size in parentheses. 'Eastern Venezuela' represents samples combined from Guiria ($N=3$) and Higuerote ($N=5$).

those west of the Andes (western Venezuela, Mexico, Costa Rica), then 82% of the genetic variation occurs among regions, 14% among populations within regions, and 4% within populations. The differences among regions, and among populations within regions are significant ($p<0.001$). Two population samples can be assumed to represent, at least approximately, the diversity of the areas from which the translocations commenced (Guyana and French Guiana; Easteal 1981). The level of nucleotide diversity in the four samples from French Guiana is 0.43% (haplotype diversity = 50%) and that from the combined eastern Venezuelan sample is 0.18% (haplotype diversity = 61%). From eastern Venezuela to French Guiana, a range greater than that of the original source populations, the level of diversity is similar (0.34%), with 5 haplotypes observed among 12 samples.

The distribution and level of net nucleotide divergence between populations (i.e. between population diversity corrected for within-population diversity) shows that the Australian and Hawaiian populations are most similar to those from French Guiana and eastern Venezuela. This is expected as these populations are the closest to the original source populations in Guyana and French Guiana. That Australia and Hawaii share the same haplotype suggests that the descendents of the 149 individuals that founded the Hawaiian population in 1932 (Easteal 1981) were predominantly of this type. There is limited divergence between the eastern Venezuela and French Guiana samples (0.15%) despite the large geographical distance (more than 1200 km) separating them. The most striking result is the extremely large divergence (mean of 7.57%) between the populations in Central America/southern North America and the populations in South America, except western Venezuela, relative to the divergences between populations within those regions (mean of 1.09%). The eastern and western Venezuelan populations are separated by a distance of only 600 km, but are 7.8% divergent.

(b) *Phylogeography of* B. marinus

The implication of a large phylogeographic break within the natural range of *marinus* is confirmed by the phylogenetic analysis of the mtDNA variants (figure 3). The dominant feature is the large and strongly supported division between the populations to the east and west of the Venezuelan Andes. Other notable outcomes are (i) a lower level phylogeographical break separating populations from Mexico and the USA from those in Costa Rica, Panama and western Venezuela; (ii) the paraphyly of the eastern/southern populations of *B. marinus* with respect to *B. paracnemis*; and (iii) the close phylogenetic relationship between the samples from the introduced range and those from French Guiana and eastern Venezuela.

The genetic distance separating the basal nodes of the eastern and western Andes' clades is 5.4%. At a rate of evolution of 2% per million years' divergence time (Wilson *et al*. 1985) this would correspond to separation of the two clades at approximately 2.7 million years, similar to the 2.74 Ma for the final uplift of the eastern Andean cordillera which extends into Venezuela and is the youngest of the Andean ranges (Helmens & van der Hammen 1994). However, the estimate of divergence time should be viewed with caution because the rate of evolution varies among mtDNA proteins (Zardoya & Meyer 1996; an approximate correction gives a divergence time of *ca*. 2.1 Ma) and may be slower in amphibians than mammals (Adachi *et al*. 1993), and also because estimates of divergence time have large errors (Hillis *et al*. 1996).

(c) *Test for selection on mtDNA*

In several species, some of the diversity in mtDNA genes has been shown to result from selection (Ballard & Kreitman 1995; Nachman *et al*. 1996). We investigated whether the distribution and level of replacement and silent substitutions in the *ND3* gene between the eastern and western Andes clades could be explained by non-neutral processes. The numbers of replacement and silent polymorphisms within the eastern Andes clade were 0 and 6, respectively, and 1 and 13, respectively, in the western Andes clade. There were 2 and 18 replacement and silent fixed differences, respectively, between the two clades. There was no significant difference in relative numbers of replacement and silent polymorphisms and fixed differences ($\chi^2=0.36$, $p>0.1$), indicating that the observed genetic variation primarily reflects neutral demographic processes rather than the idiosyncratic effects of selection.

4. DISCUSSION

(a) *Relationships and diversity of introduced populations*

Previous studies of genetic diversity in Australian and Hawaiian populations revealed substantial heterozygosity at allozyme loci (Australia, mean $H=13.6\%$; Hawaii mean $H=15.9\%$), although nine of the ten polymorphic allozyme loci contained only two alleles, which is low compared with

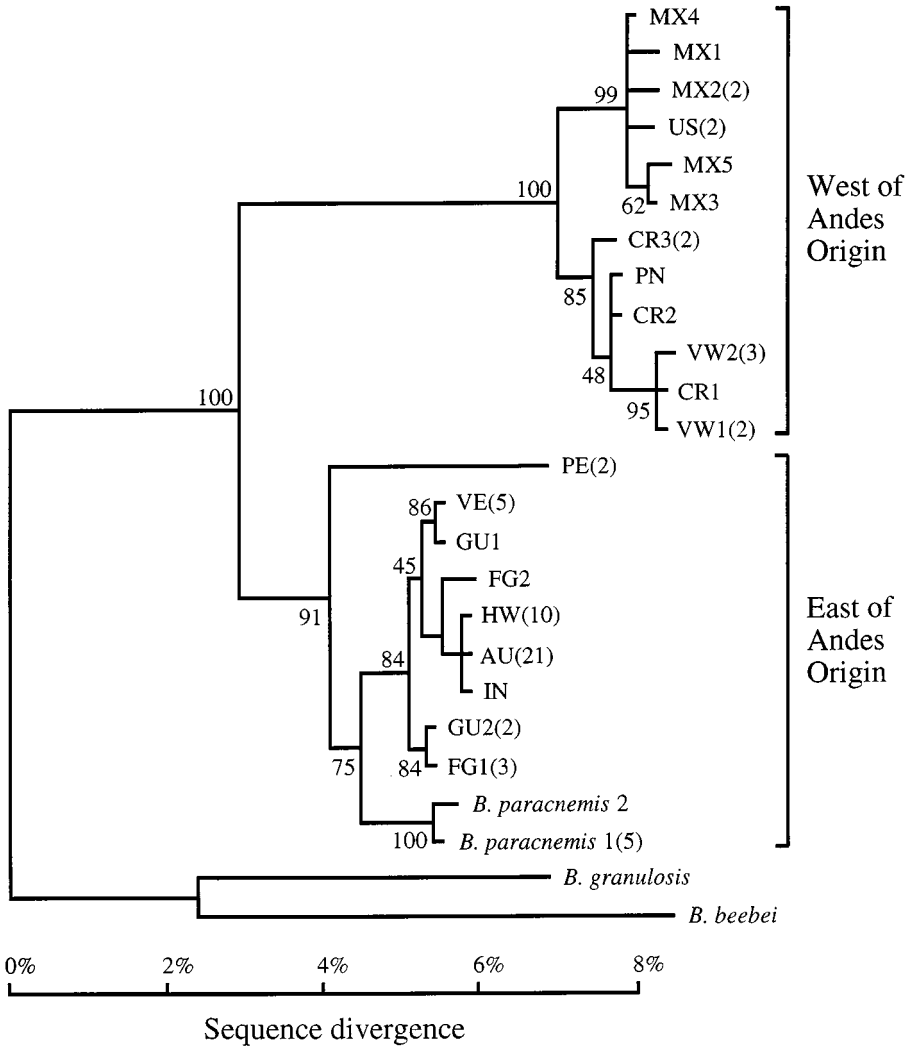

Figure 3. Neighbour-joining tree of mtDNA sequence variants observed among *B. marinus* populations and relatives. Sample location codes refer to abbreviations in table 1 with different haplotypes from each location numbered sequentially. Numbers in parentheses are the number of individuals with that haplotype. Numbers above the branches are the proportion of bootstrap pseudo-replicates in which the clade to the right appeared.

other species of amphibians with similar heterozygosity (Easteal 1988). The latter observation is consistent with the expected effects of the serial bottlenecks that have occurred during the translocation process (Nei *et al.* 1975; Easteal 1981). However, the allozyme analyses did not encompass populations from the natural range.

The present analysis of mtDNA variation is consistent with the documented introductions in two ways: (i) genetic diversity is reduced in the introduced populations relative to populations from the source areas; and (ii) the haplotype characteristic of the introduced populations is most closely related to those from French Guiana and eastern Venezuela, encompassing the geographic range of the source populations. Together with the absence of any obvious effects of selection, this gives us some confidence that the mtDNA gene tree is accurately reflecting the history of the populations.

The observed changes in genetic diversity between the source and the introduced populations are in the expected direction; however, the total absence of variation in the introduced populations precludes both the quantitative analysis of the reduction in diversity accompanying the translocation and the estimation of effective population size in the introduced range. The reduction in haplotype diversities from 0.5–0.6 to zero appears substantial. The observed nucleotide diversities within and across the populations from the source area were in the range 0.18–0.43%, but the sampling variances around these estimates are relatively large (s.e., 0.27%; Tajima 1983), giving approximate 95% confidence limits from 0–95%, which include the estimate of diversity for the introduced populations. Further, the stochastic variances would probably be substantially larger than these estimates of the sampling variance. Sampling of a more variable segment of mtDNA, more loci (e.g. microsatellites) and more populations (Lynch & Crease 1990), is needed to provide rigorous quantitative estimates of the effects of translocations on genetic diversity.

(b) *Phylogeography, biogeography, and species boundaries*

The most significant result of the mtDNA analysis is the major phylogeographic break located around the the eastern Andean cordillera which separates the western and eastern Venezuelan populations. A phylogeographic

break between the eastern and western Andes populations has previously been noted in other species in South America, for example in *Helioconis* butterflies (Brower 1994) and several species of birds (Cracraft & Prum 1988; Brumfield & Capparella 1996). The correspondence between the location of the phylogeographic break and this obvious physical feature, together with the (crude) correspondence between the minimum age of the split and timing of the final uplift of the eastern cordillera, suggest that the eastern Andes have isolated populations to the east and west. Zug & Zug (1979) comment that *B. marinus* occupies a wide range of habitats, but is rare in unbroken forest and at altitudes about 1000 m. A survey of available museum records (figure 1 in Zug & Zug 1979) did not reveal any samples from the eastern cordillera, although it is conceivable that the toads occur around the relatively xeric coastal fringe and in lower lying areas within this topographically complex region (see figure 15:11 in Duellman 1979). The results of the present study indicate a need for more detailed surveys to determine the distribution of the two genetic types of *marinus* across the Andean range in Venezuela.

The mtDNA analysis also permits some reinterpretation of the zoogeography and history of speciation in the *marinus* complex. Cei (1972) suggested that the ancestral stock of the *B. marinus* complex occurred on the Guianan shield and radiated southwards and eastwards to form other species in the complex (e.g. *paracnemis*, *poeppigii*, *rufus*, *ictericus*, *aranarum*), and subsequently (Plio-Pleistocene) *B. marinus* expanded northwards across the Panamian isthmus to occupy central America. However, fossils attributable to *B. marinus* exist from the late Miocene in Columbia and the lower Pliocene in Kansas (reviewed in Easteal 1986). The mtDNA phylogeography suggests a relatively old distribution of *B. marinus* across central and south America, with the divergence across the eastern Andes substantially predating the separation of *B. paracnemis* from the eastern/southern lineage of *B. marinus*. There is an obvious need to extend the analysis of mtDNA to other members of the *marinus* complex to see if these too are of recent origin.

From hybridization experiments it was concluded that within the *marinus* group, *marinus* and *poeppigi* formed one clade while *paracnemis*, *ictericus*, and *arenarum* formed another (Blair 1972*a*). However, these experiments, and many other studies, were conducted with *marinus* samples from various regions in Central America and North America, rather than the eastern/southern lineage (Appendix A in Blair 1972*b*). The results from the mtDNA analysis indicate that relationships within the *marinus* group need to be re-evaluated using samples representative of both major lineages within *B. marinus*. Whether or not the two evolutionary lineages of *B. marinus* should be regarded as separate species depends in part on the choice of species concepts, but in any case further studies of differences in morphology and nuclear genes are warranted. Certainly, at least one speciation event within the *marinus* group significantly postdates the split between the two *B. marinus* lineages.

(c) *Implications for biological control*

The search for *Bufo* specific pathogens that might act as biological control agents against the Australian populations of *B. marinus* have focused on populations from Venezuela. The present study indicates that populations from eastern Venezuela are genetically similar to those introduced to Australia, whereas those to the west of the Merida Andes and the cordillera de la Costa are highly divergent. We suggest that both lineages should be tested in future searches for infectious agents that may serve as potential pathogens for the biological control of Australian *B. marinus*. Parasites and pathogens are often more pathogenic in naive populations that they are introduced to than in the source populations in which they have coevolved with the host to an intermediate level of virulence; a classic example being the high level of virulence of myxoma virus in previously unexposed populations of rabbits in Australia and Europe compared with the low virulence in source populations in South America (Fenner & Ratcliffe 1965). With respect to *B. marinus*, the western Venezuelan populations may contain pathogens that can infect the Australian stock, but are novel and therefore more pathogenic.

We thank Dr Anna Goebel, Dr Leo Joseph, Ms Noel Cortinas, Dr Diane Barton, the curators of the MVZ, ASDM and USNM, and members of the CSIRO team from Australia and Venezuela for providing samples for genetic analysis. Anita Heideman provided susbtantial assistance with DNA sequencing. We thank Dr Simon Easteal and Dr Alex Hyatt for comments on the manuscript. This research was supported by grants from the CSIRO and the Australian Research Council.

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