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The phylogeny of the *Eremias velox* complex of the Iranian Plateau and Central Asia (Reptilia, Lacertidae): Molecular evidence from ISSR-PCR fingerprints

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The rapid fringe-toed lizard, *Eremias velox*, is widely distributed in the Iranian plateau and Central Asia. Several local morphotypes have so far been reported from different parts of its range, representing this taxon as a species complex. In an attempt to reveal phylogenetic relationships among various populations of this complex group, 37 specimens from 13 geographically distant localities in Iran and central Asia, covering most parts of its range, were sampled. Using Inter Simple Sequence Repeat amplification (ISSR-PCR) as a molecular marker, whole the nuclear genome of all specimens was screened. Phylogenetic analysis of the prepared data set successfully recovered seven major clades within the *E. velox* complex. Relationships among the major clades were highly resolved with remarkable statistical supports and well correspond to the geographic distribution of the populations. The reconstructed phylogeny implies that the clade as a whole has been originated in the Iranian plateau and expanded into central Asia before uplifting the Kopet-Dagh Mountains. It has then undergone a rapid cladogenesis in the latter area and produced several morphotypes. Within the Iranian clades two main groups could be defined, the foothill and highland dwellers and the open plane and desert dweller populations. The phylogenetic tree together with the estimated amounts of genetic distances among the independent lineages, provide good grounds for a fundamental revision of the taxonomic status of the *Eremias velox* complex.

Key words: Central Asia, *Eremias velox*, Iranian Plateau, ISSR-PCR, Phylogeny

INTRODUCTION

The rapid fringe-toed lizard, *Eremias velox* (Pallas, 1771) is one of the most widely distributed species of the Eurasian lacertid genus, *Eremias*. It is distributed on a large territory in Daghestan (Russia), northeastern Caucasus and Transcaucasus, Lower Volga, Kazakhstan and all of the middle Asian republics, as far east as northern Afghanistan and China. South through the Iranian plateau it occurs on the southern coast of the Caspian sea, valleys of Kopet-Dagh and scattered localities on the northern and western margin of the central plateau (figure 1.) (Szczerbak, 1974; Zhao and Adler, 1993; Anderson, 1999). Ecologically, *E. velox* inhabits a variety of surfaces. To the west, north and east of its range the preferred habitats are desert sands and loose soils with poor vegetation, to the south of its range, it occurs on foothills and river valleys with scattered vegetation and mountains up to 1900m above sea level (Eremchenko and Panfilov, 1999). *E. velox*, like many other species of the genus, is a surprisingly short-lived animal in nature. The lizard usually reaches sexual maturity at one year and has an average life expectancy of just two and half years. The reproductively is also low, laying usually two clutches of 2-5 eggs annually (Szczerbak, 2003). Morphologically, *E. velox* is a

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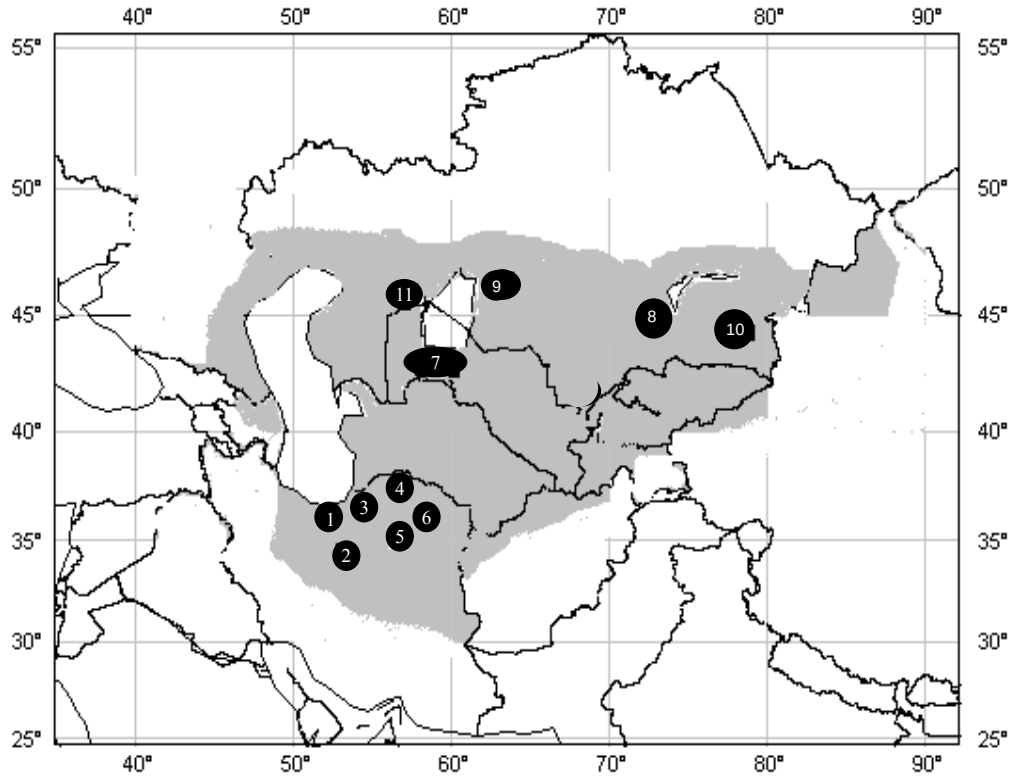

FIG.1.- The entire distribution range of *E. velox* (gray background) and 11 localities where the specimens included in the present study were collected.

medium sized lacertid with nearly 20 cm length, of which two-third is length of the tail. The juvenile radically differs from the adult in color pattern. Sexual dimorphism is also frequently observed in many populations (Szczerbak, 1974; Leviton et al., 1992; Anderson, 1999). Within the large distribution range of this taxon several isolated populations have so far been reported, representing well-differentiated units morphologically. It is generally accepted that *E. velox* as a clade in its entire range represents a species complex with several local distinguishable morphotypes (subspecies). The larger parts of its central Asian range is occupied by the nominate form which morphologically has fewer than 55 scales at mid body and dark elongated spots on the middle of the back in adults, with contrasting dark and white stripes or rows of color dots on the lateral sides of the body. The territory to the west is inhabited by the Caspian subspecies, *E. velox caucasia* (Lantz, 1928) which, as a rule, has 55 scales at midbody and the middle of the back is covered with thin dark spots, without contrasting markings on the lateral body. There are, however, rows of black-edged bark blue eye-like spots along the flanks (Szczerbak, 2003). In western China, eastern Uzbekistan and eastern Kazakhstan another subspecies, *E. velox roborowski* (Bedriagai, 1912) has been reported (Szczerbak, 1974). The adults of this subspecies have a dorsal pattern of irregular dark spots and on the lateral sides of the body there are rows of bright blue-edged, dark eye-like spots (Szczerback, 2003). Recently Eremchenko and Panfilov (1999) reported the fourth subspecies, *E. velox borkini* from the highlands of Issik-kul lake depression, Tien-Shan Mountains of Kyrgyzistan. It differs from all other subspecies in lacking sexual dimorphism, higher numbers of dorsal and gular scales. In addition, the

TABLE 1.- List of the materials used in this study with the localities and reference numbers. The number of localities corresponds to those defined in Figure 1.

Field number	Name	Locality number	*IPMB number*	*Locality*
02-74	*E. velox*	1	40605	90 km, north Tehran, northern Iran
02-75	*E. velox*	1	40606	90 km, north Tehran, northern Iran
ERP266	*E. velox*	2	41049	SE Tehran, the Central Desert of Iran
ERP267	*E. velox*	2	41050	SE Tehran, the Central Desert of Iran
ERP268	*E. velox*	2	41051	SE Tehran, the Central Desert of Iran
ERP269	*E. velox*	2	41052	SE Tehran, the Central Desert of Iran
Smp188	*E. velox*	3	٤٠٧٢٥	SE Golestan National park, Golestan province, Iran
Smp189	*E. velox*	3	٤٠٧٢٦	SE Golestan National park Golestan province, Iran
ERP250	*E. velox*	4	٤١٠٤١	10km S jajarm town, NW Khorasn, Iran
ERP251	*E. velox*	4a	٤١٠٤٢	10km S jajarm town, NW Khorasn, Iran
ERP252	*E. velox*	4a	٤١٠٤٣	10km S jajarm town, NW Khorasn, Iran
ERP253	*E. velox*	4a	٤١٠٤٤	10km S jajarm town, NW Khorasn, Iran
ERP254	*E. velox*	4a	٤١٠٤٦	10km S Jajarm town, NW Khorasn, Iran
smp267	*E. velox*	4b	٤٠٧٤٢	30km E jajarm town, NW Khorasn, Iran
smp268	*E. velox*	4b	٤٠٧٤٣	30km E jajarm town, NW Khorasn, Iran
smp266	*E. velox*	4b	٤٠٧٤١	30km E jajarm town, NW Khorasn, Iran
Smp15	*E. velox*	5	40718	15km SW Sabzevar, W Khorasan, Iran
Smp16	*E. velox*	5	٤٠٧١٩	15km SW Sabzevar, W Khorasan, Iran
Smp17	*E. velox*	5	٤٠٧٢٠	15km SW Sabzevar, W Khorasan, Iran
Smp18	*E. velox*	5	٤٠٧٢١	15km SW Sabzevar, W Khorasan, Iran
ERP189	*E. velox*	6	٤٠٨٤٠	S Naishaboor, NC Khorasan, Iran
ERP190	*E. velox*	6	٤٠٨٤٢	S Naishaboor, NC Khorasan, Iran
ERP191	*E. velox*	6	٤٠٨٤٣	S Naishaboor, NC Khorasan, Iran
ERP192	*E. velox*	6	٤٠٨٤٤	S Naishaboor, NC Khorasan, Iran
ERP198	*E. velox*	6	٤٠٨٤٥	S Naishaboor, NC Khorasan, Iran
ERP200	*E. velox*	6	٤٠٨٤٧	S Naishaboor, NC Khorasan, Iran
Ev1	*E. velox*	7	٤٠٥٦٨	S Aral Sea, Uzbekistan
Ev6	*E. velox*	7	٤٠٥٧٣	S Aral Sea, Uzbekistan
Ev7	*E. velox*	7	٤٠٥٧٤	S Aral Sea, Uzbekistan
Ev10	*E. velox*	7	٤٠٥٧٦	S Aral Sea, Uzbekistan
Ev12	*E. velox*	7	40579	S Aral Sea, Uzbekistan
Ev13	*E. velox*	7	٤٠٥٨٠	S Aral Sea, Uzbekistan
K04-6	*E. velox*	8	٤٠٩٣١	SW Balkhash Lake, Kazakhstan
K04-8	*E. velox*	9	٤٠٩٣٣	NE Aral Sea, Kazakhstan
K04-14	*E. velox*	9	٤٠٩٣٤	NE Aral Sea, Kazakhstan
K04-7	*E. velox*	9	٤٠٩٣٤	NE Aral Sea, Kazakhstan
Pp	*E. velox*	10	٤٠٩٢٥	E Kazakhstan

pattern of the dorsal part of the body is reduced and the lateral row of color ocelli is absent, instead, a row of completely dark spots runs over each side of the body. Knowledge of the Iranian populations of *E. velox* is, to a great extent, anecdotal and there are still large gaps in available material from the various parts of the plateau. However, based on some limited materials, all of the Iranian populations have been attributed to the nominate subspecies, *E. velox velox* (Anderson, 1999). Despite several morphological and ecological studies, particularly on the central and northern Asian populations, systematic status and subspecies boundaries within the *E. velox* clade are still in debate (Eremchenko and Panfilov, 1999; Szczerbak, 2003). To date, no attempt has been made to reveal intraspecific phylogeny and phylogeography of the clade based on molecular data. Origin,

distribution, fragmentation, diversification and subsequent evolution of the clade in the area are, to a great extent, unknown.

The inter-simple sequence repeats amplification (ISSR-PCR) is a relatively new technique that can rapidly differentiate closely related individuals (Gupta et al., 1994; Tsumura et al., 1996; Zietkiewicz et al., 1994). It also proved to be a powerful molecular marker to investigating intraspecific differentiation and phylogenetic relationships among closely related taxa.

ISSR analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR) containing primer (Zietkiewicz et al. 1994). This technique can be undertaken for any species that contains a sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required (Gupta et al. 1994; Goodwin et al., 1997). The primer used in ISSR analysis can be based on any of the SSR motifs (di-, tri-, tetra- or penta-nucleotides) found at microsatellite loci, giving a wide array of possible amplification products, and can be anchored to genomic sequences flanking either side of the targeted simple sequence repeats (Zietkiewicz et al., 1994). For ISSR analysis to be successful, pairs of simple sequence repeats must occur within a short distance (in base-pairs) that is amplifiable by a PCR reaction which produces a band that is resolvable on standard polyacrylamide or agarose gels (Zietkiewicz et al., 1994). The potential supply of ISSR markers depends on the variety and frequency of microsatellites, which changes with the species and the SSR motifs that are targeted (Morgante and Olivieri, 1993). If microsatellite distribution is completely random, the length of the intervening regions between simple sequences repeats of the same motif is a function of their frequency. As a result, we could hypothesize that the number of bands produced by an ISSR primer with a given microsatellite repeat should reflect the relative frequency of that motif in a given genome and would provide an estimate of the motif 's abundance different (Blair et al., 1999)

Unlike nucleotide sequences, ISSR markers describe DNA characteristics at several, mostly nuclear, chromosomal loci and thus avoid the use of gene trees as surrogates of species trees (Martin and Salamini, 2000). Revealing discontinuous markers, ISSR-PCR can provide a measure of genetic differences dispersed across the entire genome. However, it is not yet clear whether ISSR bands represent only nuclear markers or a combination of nuclear and organellar markers (Wolfe et al., 1998). The absence of a band is interpreted as primer divergence or the loss of a locus through either the deletion of the SSR site or a chromosomal rearrangement (Wolfe and Listen, 1998).

Until recently, the use of ISSR markers was restricted to cultivated plant species (e.g. Tsumura et al., 1996; Fang and Roose, 1997; Assefa et al., 2003), their pests (e.g. Kumar et al., 2001) or other animals of economic importance (e.g. Reddy et al., 1999; Nagaraju and Goldsmith, 2002). However, it is now increasingly being applied in population-level to interspecific and even intergeneric studies of natural populations of plants and animals (e.g. Culley and Wolfe, 2001; Kauserud and Schumacher, 2003; King and Ferris, 2000; Sudupak, 2004; Guicking et al., 2002a,b; Luque et al., 2002; Nagy et al., 2002; Treutlein et al., 2003; Hundsdoerfer et al., 2005a,b). ISSR markers have proven an efficient method for detecting hybridization in natural populations of plants (Wolfe et al., 1998) and animals (Wink et al., 2001). ISSR-PCR can reveal a large number of fragments, and thus many potentially polymorphic loci, in one PCR with good reproducibility. This is mostly because microsatellites are ubiquitous in eukaryotic genomes. The copy number of the tetranucleotide repeat, (GATA) n, is approximately I0000 in the mouse genome (Shafer et al., I986). It has been suggested for the human genome that one SSR occurs on average every 10 kb of DNA with a fairly random distribution (Tautz, I989). These characteristics are great advantages compared to, for example, RAPD amplification. The major advantage of ISSR-PCR over microsatellite analyses is the cost-efficiency, because no initial investment in primer design is necessary. However, a disadvantage is

that loci are usually interpreted as dominant markers, so no genotypic allele information is required, as in microsatellite analyses.

Here, we use ISSR-PCR genomic fingerprinting to investigate the molecular phylogeography of the *E. velox* complex. The main goals were to reveal (1) whether distinct evolutionary lineages exist in this morphologically species complex, (2) how these correlate to geographic regions, and (3) what inferences can be drawn from these data about the evolutionary history of the species. The data provide evidence of the persistence of several independent lineages, which are proposed as distinct taxonomic entities at species or subspecies level.

MATERIAL AND METHODS

SAMPLE COLLECTION

A total of 37 specimens of the *E. velox* complex from 13 geographically distant localities were available for the present study, which covers great parts of the species' distribution range (figure 1, table 1). The Iranian samples were collected through several fieldworks during 2002-2006. The central Asian materials were kindly provided by Ulrich Joger (State Natural History Museum, Braunschweig, Germany) and Marina Chirikova (Almaty, Kazakhstan). For details on sample localities and reference numbers of aliquots all samples included in this study are listed in table 1. The voucher specimens were deposited at the Institute of Pharmacy and Molecular Biotechnology (IPMB), University of Heidelberg, Germany.

LABORATORY PROTOCOLS

Isolation of total genomic DNA followed standard protocols (Sambrook and Russell 2001). Small aliquots of sample material were digested for 48 hours at 37°C in lyses buffer in the presence of 1% SDS and 1 mg of proteinase K. Cell fragments and proteins were precipitated with saturated NaCl solution and subsequent centrifugation or by standard phenol/chloroform extraction. The DNA was precipitated from the supernatant by adding 0.8 Vol.-% of ice-cold isopropanol. The extracted DNA was washed with 70% ethanol, dried and redissolved in TE buffer or sterile water. DNA stock solutions were kept at 4°C until further experiments.

In cases of very limited sample material and low DNA yield, the protein pellet was re-digested by addition of guanidine isothiocyanate buffer to extract DNA that was trapped in the pellet. In this case, digestion was followed by extraction twice with phenol/chloroform, then once with chloroform/isoamyl alcohol and subsequent precipitation and washing of the DNA as explained above.

To determine the approximate concentration and quality of the extracted DNA, 5 µl of each DNA solution were loaded onto a 1% agarose gel containing ethidium bromide. DNA concentration was estimated by comparison of fluorescence intensities to samples of known DNA content. For analyses for which the exact concentration of the DNA was critical to know, DNA concentration was determined photometrically.

Fragments between the microsatellites consisting of the tetra-repeat $(GACA)_n$ and $(GGTA)_n$ (and their complement $(CTGT)_n$ and $(CCAT)_n$, were amplified using the non-anchored primer, $(GACA)_4$ (Epplen et al., 1992) and $(GGTA)_4$. Each PCR was performed with about 500 ng template DNA in a 25 µl volume (10 pmol of the primer and 0.625 nmol of each dNTP, except dATP: 0.28 nmol cold dATP plus 0.1 µl radioactive 33P-dATP solution (370 MBq/ml, Amersham Biosciences), 0.1 units of Taq-Polymerase (Bioron) and water, buffered with 10 mM Tris-HCl, 50 mM KC1, 0.5% Triton X-100, 1.5 mM $MgCl_2$) and covered by two drops of mineral oil. Thermo cycling was performed with a Trio Thermo block TB1 (Biometra, Göttingen). Following the initial 5 min denaturation at 95 °C, the program consisted of 32 cycles of 50 s at 95 °C, 40 s at 53 °C for

$(GACA)_4$ and 49 °C for $(GGTA)_4$, 120 s at 72 °C and 15 min at 72 °C for final elongation. The DNA fragments were separated by PAGE (Polyacrylamid Gel Electrophoresis) in a vertical apparatus (Base Acer Sequencer, Stratagene) for 2.5 h for $(GACA)_4$ and 2 h for $(GGTA)_4$ at 65 W. The denaturating gels (6 M Urea, 100 ml Long Ranger Solution, Biozym (PA), 100 ml TBE-Buffer (lOx: 1 M Tris, 0.83 M Boric Acid, 10 mM EDTA, pH 8.6)) had a size of 45 x 30 cm and a thickness of 0.25 mm. After drying, the 11gel was exposed to an X-ray film (Fuji or Kodak) for at least 24 hours and developed (Kodak). The film was then scanned with a resolution of 300-600 dpi. The bands were analyzed visually on the film itself, but marked on an A3-sized print of the film.

The bands of the ISSR-PCR fingerprints were interpreted as representing independent characters (Assefa et al. 2003) and were visually scored into a data matrix as either absent ("0") or present ("1"). Qualitative differences in band intensity were not considered (Assefa et al. 2003). We assumed that markers from different loci did not co-migrate to the same position on the gel (Culley and Wolfe, 2001). Generally, ISSR bands are scored as dominant markers (e.g. Zietkiewicz et al., 1994), so this procedure was adopted in this analysis as well. This implies that slight differences in the retention index of apparently homologous bands or band-patterns were not scored as alternate allelic conditions or as separate characters. Since the primers used were not anchored (this does not reduce reproducibility of bands (Bornet and Branchard, 2001), slight shifts in size of fragments could also be attributed to shifts of the primer annealing on the microsatellite DNA matrix. Certain variability in electrophoretic mobility differences is widely accepted for fingerprint analyses (Jeffreys et al., 1991). By repeating the PCR of several samples a few non-reproducible bands were identified and excluded from all analyses. The bands with the highest molecular weights were not scored, due to the risk that differences in the amplification of large fragments is caused by qualitative differences in the DNA, instead of differences derived from genomic characteristics. Faint autapomorphic bands were also not scored.

The variation of the ISSR-PCR fingerprint data was characterized following Culley and Wolfe (2001). The numbers of fixed (conserved), polymorphic (variable), shared (parsimony informative), and unique (parsimony uninformative) loci were determined with PAUP* 4.0b (Swofford, 2001). No autapomorphies and only fragments that could be reliably scored and homologized between samples were included in ISSR-PCR data analysis. From the ISSR-PCR data a neighbor-joining tree with the mean and total number of pairwise character differences as distance measurement was calculated using the program PAUP 4.0b10 (Swofford 2001). Support for clades from ISSR-PCR data sets was assessed by bootstrapping (Felsenstein 1985) with 2000 replicates.

RESULTS

The gels of the ISSR-PCR fingerprints are illustrated in Fig. 2. The bands with high molecular weight (not illustrated) were excluded due to non-reproducibility. The data set did not contain missing data. In total, the ISSR-PCR fingerprints using the primers $(GGTA)_4$ and $(GACA)_4$ in 37 specimens of *E. velox* produced informative finger prints with 61 polymorphic and 54 parsimony informative bands, not considering autapomorphies. ISS profiles were more diverse with the $(GGTA)_4$ primer than with $(GACA)_4$. Both primers generated a series of population-specific bands and several individual specific bands (not included in the analysis). Pairwise comparisons of ISSR-PCR fingerprints among 37 samples resulted in 0 to 38 differences, corresponding to a maximum proportion of different fragments of 47%.

To resolve intraspecific differentiation based on ISSR-PCR data, phylogenetic trees were calculated under the Neighbor Joining (NJ) criterion with different distance measurements. All reconstructions

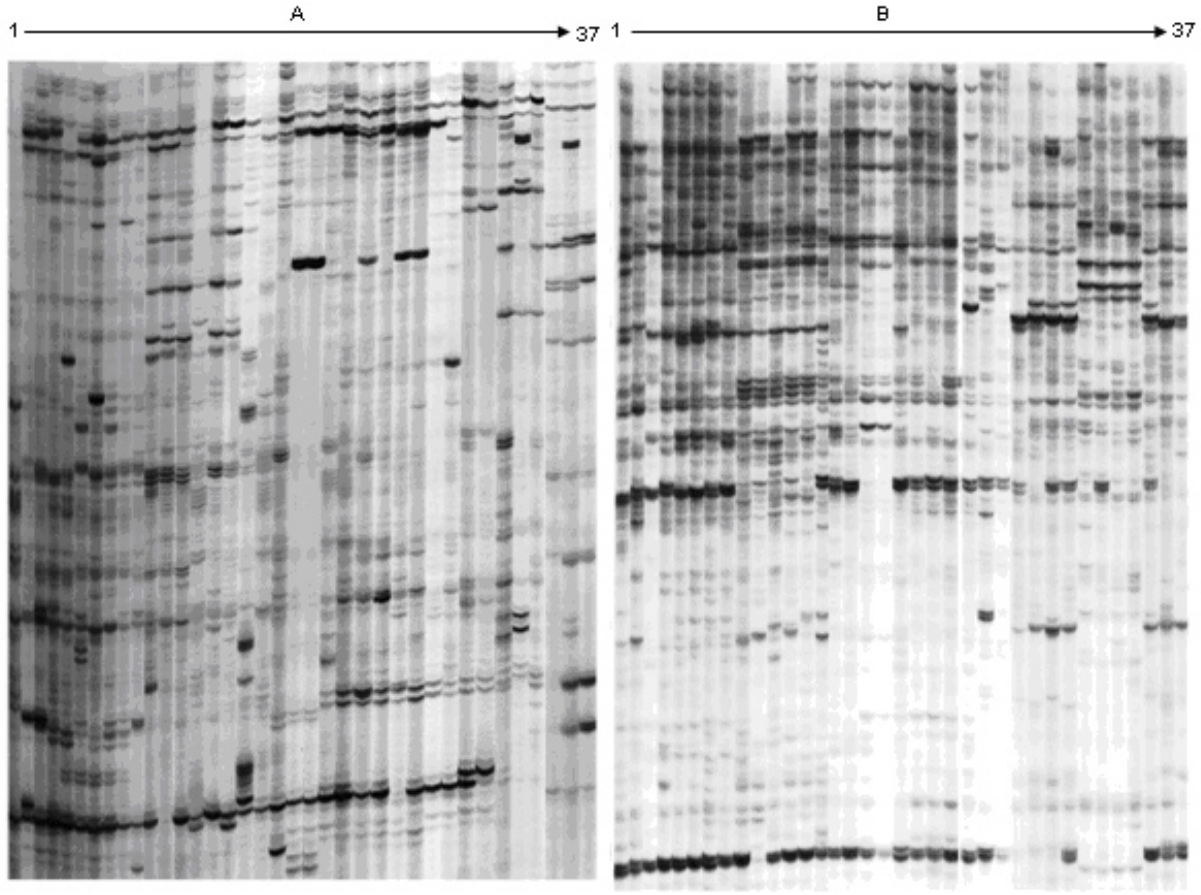

FIG.2.- Illustration of the gels of the amplified ISSR fragments (37 samples each) of the different populations of the *Eremias velox* complex (see figure 1 and Table 1) as visualized on a PA-gel by the use of radioactively labeled adenine; a). A: fragments obtained by the primer $(GGAT)_4$ and B: those obtained by $(GACA)_4$. Individuals orderly were the same in both amplifications.

yielded generally very similar topologies with small differences referring to place of the terminal nodes. One of the reconstructed trees is shown in figure 3. Relationships depicted in this tree showed no considerable discrepancy with that expressed in the NJ phylogram evaluated by total distance criteria in separate analysis.

Phylogenetic reconstruction inferred from the data set clearly supported intraspecific subdivision of the *Eremias velox* complex into the Iranian and central Asian clades with further fragmentation of the both to form several subunits leading mostly to the major clades showing in figure 3.

Seven major clades could be identified in the phylogenetic trees with high or moderate bootstrap supports, which are generally well related to geographical regions of the distribution range. These clades are designated as A, B, C, D, E, F and G in Figs 3. The most basal dichotomy in the tree separated the specimens of southwestern Sabzevar, (clade A, locality 5), with 90% of bootstrap value. The second divergence isolated the Iranian populations of southeastern Golestan (clade B, locality 3) with very high statistical support (100% bootstrap value). Surprisingly, the populations of south Jajarm in northwestern Khorasan (locality 4) along with those of around Varamin inhabited in the western margin of the Iranian central desert (Kavir-e- Markazi) constitute another well resolved

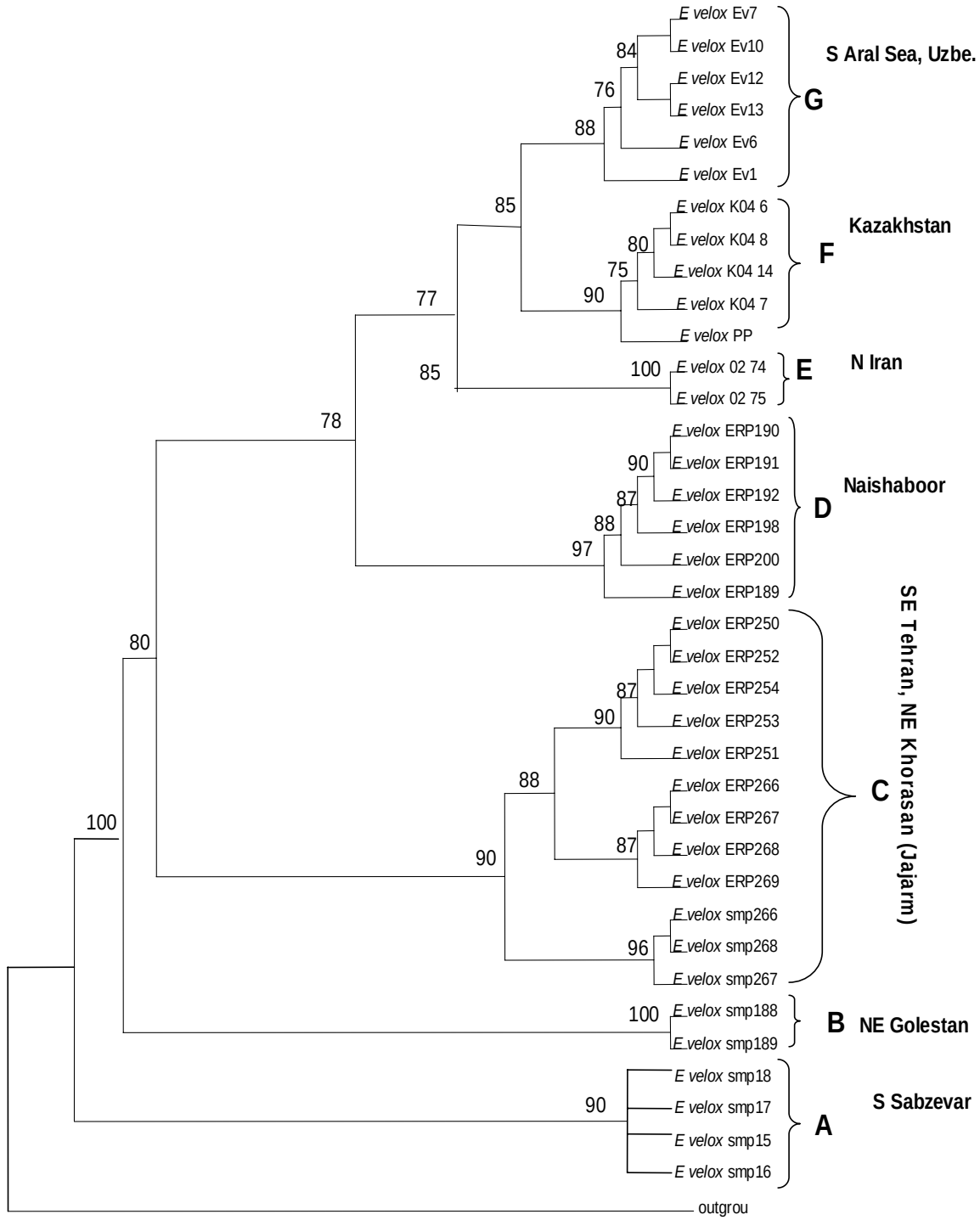

FIG.3.- A neighbor joining tree of the *E. velox* complex data, reconstructed using 61polymorphic bands. Numbers next to the nodes indicate the bootstrap values (2000 replicates). A, B, C, D, E, F and G are the highly resolved clades recovered by the ISSR-PCR profiles.

clade (clade C) which in turn contains three discernable subunits. Sister relationship between this clade and the population of northern Naishabour (clade D, locality 6) was also well established in all reconstructions. The inferred phylogeny clearly suggested a sister group relation between the highland specimens of northern Iran (clade E, locality 1) and whole the central Asian assemblages, in which the specimen of east, northeast and west Kazakhstan (clade F, localities 10, 11 and 12) forms the sister taxon for the population of southern Aral Sea in Uzbekistan (clade G, locality 7). In short, despite several problems and uncertainty associated with the phylogenetic reconstruction using any data set obtained by ISSR-PCR fingerprints (see Guicking 2004, Hundesdorfer et al, 2005a for details), the phylogenetic tree presented here is relatively robust and the bootstrap values supporting the internal nodes are mostly high or moderate. In addition, the relationships within the species complex are considerably resolved.

DISCUSSION

Data of the present study clearly indicates the occurrence of several evolutionary independent lineages within the *E. velox* complex. The length of the internal branches imply that these lineages have experienced a long period of isolation, supporting results of the previous studies based on morphology and ecology (Szczerbak, 1974, 2003; Eremchenko & Panfilov, 1999). This information may serve to elucidate the evolutionary history of the species and to provide guidelines for the revision of taxonomic status of the major lineages.

According to the results presented here, at least 7 major clades can be distinguished within the *E. velox* complex, which correlate well with geographic regions of the Iranian plateau and central Asia. Position of the southern Sabzevar and northeastern Golestan populations at the basis of the phylogenetic tree with high statistical supports may indicate a long period of isolation of these populations from the remainder**.** This differentiation is relatively well reflected in general patterns of the both populations, particularly the Golestan populations that are easily discernible from all other populations morphologically. Although populations of the clade C form a monophyletic clade they are genetically highly heterogeneous. Geographical distance between southeastern Tehran and Jajarm populations notwithstanding the habitats in which these populations were collected are highly homogeneous, this might explain the parallel evolution of these units. Indeed they are reproductively isolated because of long geographical distance, although no significant barrier can be defined between them. In addition, divergence between the eastern and southern Jajarm populations might be best explained by the possible introgression between the former population and the specimens of one of closest relatives of *E. velox*, *E. persica,* in vast deserts and steppes of the eastern Jajarm, where they can be frequently seen sympatrically. Testing this hypothesis requires a separate study on the both assemblages using appropriate molecular markers and morphological considerations.

A sister relationship of the Naishaboor specimens with those of northern area of Iran (locality 1) most likely suggests another homoplasy in the phylogenetic tree rather than the recent divergence of these clades. Indeed it looks that under the same selective pressures these populations evolved with the same fashion. Both are the highland inhabiting populations of *E. velox* in Iran. The former inhabits rugged area of the Binalood Mountains in northwestern Khorasan province and the latter on the highlands and valleys of the central Elburz. In particular, many ecological and morphological evidence support differentiation of the northern Iranian clade. These are the only records of *E. velox* in Iran inhabiting the valleys of central Elburz in elevation of over 2000 m. In addition they are morphologically well discernible from other Iranian clades of *E. velox*, with having several diagnostic features. In some morphological traits, they are closer to *E. strauchi* than to *E. velox*. In fact all the Iranian clades of the *E. velox* complex could be referred to two geographically distinct habitats in general area of the northern and northeastern Iranian plateau. Populations of the clades A, B and C

inhabit the lowlands and open plains in the altitudes lower than 1700 m. They were always observed on the open plains and desert habitats of south and southeastern Elburz Mountains in Tehran, Semnan and Khorasan provinces. In contrast, the specimens of the clade D and E inhabit the highlands, foothills and generally rugged areas of the central and eastern Elburz Mountains. During several long term expeditions in the area, they have never been observed in altitude lower than 1800m.

The branching pattern recovered in the phylogenetic reconstruction supports the monophyly of the Central Asian populations and separated them well outside of the Iranian specimens. Further, the population of southern Aral Sea in Uzbekistan was isolated from the specimens of Kazakhstan in forming the clades F and G. However, the branch lengths separating these units are relatively short suggesting a recent splitting event. In particular, notable is the position of the specimens of northeast Aral Sea and those of eastern Kazakhstan in forming the clade F, despite very long geographical distance between them.

The fact that phylogenetic inference clearly assigned the basal status to the Iranian clades on one hand and considering the high level of interpopulation differentiation among these clades on the other, imply that the rapid fringe-toed lizard originated in the Iranian plateau and invaded central Asia just recently. Sister relationship between the highland dweller populations of Iran (localities 1 and 6) and whole the Central Asian clades suggest that these lineages may have diverged only recently. The Central Asian populations now mainly inhabit the lowlands and deserts of the area while their closet relatives in Iran are highlands and mountain dwellers; in addition they are also morphologically well distinguishable, suggesting that the divergence time still is not long enough to fix the mutations into their genomes. Taking into account highly resolved relationships among the lineages recovered in this study as well as lack of polytomy and zero branch length in the tree; indicate that the early divergence within the *E. velox* complex should have not produced more than two lineages simultaneously. In a comprehensive study of the phylogenetic relationships within this species complex using mitochondrial markers (Rastegar Pouyani et al., submitted), which is generally supported by the present study, possibly one or two independent lineages invaded central Asia via the land bridge between Caspian and the high plateaus of Badkyz and Pamir, a region, which started to emerge when the Caspian Sea started to retreat some 7-8 mya. With the late Miocene the invaders seem to have dispersed in the area relatively rapidly due to enormous unoccupied niches and lack of competition. Therefore rapid cladogenesis and explosive radiation resulted in producing several morphologically distinct forms, whereas these morphotypes could not be traced by genetic markers with high resolution. This is possibly responsible for lower genetic divergence and relatively less resolved relationships among the central Asian clades, in spite of a vast distribution range. The case is not restricted to *E. velox*, it has also been shown in a phylogenetic study of the agamid lizard *Trapelus agilis*, which is widely distributed in the same general area (Macey and Ananjeva, 2004). The low resolution of the phylogenetic relationships among the central Asian clades is more pronounced in the tree suggested by ISSR-PCR data set than to the trees reconstructed by mitochondrial DNA sequences (Rastegar Pouyani et al., submitted). It is possibly due to slower lineage sorting in nuclear than in mitochondrial DNA (Moore 1995; Moritz and Hillis 1996). Furthermore the central Asian lineages are mainly isolated just by distance, whereas significant geographical barriers could be defined between the Iranian lineages.

The data presented here provide a basic evidence for intraspecific subdivision in the *E. velox* complex. Several distinct lineages can be recognized that most likely evolved independently for more than five million years. To account for this high intraspecific diversity and long divergence times, it seems desirable to assign species or at least subspecies rank to some of the genetic lineages. The Iranian lineages (clades A to E) are well discernible genetically and even ecologically. Particularly, the clades E and F are well distinguishable from all other Iranian lineages genetically, ecologically and

morphologically. To some extent, it is also true of other Iranian lineages when comparing them genetically (clades A to D). The fact that several genetic units are discernable within the clade C should not be used as grounds to partitioning them into several distinct species or subspecies. Morphologically, the group is well defined. The populations of localities 2, 4a, and 4b are easily recognized as belonging to the same clade, but this is not strictly true of the subunits, which are difficult to define morphologically. The central Asian lineages as a whole represent a well distinguishable unit from the Iranian lineages, genetically, geographically and, to a great extent, morphologically. Hence, the central Asian clade should also be recognized as distinct species. The data presented here provide good evidence to identify two independent lineages within the central Asian clade corroborating to the previously recognized morphotyeps (subspecies) in the area (Szczerbak, 1974). The eastern and northeastern Kazakhstan populations, grouped within the clade F, re-identify the traditional subspecies of *E. velox roborowskii*, as do likewise the clades E in re-identification of *E. velox velox*. Since the type locality of *E. velox* locates in central Asia, thus, all the central Asian clades should be assigned to *E. velox* and the Iranian lineages should await new scientific names. However, as taxonomic decision making based solely on ISSR data is not recommended (Martin and Salamini, 2000; Culley and Wolfe, 2001; Kauserud and Schumacher, 2003) therefore separation of the lineages recovered in this study as distinct taxonomic entities at species or subspecies level remains uncertain. Increased sampling all over the distribution range, extension of molecular investigations, providing diagnostic morphological data and considering ecological traits of all lineages seem desirable and necessary to define geographic boundaries between evolutionary lineages more precisely. Until such a study possibly sheds more light on this enigma, we consider *Eremias velox* clade as a species complex. There is evidence that the clade as a whole started to diverge by the Late Miocene and most likely one or two lineages of this complex group invaded Central Asia before uplifting of the Kopet-Dagh Mountains some 6-8 mya. At the present, the group comprises several independent evolutionary lineages that possibly some of them deserve raising to species or subspecies rank.

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