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Single hybrid population but multiple parental individuals at the origin of parthenogenetic rock lizards *Darevskia sapphirina* and *D. bendimahiensis* Schmidtler, & Eiselt Darevsky (1994) endemic to the area of Lake Van in East Turkey

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ABSTRACT

Among vertebrates, obligate parthenogenesis is only found in Squamata, where it always has a hybrid origin and a few lizard genera contain most of the known hybridogenous parthenogenetic taxa. Parthenogenesis thus seems to be pre-conditioned at the genus level, but it is not clear how often the encounter between two parental sexually reproducing species can result in the parthenogenetic offspring, nor whether the success of such hybridization event requires certain conditions or the specific time frame. To address this question, we studied the rock lizards of genus *Darevskia*, where a pair of parental species, *D. valentini* and *D. raddei*, as well as their parthenogenetic daughter species *D. bendimahiensis* and *D. sapphirina*, are found in close proximity NE of the Lake Van in East Anatolia. Using ddRAD-seq genotyping on 19 parental and 18 hybrid individuals, we found that (i) all parthenogenetic individuals from both *D. bendimahiensis* and *D. sapphirina* have a monophyletic origin tracing back to a single initial hybrid population, but their current genetic variation is geographically structured; (ii) unlike the most probable paternal ancestor, the genetically closest extant population of the maternal ancestor is not the geographically nearest one; and (iii) in the parthenogens, about 1% of loci carry multiple haplotypes, frequently differentiated by multiple substitutions. This pattern, in addition to biases in the relative frequency of haplotypes of maternal and paternal origin, does not appear compatible with a scenario of the entire parthenogenic clonal population having descended from a single pair of parental individuals. Instead, the data suggest that multiple parental individual ancestries still persist in the parthenogenetic gene pool. This supports the notion that although hybridization leading to parthenogenesis is generally rare at the level of species, it may be more common at the individual/population level once the right conditions are met.

1. Introduction

The genus of rock lizards *Darevskia* Arribas, 1997 was the first group of terrestrial vertebrates where obligate parthenogenesis had been discovered (Darevsky, 1957). To date, seven unisexual forms have been described (Darevsky 1967; Darevsky & Danielyan, 1977; Murphy et al., 2000). Each of these parthenogenetic forms appears to have arisen from hybridization between two distant phylogenetic branches within the genus, which have been estimated to have diverged at least 13 Myr ago based on a molecular clock analysis (Murphy et al. 2000, Yanchukov et al. 2022). For each parthenogenetic species, the paternal parent always belongs to the clade "rudis" and the maternal parent to the clade "caucasica" (Murphy et al. 2000; Tarkhnishvili et al. 2020a, Yanchukov et al. 2022). While the genus *Darevskia* has a broad distribution ranging from the Caucasus to SE Europe (Darevsky et al., 1985; Arnold et al., 2007), all parthenogenetic forms are found in a relatively small area shared by Georgia, Armenia, Azerbaijan and Eastern Turkey (Tarkhnishvili, 2012).

One of the most disputed questions in the > 50 years history of research on *Darevskia* was the timing and the number of so-called "hybridization events" that occurred at the origin of extant parthenogenetic hybrid lineages (Darevsky 1967). One might assume that the

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parthenogenetic hybrids appeared when the parental species were found in sympatry in the past. During such periods of contact, the genetic diversity of the parthenogenetic populations would be at its highest due to constant influx of new gene variants from the parents. Under this scenario, the hybridization event roughly coincides with the period spent in sympatry. In the absence of immediate contact between parental species, however, the genetic drift in the non-recombining parthenogenetic populations is generally expected to reduce the initial diversity, with mutations within the clonal lineages being the only (rather weak) driving force generating polymorphism. All bisexual parental species in *Darevskia* system are presently allopatric (Tarkhnishvili et al. 2023). It is also possible that even in sympatry, successful hybridization between two highly divergent parental *Darevskia* species is a very rare event conditioned on the specific complement of genetic or ecological factors (Freitas et al., 2022). In such scenario, a much more narrow definition of the hybridization event applies to the actual mating between parental individuals that creates a successful hybrid parthenogenetic lineage. In a similar hybridogenous parthenogenetic system of the North American teiid lizards, *Aspidoscelis* sp., which features even larger number of distinct parthenogenetic forms, attempts to re-create a diploid parthenogenetic lineage in captivity, by cohabiting their parental, sexually reproducing species for over 29 years, only resulted in sterile offspring with multiple developmental abnormalities (Cole et al. 2010).

Finally, it has been proposed that some clonal unisexual populations of *Darevskia* could backcross with one of the parental species, or even with another bisexual species from the same paternal lineage (Tarkhnishvili et al. 2017). Such a scenario would certainly lead to the enrichment of the unisexual gene pool and / or could have lead to the formation of a new parthenogenetic form (Tarkhnishvili et al. 2017; Tarkhnishvili et al. 2020a). Natural parental backcross, i.e. the union of diploid oocytes from the parthenogenetic lineage and haploid sperm from bisexual progenitor, is known to have resulted in new triploid parthenogenetic species *Aspidoscelis netesselata* and *A. exsanguis* (Taylor et al. 2015). Moreover, Lutes et al. (2011) reported laboratory syntheses of four parthenogenetic, tetraploid lineages by crossing triploid *A. exsanguis* with haploid sperm from the bisexual species *A. inornata*. No similar polyploid and self-perpetuating unisexual forms are known in *Darevskia*, although apparently sterile triploid and tetraploid hybrids commonly result from the backcross of parthenogenetic *D. armeniaca* and *D. unisexualis* with their paternal *D. valenitini* (Darevsky & Kulikova, 1964; Arakelyan et al., 2008).

Addressing the above questions empirically is possible by examining the number of independent clonal lineages that constitute the all-female unisexual population (Tarkhnishvili et al., 2020a). The "clonal lineage" is thereby defined as (i) having originated from a single hybrid (F1) genotype, possibly a single parthenogenetic individual, and (ii) one that derives all its genetic variation from the accumulation of mutations after the onset of parthenogenesis. Assuming there were multiple clonal lineages in the past, the genetic differences among such lineages should still reflect the differences among the multiple parental individuals. Hence, the different clonal lineages should be identifiable in the parthenogenetic populations today if sufficient sequence length is combined with large sample sizes (and assuming large enough genetic differences between the ancestral parents). In previous work, extensive variation in the flanking regions of a few microsatellite loci was taken as evidence of the existence of several clones in parthenogenetic *D. armeniaca, D. dahli* and *D. unisexualis* (Vergun et al., 2014; Vergun et al., 2020; Girnyk et al., 2018), but only a single one in *D. rostombekowi* (Ryskov et al., 2017).

The parthenogenetic *D. bendimahiensis* and *D. sapphirina* (*D.b.* and *D.s.* from now on) are the last two to have been discovered and given taxonomic species status (Schmidtler et al., 1994). They are the rarest among the seven parthenogenetic forms, with each species only known from two respective localities in the basin of Lake Van in Eastern Turkey ((Schmidtler et al., 1994) Fig. 4.1). The current ranges of the nominal species *D.b.* and *D.s.* are allopatric to all other parthenogenetic forms as well as to each other (i.e. ~ 50 km between the respective type localities, Fig. 4.1). According to allozyme, mtDNA, microsatellite, and Z-linked genetic marker data, all parthenogenetic lizard populations in the Lake Van basin had originated from hybridization between the local populations of maternal *D. raddei vanensis* Eiselt, Schmidtler and Darevskiy, 1993 , and the paternal *D. valentini* Boettger, 1892 Murphy et al., 2000, Tarkhnishvili et al. 2020a, Yanchukov et al. 2022). The Lake Van population of *D. valentini* is represented by a distinct clade, which recently has been elevated to species status (*D. josefschmidtleri* Arribas et al. 2022), but for the purpose of this study, we prefer to use the old taxonomic designation.

Given a high degree of geographic endemism of *D.b.* and *D.s.*, as well as their putative parental populations, these lineages provide an excellent opportunity for investigating the evolution of hybridogenetic parthenogenesis in fine detail. In this study, we present a novel genome-wide analysis of several populations of *D. raddei vanensis, D. valentini, D. b. and D.s.* endemic to the Lake Van area. We aim to gain better insight into the evolution of the hybrid species, asking two main questions:

1.1. Are D. bendimahiensis and D. sapphirina reciprocally monophyletic, and which maternal and paternal populations contributed to their ancestry?

When *D.b.* and *D.s.* were first described as separate species by Schmidtler et al., (1994), the authors only relied on minor morphological differences and the fact that their distribution is allopatric. However, both parthenogens share from 51% to up to 67% of their microsatellite genotypes and an identical haplotype of the 683 bp fragment of the mitochondrial cytochrome *b* gene (Tarkhnishvili et al. 2020a). Later on, we showed that these two cannot be clearly distinguished based on the RAD-seq markers linked to chromosome Z (Yanchukov et al., 2022). The latter study also revealed that the local population of *D. valentini* from Çaldıran, NE of Lake Van, has the highest Z-chromosome sequence similarity with both parthenogens. Earlier comparison of partial mtDNA sequences revealed that several populations of *D. r. vanensis*, sampled over a large area southeast of Lake Van and extending into Iran, are equally similar to *D.b.* and *D.s.* (Freitas et al. 2016). Here we readdress the details of both parents' contributions to the parthenogenetic hybrid pool, using genome-wide ddRAD-seq markers.

1.2. Have D. bendimahiensis and D. sapphirina descended from a single pair of parental individuals?

The two nominal species *D.b.* and *D.s.* were shown to share, on average, > 60% of the microsatellite genotypes, which prompted a suggestion that they might originate from a single hybridization event (Tarkhnishvili et al. 2020a). The authors did note, however, that the resolution provided by a handful of the microsatellite loci might not be sufficient to reveal the particulars of the hybrid origin, and suggested that genome-level genotyping methods are better suited for future studies. Both forms formed a single monophyletic clade in the phylogeny constructed using only Z-linked (i.e. paternally inherited) genetic markers (Yanchukov et al. 2022). Although the *D.b.* is found in close proximity or sometimes in the same habitat with both of its parental species, no backcross hybridization has ever been reported there. In this study, we check for the presence of multiple clonal lineages in *D.b.* and *D.s.*, thus testing the null hypothesis that a single pair of parental individuals lies at the origin of the entire present-day gene pool of parthenogenetic Lake Van rock lizards.

2. Methods

2.1. Collection of samples

We collected samples (lizard tail tips) from two populations of

D. valentini, five populations of *D. raddei vanensis*, two populations of *D. b.*, and two populations of *D.s.* from the locations near Lake Van in Eastern Türkiye (Fig. 1) as shown in Table 1 (see Table S1: Supplementary Material for coordinates), and previously reported in Tarkhnishvili et al. (2020a). The entire region has been rigorously surveyed to ensure that no other populations in the region remain undiscovered. *Darevskia raddei, D. valentini* and the *D.s./ b.* lizard species, are easily identifiable using morphological characters (Arnold et al., 2007). Since the morphological description of *D.s.* and *D.b.* is nearly identical (Schmidtler et al., 1994), the geographic locations of the above taxa as reported in the literature were used to assign the species to the samples collected (Fig. 1, Schmidtler et al., 1994; Tarkhnishvili et al. 2020a, 2020b; Yanchukov et al., 2022). External traits such as dorsal and side coloration and the presence of femoral pores were used to determine the sex of adults (later confirmed by genotyping of all individuals). DNA was extracted from the tail tips taken with negligible harm to the animals, as described in Tarkhnishvili et al., 2020a. In one location (Muradiye), both maternal *D. raddei vanensis* and the daughter *D.b.* occur sympatrically, and in another location (Çaldıran), the populations of *D.b.* and the paternal parent *D. valentini* are only ~ 3 km away from each other.

2.2. ddRAD-seq library preparation and sequencing

The extraction of genomic DNA of samples from alcohol-preserved tail tips was carried out following the methodology outlined by Tarkhnishvili et al. (2020a). ddRAD library preparation and sequencing (PstI + MseI restriction enzymes, single-end reads, 100 bp on Illumina HiSeq 2500) were carried out by a commercial provider Floragenex OR, USA. The details of the library preparation protocol are described in Yanchukov et al. (2022).

2.3. Bioinformatic analysis

We demultiplexed the raw data using the "process_radtags" program in Stacks v. 2.54 (Rochette et al., 2019) and performed quality control for the raw reads of each sample using FastQC v0.11.9 and we used MultiQC v1.14 (Ewels et al., 2016) for visualization of the quality scores per sample. We then aligned the reads twice, using 1) the scaffold-level reference genome of *Darevskia valentini*, Ochkalova et al., 2022; NCBI accession number: GCA_024498535.1) and (2) the chromosome-level reference genome of *Podarcis muralis* Laurenti, 1768 (Andrade et al., 2019; NCBI accession number: SIRZ00000000) using Bowtie2 v.2.4.1 (Langmead & Salzberg, 2012) with the conservative "–end-to-end" setting (i.e. alignment scores calculated for the entire read matching the reference with default parameters: −6 score penalty for a mismatch, −11 score penalty for a 2-bp gap, −0.6–0.6*L total score threshold required to retain the read, where L is the read length). Next, we filtered out the reads with an alignment quality score < 30 using Samtools v.1.11 (Li et al., 2009) and calculated the ratio of filtered reads (see Supplementary Material, section II). The complete data analysis flowchart is presented on Fig. 2.

The reference genome of *D.valentini* belongs to an individual collected in Armenia, and is a member of a different phylogenetic clade within this species (Candan et al. 2021). However, it is still by far the closest reference genome to all *Darevskia* and the respective alignments were used in all subsequent analyses. We only used the outgroup genome of *Podarcis muralis* twice (i) to compare the number of reads aligned to chromosome Z with the number of autosomal reads, in order to confirm the ploidy of parthenogenetic individuals; and (ii) to examine the asymmetry in the numbers of paternally *vs* maternally derived haplotypes (Supplementary Material, section II, Fig. S2). The lacertid lizards are characterized by remarkable level of chromosome synteny

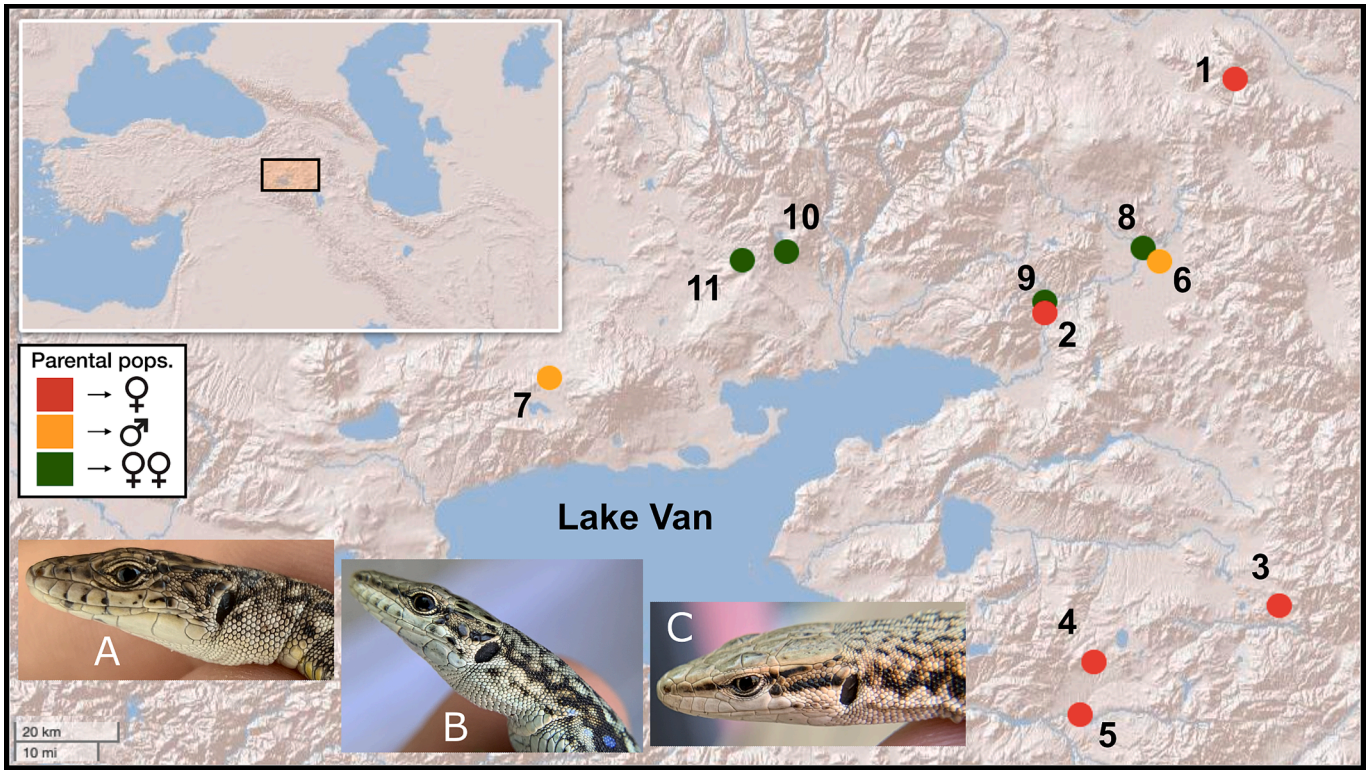

Fig. 1. Sampling locations of *D. valentini, D. raddei, D. bendimahiensis* and *D. sapphirina* around Lake Van (♀: Maternal species, ♂: Paternal species, ♀♀: Parthenogenetic species. The inset pictures: A - *D. valentini (=josefschmidtleri);* B - *D. sapphirina;* C - *D. raddei vanensis*). Numbers on the map indicate the following: *D. raddei*: 1: Doğubayazıt, 2: Muradiye, 3: Saray, 4: Umut, 5: Çörekli; *D. valentini*: 6: Çaldıran, 7: Kızılyusuf; *D. bendimahiensis*: 8: Çaldıran, 9: Muradiye; *D. sapphirina*: 10: Pınarlı, 11: Van/Ağrı. The two parental and their parthenogenetic daughter species are colored on the location map as: red (maternal), yellow (paternal) and green (parthenogenetic hybrid). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Samples and populations used in the analysis (* the sampling locations of D. sapphirina and D. bendimahiensis were also reported by Tarkhnishvili et al. 2020a; Yanchukov et al. 2022), and the mean number of alleles per locus and allelic richness in hybrid and parental species. Location numbers correspond to the map on Figs. 1 and 5.

Species	Role in the hybridization	Location name, sample size (n)	Mean number of alleles per locus and individual	Allelic richness
♀ *D. raddei vanensis*	Maternal parent	(1) Doğubayazıt (n = 4) (2) Muradiye (n = 2) (3) Saray (n = 3) (4) Umut (n = 1) (5) Çörekli (n = 1)	1.38	1.16
♂ *D. valentini* (=*D. josefschmidtleri*)	Paternal parent	(6) Çaldıran (n = 4) (7) Kızılyusuf (n = 4)	1.39	1.19
♀♀ *D. bendimahiensis*	Parthenogenetic daughter	(8) Çaldıran (n = 7) (9) Muradiye (n = 3)	3.86	1.75
♀♀ *D. sapphirina*	Parthenogenetic daughter	(10) Pınarlı (n = 3) (11) Van/Ağrı (n = 5)		

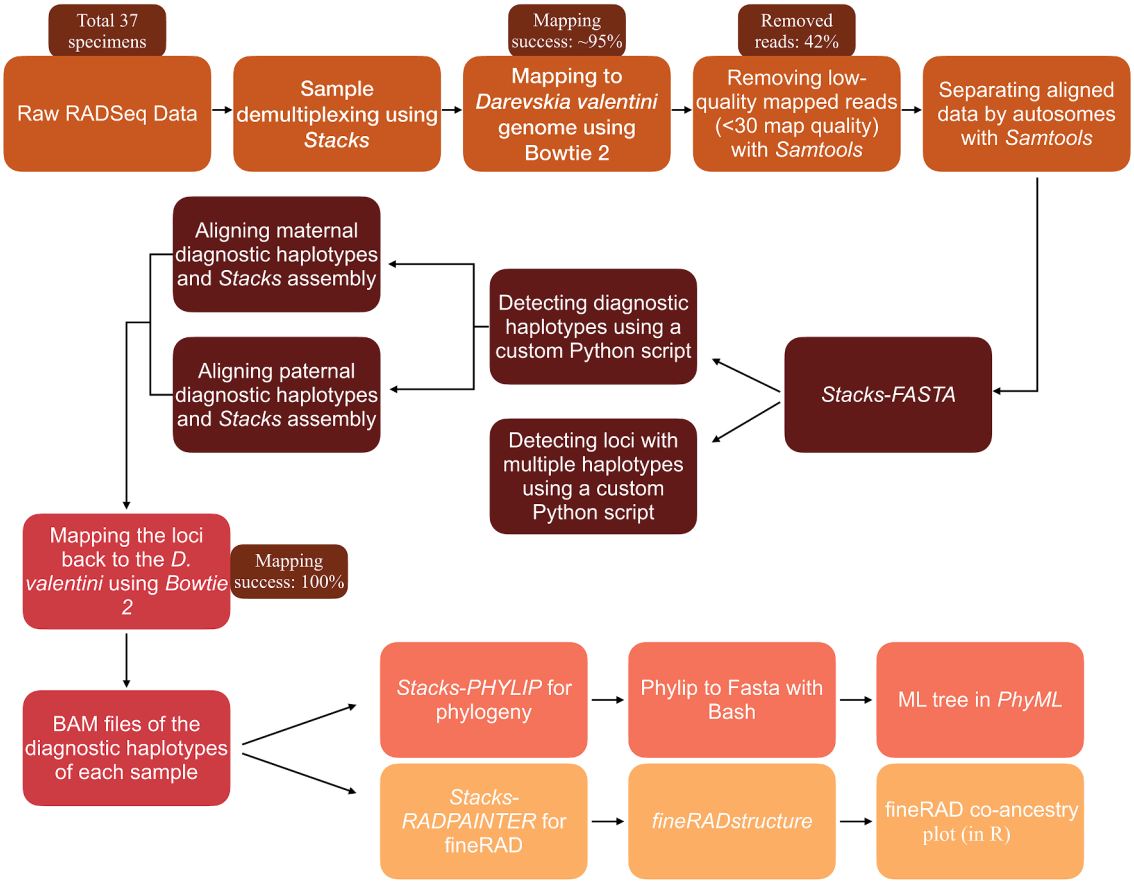

Fig. 2. The step-by-step bioinformatic and population genetic / phylogenetic analysis performed in the study.

conservation (Rovatsos et al. 2019), and the *P. muralis* reference was successfully used for the chromosome Z-based phylogenetic inference in *Darevskia* before (Yanchukov et al., 2022). The individual ddRAD loci were assembled and cataloged in Stacks v2.54. To maximize the amount of data, we did not set any additional population filters, effectively using all loci in the analysis. However, for the purpose of testing the hypotheses of single *vs* multiple parental pairs at the origin of the parthenogenetic populations, we discarded the bi-allelic loci and focused on the Loci with Multiple (>2) Haplotypes (LOMHAs).

2.4. Population structure and haplotype diversity of the parthenogens

We used all variable sites concatenated in a supermatrix (–phylip flag output in Stacks 2) to build a maximum likelihood (ML) tree using all RAD loci in all parthenogenetic individuals, using FastTree 2.1.11 version (Price et al., 2010). The General Time Reversible (GTR) nucleotide substitution model and gamma distribution was used, and we note that the Jukes-Cantor (JC) model resulted in the exact same topologies. We calculated Fst among the parthenogenetic populations (among the four extant geographic populations and between two species of *D.b.* and *D.s.*) based on all ddRAD alleles, both per SNP and across the genome, using Stacks 2. To assess the significance of the Fst values, we performed 500 random permutations of individuals across taxa using a custom R code (see Supplementary Material, section VIII).

We calculated the allelic richness in both the parental and hybrid species using the R package *heirfstat* version 0.5.11 (Goudet, 2005) and the mean number of alleles (i.e. haplotypes) per locus in these by dividing the number of haplotypes in the populations by the number of

loci (Supplementary Material, section VIII). Allelic richness is defined as (r(n)) the number of different alleles in a sample of n genes when a total of N (>n) genes are examined at this locus. This estimation is obtained using the rarefaction method proposed by Hurlbert (1971) (see equation (1)). In this method, *Ni* represents the number of occurrences of the *i*th allele among the *N* sampled genes. This formula enables the computation of the expected number of different alleles when n genes are sampled in the total population (El Mousadik and Petit, 1996). To assess the significance of the allelic richnesses, we performed 100 random permutations of individuals across *D. valentini* and *D. raddei* species using Stacks 2 and custom R script (Supplementary Material, section VIII).

$$\widehat{r} = E[r(n)] = \sqrt{\sum_{i}[1 - \frac{\binom{N-N_i}{n}}{\binom{N}{n}}]} \quad (1)$$

Finally, the hierarchical AMOVA was performed in R package poppr (Kamvar et al. 2015) to find out whether the genetic distances at the level of two nominal species (*D.b.* and *D.s.*) exceed those between the four populations.

2.5. Discerning maternal and paternal ancestry using diagnostic parental haplotypes

In each parthenogenetic individual, we focused on the heterozygous loci where one allele is shared exclusively with the maternal parent, and the other allele with the paternal parent (i.e. diagnostic haplotypes, Fig. 3; Supplementary Material, section IV). A similar approach was used by Grismer et al. (2014) to phase the nuclear gene haplotypes of the hybrid unisexual *Leiolepis* lizards.

The reads that composed such maternal and paternal diagnostic haplotypes were combined with all reads from the respective parental species. Maximum likelihood (ML) phylogenetic trees were built using PhyML v. 3.3_1 with the "optimise parameter" option, which adjusts parameters such as branch lengths and substitution rates in order to maximize the likelihood of the tree topology (Guindon et al., 2010; Lemoine et al., 2018). We visualized the trees using the web tool iTOL (Letunic and Bork, 2021). The fineRADstructure (Malinsky et al., 2018) was used to construct the co-ancestry matrices and the fineRADstructure plots were visualized using the R script "fineRADstructurePlot.R" (https://github.com/millanek/fineRADstructure/blob/master/fineRADstructurePlot.R) and the R library "FinestructureLibrary.R" (https://github.com/millanek/fineRADstructure/blob/master/FinestructureLibrary.R) in R v. 4.0.0 (R Core Team, 2020a) and R Studio v. 2022.12.0 + 353 (RStudio Team, 2020b).

3. Results

We produced ddRAD data from 37 individual lizards collected in the Lake Van basin. After demultiplexing, we obtained an average of ~ 1.4 million raw reads per sample (~1–2 m) across 11 *D. raddei*, 8 *D. valentini*, 8 *D.s.* and 10 *D.b.* individuals (see Table S2 and Fig. S1 in Supplementary Material for average quality score distribution). Aligning this data to the *D. valentini* genome and running Stacks (Rochette et al., 2019), we obtained a total of 1,634,453 loci (size ~ 95 bp). Of these, on average ~ 480 k were genotyped in a single individual and ~ 73 k were genotyped in all individuals. These in total included 831,605 SNPs. The mean coverage per genotyped locus per individual was 3.6x.

We confirmed the sex of individuals by estimating the ratio of Z-chromosomal reads to the autosomal reads using the alignment to the chromosome-level reference genome assembly of *Podarcis muralis* (Table S3: Supplementary Material). We found a clear distinction between the males (ZZ) of the parental species, where this ratio approached ~ 1, and the females including both the parental and the parthenogenetic individuals, which exhibited a ratio close to ~ 0.5 (Fig. S2: Supplementary Material), thus confirming that the latter were diploid females (ZW).

3.1. Population structure of D. bendimahiensis and D. sapphirina

We next studied the population structure among the four parthenogenetic *D.b.* and *D.s.* populations. A maximum likelihood tree clustered all individuals by their respective population, with full bootstrap support (Fig. 4). In contrast, co-ancestry analysis using fineRADstructure suggested modest clustering by nominal species, while some individuals showed higher affinity to individuals from other populations (Fig. S4: Supplementary Material). At the same time, the genetic differences between the two nominal species did not exceed those among the four sampled populations (2.4% and 3.2% of total variance explained respectively in the hierarchical AMOVA). The variances at either level were significant ($p = 0.01$, Table S10: Supplementary Material). To investigate this further, we calculated Fst among populations across all SNPs (n = 396,312 SNPs identified across n = 1,032,862 ddRAD loci). Average Fst values were low (0.04–0.06), but marginally significant compared to random permutations ($p < 0.06$) (Fig. S3; Table S6: Supplementary Material). Moreover, a small fraction (~1%) of SNPs were

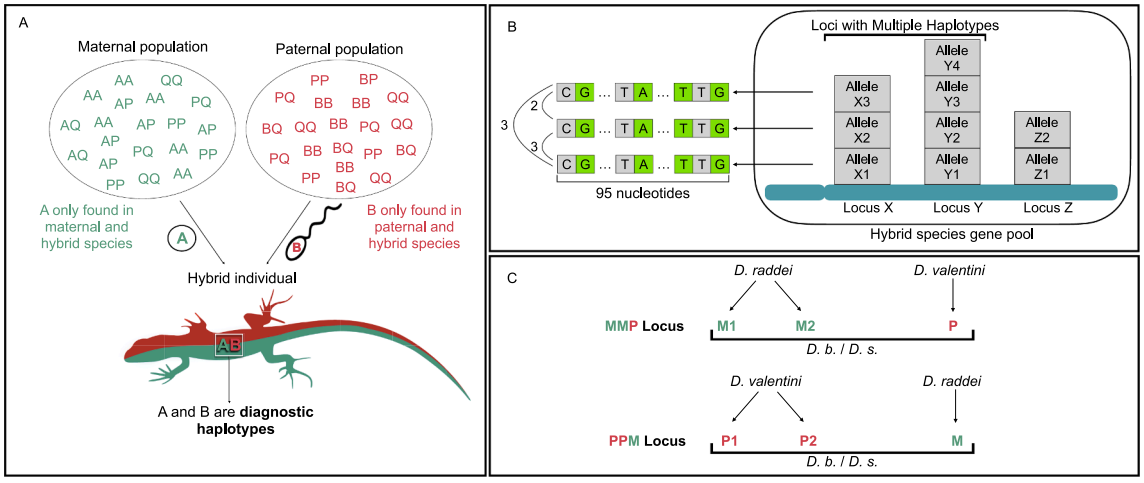

Fig. 3. Panel A: Identification of diagnostic haplotypes at a ddRAD locus (A and B are haplotypes unique for only one parental side, P and Q are shared haplotypes in both parents). Panel B: Hypothetical examples of loci with multiple haplotypes (LOMHAs). LOMHAs (right), and the number of nucleotide differences between haplotypes of LOMHAs (green positions are polymorphic sites) (left). Panel C: Two types of three-haplotypic LOMHAs used in the binomial test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

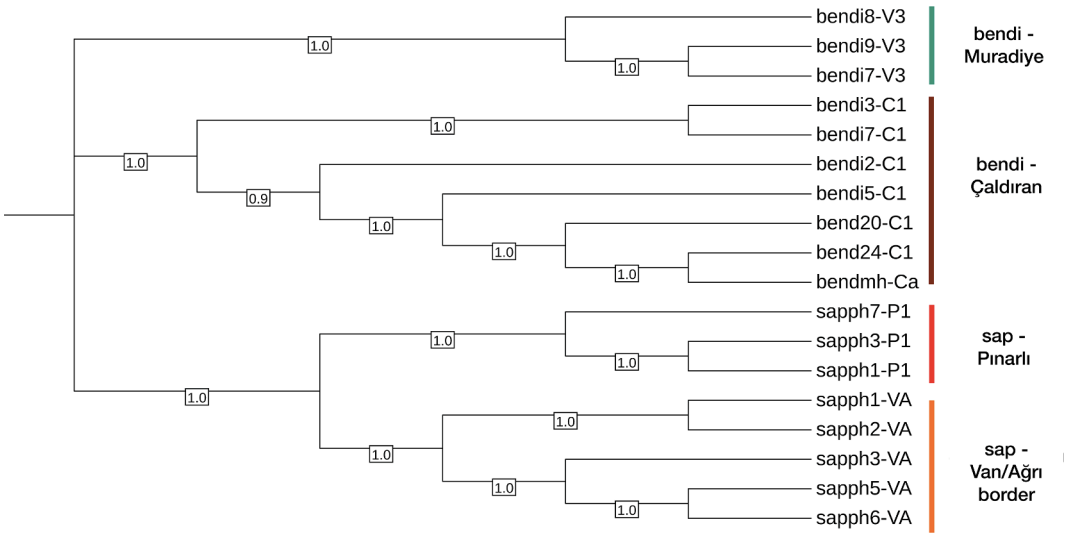

Fig. 4. The ML phylogeny based on all ddRAD-seq loci (number of loci: 1,032,862) of *D. bendimahiensis* and *D. sapphirina* populations near Lake Van. The bootstrap values are indicated on the branches.

fixed between populations. These results suggest the presence of weak structure among the four parthenogenetic populations, which may be partly obscured in some analyses due to the low coverage of our data (see Discussion).

3.2. Parental source populations of *D. bendimahiensis* and *D. sapphirina*

We then sought to identify the maternal and paternal lineage populations genetically closest to the parthenogenetic *D.s.* and *D.b.* lineages,

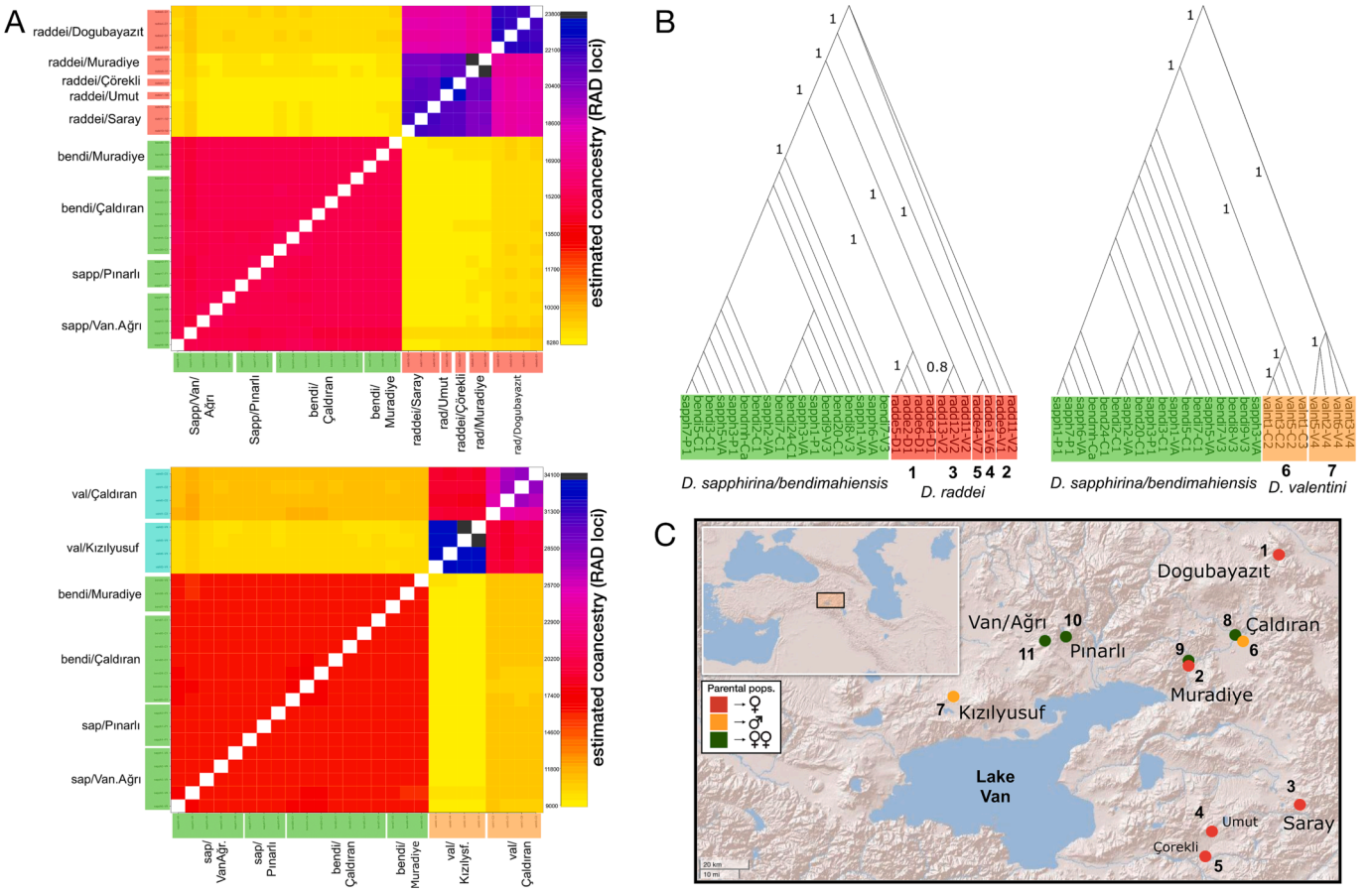

Fig. 5. Panel A: The fineRADstructure co-ancestry matrix based on all RAD-seq alleles of *D. bendimahiensis, D. sapphirina* populations together with *D. raddei* populations (upper) and *D. valentini* populations (lower) around Lake Van. Panel B: A maximum likelihood phylogeny based on diagnostic haplotypes of *D. bendimahiensis, D. sapphirina, D. raddei* and *D. valentini* populations around Lake Van. The numbers on branches are branch supports (approximate Bayes branch support); support values close to zero are not shown. The trees were built using n = 34,271 maternal and paternal diagnostic haplotypes. Panel C: Sampling locations map, locations numbers, symbols and colors correspond to Fig. 1 and Table 1.

and thus best representing maternal and paternal sources. Our naive hypothesis was that if hybridization was a relatively recent event, the genetically closest parental groups would also be geographically closest to the parthenogenetic populations (Freitas et al. 2016, Yanchukov et al. 2022).

We started by investigating the maternally-related populations of *D. raddei vanensis* in the Lake Van basin. After including 29 individuals of *D.s.*, *D.b.* and *D. raddei vanensis*, in the fineRADstructure co-ancestry matrix, we found that *D. raddei* population sampled near Doğubayazıt showed the highest coancestry compared to the rest of *D. raddei* populations around Lake Van (Fig. 4A). This pattern was also observed when we analyzed the same sample but only using maternal diagnostic haplotypes (Methods): both fineRADstructure (Fig. S5: Supplementary Material) and a maximum likelihood phylogenetic tree showed *D. raddei* from Doğubayazıt clustering with *D.b.* and *D.s.* with full support (Fig. 4B). This suggests that all four parthenogenetic populations may derive from the same maternal source. Also interestingly, we found that the *D. raddei* from Muradiye was genetically most distant to *D.b.* and *D.s.*, despite being the geographically closest maternally-related population (Fig. 4C).

We next investigated the paternal sources of *D.s.* and *D.b.* lineages among the two *D. valentini* populations in the Lake Van basin, comparing a total of 26 individuals. Both fineRADstructure heatmap built using only paternal diagnostic alleles (Fig. S6: Supplementary Material), as well as a maximum likelihood tree (Fig. 5B) indicated that *D. valentini* from Çaldıran has a higher genetic similarity with both *D.b.* and *D.s.* populations relative to the other paternally-related population (*D. valentini* in Kızılyusuf). Hence, *D. valentini* from Çaldıran is the best paternal source of all four parthenogenetic populations. We note that compared to Kızılyusuf, the Çaldıran population is also geographically more proximate to the best maternal source population (*D. raddei* in Doğubayazıt, Fig. 5C).

Similar to the co-ancestry analysis, the phylogenetic trees constructed using only diagnostic haplotypes (on both parental sides) did not reveal any obvious clustering among *D.b.* and *D.s.* (Fig. 4A, 4B; S6 and S7 in Supplementary Material). Note that the sequencing depth coverage of the diagnostic haplotypes was only slightly lower than that for all loci (3.2x *vs* 3.6x, respectively).

3.3. Multiple same-locus parental haplotypes in D. bendimahiensis and D. sapphirina do not support their origin from a single parental pair

A major question on the evolution of *Darevskia* parthenogenetic populations is the number of hybridization events that created them. Given the close genetic similarity among the four *D.b.* and *D.s.* populations and the fact that their maternal and paternal sources appear the same, we investigated whether they might have all arisen from a single parental pair, or whether they could have resulted from multiple hybridizations involving different individuals from the same populations.

To tackle this, we focused on loci with multiple (>2) haplotypes (LOMHAs) in the hybrid gene pool. We reasoned that a hybridization between only two parental individuals would lead to a maximum of two haplotypes per locus among hybrids; third haplotypes could only arise due to *de novo* mutations or technical artifacts. Meanwhile, multiple founder hybridization events could lead to widespread LOMHAs in the hybrid gene pool, their frequency possibly depending on the level of genetic dissimilarities between the paternal sources and between the maternal sources. Hypothetical paternal backcrosses (see Introduction) could also lead to LOMHAs.

Across 1,345,024 RAD-seq loci we identified 18,066 LOMHAs (1.3%) among the 10 *D.b.* and 8 *D.s.* individuals, the majority (n = 16,096, or 89%) with 3 haplotypes (Fig. S7: Supplementary Material). Of these, 23% and 26% were only present in the maternal (*D. raddei*) or paternal (*D. valentini*) species, respectively. Among 3-haplotypic LOMHAs we also determined 310 cases where the closest haplotypes were $\geq$ 2 nucleotide substitutions distant to each other. Observing LOMHAs haplotypes shared with only the maternal or paternal gene pools or having $\geq$ 2 differences to alternative haplotypes suggests that at least some LOMHAs may have been transmitted via hybridization, rather than having arisen by *de novo* mutations/artifacts. We also tested the possibility that LOMHAs could actually represent hidden paralogs. For this, we compared the mean sequencing depth of LOMHAs with that of loci with 2 haplotypes (Tables S8-S9: Supplementary Material). This indeed revealed a slightly lower mean depth for the former than the latter (2.34 *vs*. 2.33; Mann-Whitney *U* test p = 0.001), although the small effect size did not suggest this was a major source of 3rd alleles.

We then reasoned that, if all hybrids descended from a single parental pair, *de novo* mutations/artifacts should equally affect the two maternal and paternal haplotypes at each locus, such that the third haplotypes of maternal or paternal origin should be equally frequent. Instead, if multiple hybridizations occurred, third haplotypes could be biased towards maternal or paternal origins depending on the genetic differences between the maternal parents or between the paternal parents. Hence, if we can reject the symmetrical distribution of third haplotypes among maternal and paternal gene pools, this would support the multiple hybridization hypothesis.

To test this, we compiled the list of 3-haplotypic LOMHAs with two haplotypes unique to one parent and the other haplotype unique to the other parent, calling these "MMP" for 2 maternal + 1 paternal, and "PPM" for 1 maternal + 2 paternal haplotypes. Among n = 815 such loci, 334 and 481 were determined as MMP and PPM, respectively, which significantly deviates from the 1:1 expectation (binomial test p-value < 1e-6). This result supports the possibility that the 3rd haplotypes may have been introduced via secondary hybridizations. We note that the difference between MMP and PPM frequencies is likely not due to the differences in our statistical power in detecting maternal- or paternal-specific haplotypes, because the allelic diversity estimates in the maternal and paternal samples were highly similar (Table 1; permutation test for difference p = 0.01).

To ensure that the asymmetry in MMP and PPM numbers are not related to the use of *D. valentini* (the paternal parent) as a reference genome, we repeated the same analysis using data aligned to the outgroup *P. muralis* genome. Among 11,667 3-haplotypic loci detected, we again identified a higher number of PPM than MMP loci (371 *vs*. 306; binomial test p-value = 0.013).

Under the multiple hybridization scenario, we may further expect the relative frequencies of the 3rd haplotypes to be correlated between the parental and the hybrid species. This would not be expected if the 3rd haplotypes resulted from *de novo* mutations/artifacts. However, we found that the frequencies of 3rd haplotypes in the hybrid and in the parental species were not correlated: r = 0.1 across n = 481 paternal haplotypes and r = -0.05 across n = 334 maternal haplotypes (Spearman correlation test p-values: 0.07 and 0.27 respectively).

4. Discussion

4.1. Maternal and paternal ancestry of D. bendimahiensis and D. sapphirina

In this study, we analyzed a large number of short ddRAD-seq loci in hybrid parthenogenetic and parental sexually reproducing populations of rock lizards in the area of Lake Van. Among the several geographic populations of both parents surveyed, two with the shortest genetic distance to the hybrids were identified unambiguously. Evidence that the local parental species populations are the most likely candidates for their respective parthenogenetic daughter species has been obtained before at the scale of the entire geographic range of the latter (Freitas et al. 2016, Tarkhnishvili et al. 2020a; 2020b). However, these studies did not have sufficient genotype resolution to pinpoint the exact populations. Our previous analysis of chromosome Z-linked genetic markers identified the most likely paternal ancestors of all seven parthenogenetic forms in *Darevskia* (Yanchukov et al. 2022). The current results based on

the genome-wide markers are in perfect agreement with this study, confirming that the population of *D. valentini* in Çaldıran shares the highest proportion of haplotypes with the actual ancestor of *D.b.* and *D.s.* (Fig. 4 and S7: Supplementary Material). A different pattern, however, was observed on the maternal side. The population of *D. raddei vanensis* from Doğubayazıt is separated by 56 km of complex terrain (a 2212 m a.s.l. Tendurek mountain pass) from the nearest known location of *D.b.* This population was nevertheless genetically much closer to *D.b.* and *D.s.*, compared to *D. r. vanensis* north-east and east of Lake Van, even despite the latter living in sympatry with D.b. (Fig. 5 and S6: Supplementary Material). This finding could reflect a complex history of distribution range shifts in either maternal or parthenogenetic daughter populations, possibly driven by the changes in climate since hybridization (Yanchukov et al. 2022), or by accumulated ecological nich incompatibilities between species (Tarkhnishvili and Iankoshvili 2023). The timing of this event was independent of the origin of all other parthenogenetic forms and has been dated to be as recent as 18–204 kyr (Freitas et al., 2016) or as early as 1–2 Myr (Yanchukov et al., 2022).

4.2. Divergence of parthenogenetic populations after the initial hybridization

Our phylogenetic analysis supports the origin of all parthenogenetic individuals from a single initial hybrid population (Fig. 5). Incidentally, we could not detect any meaningful structure in *D.b.* and *D.s.* while examining their diagnostic parental haplotypes. This result implies the common origin of both parthenogenetic taxa, given that we only retained those diagnostic haplotypes that were identical between the parental and the hybrid daughter populations. Imposing such strict criteria effectively removed any variation that accumulated after the hybridization had occurred, and exposed the state of the initial, single hybrid population that preceded the current parthenogenetic gene pool. We note that while we used a small number of parental populations/individuals, we also find indication of population structure in both parental groups (strong bootstrap support between individuals from different locations), which implies that single individuals are sufficiently representative of their respective populations. Finally, the relatively low coverage of our ddRAD sequences could lead to missing data, and/or heterozygous SNPs being erroneously called as homozygotes due to just one allele in a locus being sequenced at random. The latter could not have any major effect in our analyses based on diagnostic haplotypes, where only heterozygous positions were considered in the first place. Similarly, the false homozygotes could have caused slight overestimation of the population structure (i.e. Fst) among the parthenogenetic populations, which does not affect our overall conclusions. Most importantly, errors due to low coverage are extremely unlikely to increase the number of unique haplotypes shared between different species or populations. We also limited the impact of sequencing error by using minimal coverage thresholds.

We did, however, detect a weak but significant population structure once all ddRAD loci were analyzed together. The variation among *D.b.* and *D.s.* perfectly corresponded to the geographic locations where two species were sampled (Fig. 1 and Fig. 5C) with a number of SNPs fixed for the alternative alleles between the respective populations (Fst = 1, Fig. S3 in Supplementary Material). These differences most likely appeared once the initial hybrid population had spread from the point of its origin, and then fragmented into isolated enclaves (the four isolated populations of both *D.b.* and *D.s.* we sampled are the only ones known). Geographic variation manifested at the level of microsatellite genotypes has been reported in another parthenogenetic species, *D. armeniaca* (Fig. 5 in Tarkhnishvili et al. 2020a), which is thought to originate later than *D.b.* and *D.s.* (Yanchukov et al. 2022). The amount and quality of our ddRAD-seq data were not sufficient to infer the timing of the demographic history of parthenogenesis in the Lake Van area, and future studies with higher genomic resolution would be necessary to address this question. At the same time, it is obvious that the current taxonomic status of *D.b.* and *D.s.* as two different species is not warranted, given (i) almost identical morphological description given by Schmidtler et al. (1994), (ii) their monophyletic origin can be traced to a single initial hybrid population and (iii) very low level of genetic divergence (also see Tarkhnishvili et al. 2020b; Yanchukov et al. 2022). Following the order in which the two taxa were described in Schmidtler et al. (1994), we suggest that the name *D. bendimahiensis* is used only as a synonym of *D. sapphirina*.

Our genotyping approach and the corresponding data analysis bear many parallels with the study of Barley et al. (2022), who investigated genome-wide diversity in the parthenogenetic teiid lizard *Aspidoscelis laredoensis* in North America. There, two independent hybridization events between the same sexually reproducing parental species were detected in the gene pool of a single unisexual species. Unlike *D.b.* and *D.s.*, the two clonal lineages in parthenogenetic *A. laredoensis* can be clearly distinguished at the morphological as well as genetic level, which is likely the result of slightly different parental lineages involved in two respective hybridization events.

It was earlier suggested that all other parthenogenetic species of *Darevskia*, except one (*D. rostombekowi*), are also composed of multiple clonal lineages (Ryskov et al. 2017, Girnyk et al., 2018). In these studies, the conclusions were drawn after examining the variation in the flanking regions of a handful of microsatellite loci. The authors confirmed, visually, the presence of two types of "clones" - (1) those that differed from each other by ~ 1 substitution and (2) those that involved multiple substitutions. Consequently, the type 1) clones were thought to be the result of mutations which occurred after the hybridizations event(s) and type (2) were regarded as evidence for ancestral polymorphism in the parental populations, and, therefore, multiple hybridization events. This approach is fundamentally similar to the one we have taken in our study, though we were able to perform the analysis on a much larger genomic scale.

4.3. Multiple parental haplotypes contributed to the current genetic pool of D. bendimahiensis and D. sapphirina

Our results show that *D.b.* and *D.s.* are unlikely to have originated from a single pair of parental individuals. Instead, a scenario involving multiple parental pairs with highly similar genetic backgrounds (i.e. with similar maternal ancestries and similar paternal ancestries) is supported by our analysis of 3-haplotypic ddRAD loci. In particular, we find (i) the presence of 310 loci with three haplotypes differing from each other by > 1 substitution and (ii) a significant number of loci with two haplotypes shared with one or the other parental species (i.e. MMP and PPM-type loci) (Methods). These observations are difficult to explain by random mutation accumulation or by PCR or sequencing errors. Erroneous paralog assembly (hidden paralogs) could have also resulted in 3rd haplotypes, although this appears plausible for only a small proportion of sites, since the mean depth of the 3-haplotypic loci only exceeds that of the 2-haplotypic loci by a negligible factor of 1.004. We also note that the reference genome of *D.valentini* does not contain large duplications or repetitive regions (Ochkalova et al., 2022), nor are there any large duplicated regions in the reference genome of *P. muralis* (Andrade et al., 2019), which further suggests that the vast majority of reads in our study were assembled to their correct locations.

We further find that the paternal parent, *D. valentini*, has more 3rd haplotypes shared with the parthenogens compared to maternal *D. raddei*. This asymmetry in shared haplotype numbers between paternal *vs.* maternal species further undermines the "single parental pair" scenario, under which 3rd haplotypes would result from *de novo* mutations or technical artifacts. At the same time, the absence of correlation in the frequencies of the 3rd (minor) haplotypes between the parental and the parthenogenetic populations is consistent with either scenario, since any initial correlation could simply be lost over time passed after hybridization.

Two caveats need to be discussed here. First, the fact that the

reference genome used in our study belongs to *D.valentini* (though a different lineage within the species), may have caused an ascertainment bias, i.e. the paternally-inherited haplotypes having a higher probability of successful alignment to the reference, compared to the maternally-inherited ones, and consequently higher statistical power in identifying paternal alleles. However, using the outgroup *P. muralis* genome resulted in a similar asymmetry, which cannot be explained by the ascertainment bias in favour of just one of the parental species.

The second caveat refers to the slightly higher haplotype (=allelic) diversity in the *D. valentini* populations sampled in our study (Table 1), as well as a higher proportion of the private haplotypes shared with both parthenogenetic species (Table S7: Supplementary Material). If some 3rd haplotypes were the result of homoplasy (parallel *de novo* mutations or PCR/sequencing error) in parthenogenetic species, higher allelic diversity in *D. valentini* could increase the probability of these being also found in that gene pool. However, homoplasy would not lead to higher ratio of paternal to maternal haplotypes in the parthenogens compared to that in the parental gene pool.

The alternative scenario involves multiple parental individuals hybridizing to produce the multiple founders of present-day *D.b.* and *D.s.*, and the resulting multiple parthenogenetic lineages surviving to the present day despite an inevitable reduction of polymorphism due to genetic drift. The current distribution range of *D.b.* and *D.s.* is highly fragmented, and both nominal species possess a large proportion of private haplotypes at PPM and MMP loci (up to 28% in *D.b.*). This pattern can therefore reflect the history of alternative alleles becoming fixed in geographically isolated populations and/or being fixed by gene conversion, while preserving the overall diversity of the parthenogens. However, the same results can arise from a high proportion of missing loci/haplotypes in our ddRAD-seq data (a common problem with reduced representation genomic approach, Andrews et al. 2016).

Because *D.b.* and *D.s.* lineages are highly similar, a multiple hybridization scenario would also require hybridization events involving parents with similar backgrounds, likely around the same time. This implies that conditions allowing hybridization may be generally constrained, but once favourable circumstances appear, multiple hybridizations were possible.

Finally, our results neither disprove nor support the possibility of backcross gene exchange between the parental and the parthenogenetic species (Tarkhnishvili et al., 2020a). Such backcross gene flow could certainly generate and maintain the multiple haplotypes inherited from the same (e.g. paternal) parent, which is consistent with our data. However, it would also need to include yet unknown genetic mechanism, e.g. the reduction of ploidy if the triploid backcross offspring is involved, or the production of haploid gametes by the parthenogenetic individuals (Tarkhnishvili et al., 2020a). An even more speculative scenario involving occasional sex and recombination within the parthenogenetic populations could also contribute to the maintenance of haplotype diversity. Recent studies in other organisms, which were long thought to be obligate parthenogenetics, have unambiguously demonstrated that rare sex and recombination do occur and play an important role in the maintenance of genetic diversity (Vakhrusheva et al. 2020).

In conclusion, our results suggest a hybridization scenario where only two ancestral populations of the respective parental species *D. raddei* and *D. valentini* are responsible for the origin of the entire parthenogenetic gene pool of *D.b.* and *D.s.* Under a broad definition (see Introduction), this was a single hybridization event at the species level. At the same time, a single pair of parental individuals could not be responsible for the amount and pattern of genetic diversity we currently observe in the parthenogens. While more detailed scenarios remain speculative, we suggest that many, if not most of the questions raised in our study can be answered by employing more advanced genotyping methods. In particular, longer sequencing reads and higher genomic coverage can easily resolve the number of extant clonal lineages in the parthenogenetic populations and/or verify the validity of the backcross gene flow scenarios. Unfortunately, in the case of *D.b.* and *D.s.*, the ongoing destruction of habitat is having a significant negative impact on the parthenogenetic populations near Lake Van (Akman et al., 2016). Future genomic and ecological investigations on the origin and maintenance of asexual reproduction in this exceptional system will need to take into account the real risk of rapid extinction of parthenogenetic *Darevskia* in the Lake Van area.

5. Ethics Approval

Fieldwork and collection of samples were done under permit no. E.1284045 from the Ministry of Agriculture and Forestry of Türkiye and no. 91330202 from the Animal Research Ethics Committee of Zonguldak Bülent Ecevit University.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data and code are provided in the Supplementary Material

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107925.

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