Conservation biology of three threatened *Limonium* species endemic to Zakynthos Island (Ionian Islands, Greece)

Anna-Thalassini Valli[1], Charikleia Papaioannou[2], Eleni Liveri[3]
Vasileios Papasotiropoulos[4] and Panayiotis Trigas*[1]

Abstract Knowledge of the life history traits, reproductive biology and demography of rare species is fundamental for their conservation, yet plant population monitoring is uncommon. The restricted ranges of the *Limonium* species endemic to the Mediterranean area, combined with the vulnerability of their specialized littoral habitats, indicate the need for appropriate conservation measures. We evaluate the conservation status and estimate the future extinction risk of three *Limonium* species endemic to Zakynthos Island in the Ionian Islands, Greece (*Limonium korakonisicum*, *Limonium phitosianum* and *Limonium zacynthium*) using 5 years of monitoring data. We compile information on their geographical distribution, population dynamics, reproductive biology and genetic diversity. Population sizes and survival rates of seedlings exhibited marked annual fluctuations, although fecundity and relative reproductive success remained high throughout the monitoring period. We observed a dominance of mature individuals in all three species, indicating their increased tolerance to salinity. Three subpopulations each of *L. phitosianum* and *L. zacynthium* were genotyped using five microsatellite loci. The observed number of alleles and the low gene flow value potentially indicate reduced genetic diversity, inbreeding, and limited gene flow within and among subpopulations of both species. Based on the IUCN categories and criteria, we assess *L. korakonisicum* as Critically Endangered, *L. phitosianum* as Near Threatened and *L. zacynthium* as Endangered. Population viability analyses predict that, among the three species, *L. zacynthium* will face the highest risk of extinction within the next 50 years. Knowledge of the biology of these species provides data essential for identifying critical factors for their survival and for proposing targeted conservation measures.

*Corresponding author, trigas@aua.gr
[1]Laboratory of Systematic Botany, Department of Crop Science, Agricultural University of Athens, Athens, Greece
[2]Laboratory of Genetics, Department of Biology, University of Patras, Patras, Greece
[3]Laboratory of Botany, Department of Biology, University of Patras, Patras, Greece
[4]Laboratory of Plant Breeding and Biometry, Department of Crop Science, Agricultural University of Athens, Athens, Greece

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Introduction

Knowledge of the life history traits, reproductive biology and demographic characteristics of rare species is fundamental for their protection and restoration (Schemske et al., 1994; Yates & Broadhurst, 2002). Monitoring, including assessing range size, population dynamics and exposure to anthropogenic threats, is one of the core activities of conservation biology and provides predictive power (Tienes et al., 2010). Long-term monitoring of marked individuals can provide information on vital rates (e.g. survival, transition between life stages) for estimating population growth rates and probability of extinction through matrix population modelling (Morris & Doak, 2002; McCaffery et al., 2014).

Genetic information is important for managing threatened populations and species, and variability within populations is related to their evolutionary potential, which is often higher in more genetically variable populations (Hoffmann et al., 2017; Ørsted et al., 2019). Consequently, evaluating genetic variability is crucial when making management decisions for threatened species (Weeks et al., 2011; Hoffmann et al., 2020). Genetic studies enhance conservation effectiveness by identifying populations with low genetic diversity and predicting genetic drift effects (Nicoletti et al., 2012; Augustinos et al., 2014).

Plant population monitoring is uncommon because it is time- and resource-demanding (Heywood & Iriondo, 2003). In Greece, for example, despite the high number of endemic and threatened plants (Phitos et al., 1995, 2009; Dimopoulos et al., 2013), studies of their conservation biology is scarce (but see Valli et al., 2021a,b). *Limonium* Miller is one of the largest genera in the Mediterranean area, which is the centre of diversity of the genus (Brullo & Erben, 2016). The high diversity and complexity of *Limonium* is a result of the reproductive behaviour of the genus, which encompasses sexual and apomictic reproduction, frequent hybridization, and polyploidy (Georgakopoulou et al., 2006; Brullo

& Erben, 2016; González-Orenga et al., 2021). The *Limonium* species of the Mediterranean area often have punctiform distributions (Brullo & Erben, 2016; Buira et al., 2017).

Limonium species typically grow in littoral habitats, and they are adapted to the environmental stress of rocky and sandy seashores and salt marshes (Erben, 1993; Caperta et al., 2014). However, a substantial number of these littoral species are threatened (van der Maarel & van der Maarel-Versluys, 1996), primarily as a result of anthropogenic impacts in coastal regions. Fourteen *Limonium* species endemic to Greece are already considered threatened (Phitos et al., 1995, 2009). The Greek Ionian Islands host 14 *Limonium* species, nine of which are endemic to this archipelago (Dimopoulos et al., 2013; Flora Ionica Working Group, 2016). Among these, three species (*Limonium korakonisicum* R. Artelari & Valli, *Limonium phitosianum* R. Artelari and *Limonium zacynthium* R. Artelari) are endemic to Zakynthos Island. The narrow ranges of the endemic *Limonium* species in the Ionian Islands combined with the high vulnerability of their specialized habitats indicate the need for appropriate conservation measures.

Here we combine demographic and genetic approaches to assess conservation status and population trends, and predict the extinction risk of the three *Limonium* species endemic to Zakynthos Island. We aimed to (1) define the distribution of the species by exploring all potentially suitable habitats, (2) assess their population dynamics and reproductive biology, (3) estimate genetic diversity and potential gene flow within and among subpopulations of *L. phitosianum* and *L. zacynthium*, and (4) propose conservation measures for the management and maintenance of the three species.

Methods

Studied species

Limonium korakonisicum, L. phitosianum and *L. zacynthium* are endemic to Zakynthos Island (Plate 1). Although endemism in *Limonium* is usually associated with apomixis (Erben, 1978; Artelari, 1984a; Castro & Rosselló, 2007; Brullo & Erben, 2016), most of the Ionian endemics, including *L. phitosianum* and *L. zacynthium,* are sexually reproducing diploids ($2n = 18$; Artelari, 1984a,b; Artelari & Kamari, 1986). Other *Limonium* species are mostly polyploids and typically apomictic, and *L. korakonisicum* is the only endemic apomictic polyploid species (6×) known in this area (Valli & Artelari, 2015). The latter species forms a small population in the Korakonisi area, where it coexists with *L. phitosianum* (Valli & Artelari, 2015). *Limonium phitosianum* and *L. zakynthium* are included in The European Threatened Plant List (Sharrock & Jones, 2009). A record of *L. phitosianum* from the Stamfani islet in the Strofades Islands c. 46 km south-south-east of Zakynthos (Brullo & Erben, 2016) is based on a single incomplete specimen (Messenien, Insel Stamfani, 14 September 1980, Pieper s.n. (Herb. Greuter)), and its occurrence there is considered doubtful. All subsequently collected *Limonium* specimens from Strofades Islands belong to *Limonium virgatum* (Willd.) Fourr., and therefore *L. phitosianum* is considered endemic to Zakynthos.

Geographical distribution and spatial data

To document distribution, we conducted a 5-year survey (2014–2018) encompassing all suitable habitats (i.e. calcareous maritime cliffs, rocky and sandy coastal areas) across Zakynthos Island. We mapped each species once per year, during flowering, using a GPS, and calculated extent of occurrence (EOO) for each species and the local extent of occurrence (local EOO) of each subpopulation using *ArcGIS 10.5.1* (Esri, USA), and estimated area of occupancy (AOO) as the sum of the occupied 2×2 km grid cells per species (IUCN, 2022a). Estimated EOO and AOO were cross-checked using *GeoCAT* (Bachman et al., 2011). Local EOO was calculated following Andreou et al. (2011) as the smallest polygon or polygons encompassing all plant colonies uninterrupted by unsuitable habitat at each subpopulation location. We chose this approach because a 2×2 km grid is too coarse for species with restricted geographical distributions, particularly littoral species, and provides a more precise measure of a species' spatial extent within its fragmented habitats, often yielding smaller values than the traditional EOO. In 2023, we revisited the locations of all three species to ascertain whether there had been any changes in EOO or AOO since 2018.

Population size

The terminology used (mature individual, population, subpopulation, population size and location) follows IUCN (2022a). To estimate population size, a count of all mature individuals was conducted during flowering and fruiting across all subpopulations of each species. For *L. korakonisicum*, we completely censused mature individuals in 5 consecutive years (2014–2018; Table 1). For *L. phitosianum*, we counted mature individuals in 20 random 5×5 m plots in subpopulations Lp1, Lp2, Lp8, Lp9 and Lp11, and in complete censuses in other subpopulations over the same 5-year period, except for Lp13 and Lp14 (only in 2018). For *L. zacynthium*, we counted mature individuals in four random 5×5 m plots in subpopulation Lz1, and in complete censuses in other subpopulations for the same 5 consecutive years, except for Lz2 and Lz3 (4 years of counts). The number of plants per m^2 provided an approximate estimate of plant density (Andreou et al., 2015), calculated by dividing the number of mature individuals by the local EOO for each subpopulation. To assess the stage-structure

PLATE 1 The three species and their habitats: (a) *Limonium korakonisicum*, (b) *Limonium phitosianum* and (c) *Limonium zacynthium*.

distribution, plants were categorized as seedlings, non-reproductive (juveniles, immatures, non-flowering), and reproductive (flowering/fruiting). *Limonium korakonisicum* seedlings were distinguishable from those of the sympatric *L. phitosianum* by morphological features (Valli & Artelari, 2015). Discrimination between *L. zacynthium* and *L. phitosianum* seedlings was not feasible, resulting in their exclusion from life stage recordings in subpopulations where both species coexist (i.e. Lz5/Lp8, Lz1/Lp9, Lz4/Lp11). The population size of all three species was re-evaluated in 2023.

Reproductive biology

The reproductive characteristics of the species (fertility: the mean number of seeds produced per individual; Burns et al., 2013; relative reproductive success: the per cent of ovules developing into seeds), were determined by tagging 10 randomly selected mature individuals per subpopulation at the onset of flowering and monitoring them until the end of fruiting. *Limonium korakonisicum* and *L. zacynthium* were monitored for 4 years (2015–2018) and *L. phitosianum* for 5 years (2014–2018), except for Lp13 and Lp14 (only in 2018).

We recorded the number of flowering/fruiting stems per individual and the number of flowers and fruits (caryopses) per stem weekly throughout flowering and fruiting. To determine the number of viable seeds per flower and per stem, we collected two stems during each fruiting period from tagged individuals and examined them under a stereoscope to identify sound seeds devoid of morphological alterations or infestations. We calculated relative reproductive success by dividing the actual sound seed production by the maximum potential seed yield, and estimated seed rain by multiplying the estimated seed number per individual by the number of mature individuals in each subpopulation and dividing this by the local EOO for each subpopulation (Andreou et al., 2015). For assessing seedling survival rate, we randomly tagged seedlings during August and checked viability during the following breeding season, for the single known subpopulation of *L. korakonisicum* and for subpopulations Lp1, Lp2, Lp10 of *L. phitosianum* during 2015–2018, and for subpopulations Lz2 and Lz3 of *L. zacynthium* during 3 years (2016–2018).

TABLE 1 Geographical data for the subpopulations of *Limonium korakonisicum*, *Limonium phitosianum* and *Limonium zacynthium*, with area of occupancy (AOO) and number of individuals sampled for genetic analyses.

Subpopulation (by species)	Location	Altitude (m)	Slope (°)	Longitude	Latitude	AOO (km^2)	No. of sampled individuals
L. korakonisicum							
	Korakonisi	7–20	70–80	20.730061°	37.717237°	4	
L. phitosianum							
Lp1	Korithi	6–27	18	20.700863°	37.932599°	4	10
Lp2	Megalo nisi	1–8	12–19	20.708932°	37.910338°	4	
Lp3	Mikro nisi	0–5	20	20.721823°	37.882508°	4	
Lp4	Aghios Petros	2–14	15–20	20.733364°	37.872918°	4	
Lp5	Xigia	1–12	12–27	20.737938°	37.864306°	4	
Lp6	Makris Gialos	1–9	18–21	20.724814°	37.879654°	4	
Lp7	Aghios Nikolaos (Vasilikos)	3–13	2–19	20.991782°	37.728830°	4	10
Lp8	Porto Roma	3–9	9–16	20.990928°	37.710977°	4	
Lp9	Marathias	2–7	5–16	20.845326°	37.677607°	8	
Lp10	Korakonisi	8–18	16–21	20.729977°	37.717501°	4	9
Lp11	Porto Limnionas—Roxa	7–15	14–21	20.701308°	37.739396°	4	
Lp12	Porto Vromi	2–7	15	20.633498°	37.822426°	4	
Lp13	Plakaki	10–12	40–70	20.770373°	37.684366°	4	
Lp14	Stenitis	10	20–40	20.639588°	37.810444°	4	
Total						60	
L. zacynthium							
Lz1	Marathias	1–5	20–30	20.845198°	37.678225°	12	8
Lz2	Marathonisi	1–7	50–80	20.866335°	37.685619°	4	10
Lz3	Pelouzo	1–9	70–90	20.944736°	37.705409°	4	9
Lz4	Porto Limnionas-Roxa	12	50–70	20.700092°	37.739530°	4	
Lz5	Porto Roma	9–10	20–40	20.992023°	37.712677°	4	
Total						28	

The duration of flowering and fruiting across all species and subpopulations was monitored at intervals of 1–2 weeks over the 5 consecutive years. Correlation of the duration of flowering/fruiting with mean annual temperature, maximum and minimum temperatures, and precipitation was examined with stepwise multiple linear regression analysis. Meteorological data were obtained from the Hellenic National Meteorological Service. Comparisons of reproductive data were assessed using One Way ANOVA, and differences among pairs of means were validated using Tukey's Method in *Statistica 8.0* (StatSoft, Germany).

Population viability analysis and conservation status assessment

We conducted a population viability analysis in *RAMAS Ecolab 2* (Rexstad et al., 2000), using the simple, unstructured population model. Initial abundance was based on population size in the first year, and seedling survival rate was used as the survival rate. Population growth rate (R), reflecting population size interannual variation ($N(t+1)/Nt$), where N is number of individuals in year t, was characterized by mean and SD values, accounting for environmental stochasticity. We used the demographic stochasticity option facilitating the consideration of variations in annual recruitment, growth and mortality rates, even under stable environmental conditions. The model assumed density-independent, exponential population growth until reaching ceiling K, at which point the growth rate abruptly reduced to 1.0 (Akçakaya et al., 1999) because of uncertainties regarding density dependence in the three *Limonium* species. Projections were to 10 and 50 years from 2018, and simulations ran with 1,000 replications, with 95% confidence intervals based on the Kolmogorov–Smirnov dispersion test (Sokal & Rohlf, 1981). For *L. phitosianum* and *L. zacynthium*, analyses were conducted for both total populations and individual subpopulations. Conservation assessment followed IUCN (2022a).

Threats

Any direct threats to the habitats and/or individuals of the three species and any stresses they cause to the species were recorded in situ and classified according to IUCN (2022b).

Genetic analyses of the diploid *Limonium* species

We sampled a total of 56 individuals from three subpopulations of *L. zacynthium* and three subpopulations of *L. phitosianum* in September 2021 (Table 1). Fresh leaf material was immediately placed in silica gel to dry. To ensure sampling of separate individuals, material was only collected from plants at least 5 m apart. Total genomic DNA was extracted from 20 mg of dried leaf samples using the NucleoSpin Plant II kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The quantity and quality of the extracted DNA were assessed by agarose gel electrophoresis and spectrophotometer. We stored DNA samples at −20 °C until used.

Of the eight microsatellite markers initially selected for genotyping (Supplementary Table 1), five (Ln39, Ln68, Ln115, Ln152, Ld423) that showed correct amplification pattern by polymerase chain reaction (PCR) testing were selected and genotyped. Multiplex PCR reactions were carried out in 96-well plates containing c. 10 ng of template DNA, 0.2 μM forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 × KAPA Taq buffer and 1 U of KAPA Taq DNA Polymerase (Kapa Biosystems, USA). Cycling conditions consisted of an initial 94 °C denaturation step for 5 min, followed by 30 cycles of 40 s at 94 °C, 50 s at 54/64 °C (depending on the primer pairs used) and 50 s at 72 °C, with a final extension at 72 °C for 7 min. PCR products were separated on SeqStudio Genetic Analyzer (Applied Biosystems, USA). Allele sizes were determined using *STRand 2.4.110* (Toonen & Hughes, 2001).

We used *GenAlEx 6.5* (Peakall & Smouse, 2012) and *GENEPOP 4.7.5* (Raymond & Rousset, 1995; Rousset, 2008) to calculate the numbers of alleles (Na) and effective alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, total mean fixation index (F_{ST}), inbreeding coefficient (Fis) and gene flow (Nm).

Results

Distribution

Limonium korakonisicum is currently known only from its type locality in Korakonisi, south-west Zakynthos (Fig. 1). Its EOO and AOO are 4 km², and its local EOO is 463 m² (Tables 1 & 2; values of local EOO for the three species are for 2018).

The population of *L. phitosianum* comprises 14 subpopulations (Table 1, Fig. 2). We discovered several new subpopulations (Lp2, Lp4, Lp9, Lp10, Lp12, Lp13, Lp14), expanding the known range of this species. Habitat loss has occurred, however, as a result of anthropogenic activities, including the construction of a port that led to the extinction of a subpopulation in Aghios Nikolaos (Volimes). The EOO of *L. phitosianum* is 460 km², the AOO is 60 km² and the local EOO is 83,443 m² (Fig. 2).

We confirmed *L. zacynthium* at all of its known localities except its locus classicus on Keri Lake beach, which has suffered habitat destruction from a tourism-related

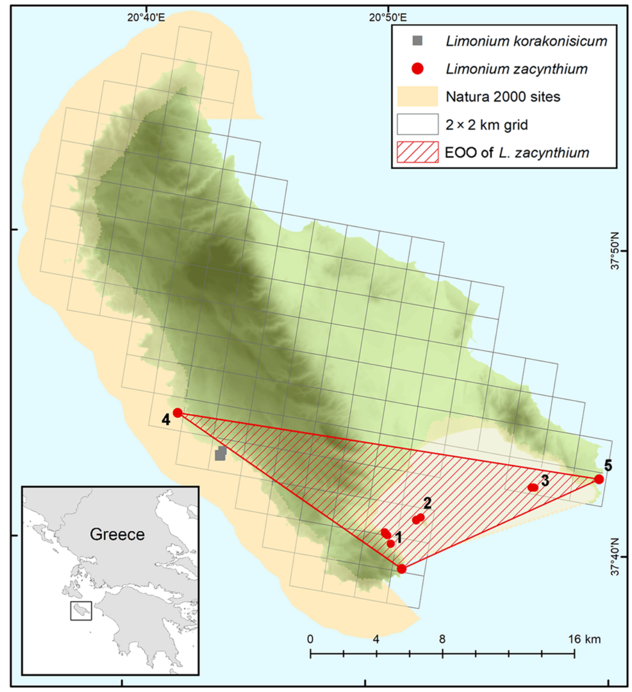

FIG. 1 Distribution of *Limonium korakonisicum* and *Limonium zacynthium*, their area of occupancy (i.e. the number of occupied 2 × 2 km grid cells), and the estimated extent of occurrence (EOO) of *L. zacynthium*. Numbers indicate subpopulations (Lz1, etc.; Table 1).

development. We discovered one new subpopulation, Lz3 (Pelouzo islet). The species has three large subpopulations (Lz1, Lz2, Lz3) but subpopulations Lz4 and Lz5 comprise only 2–5 individuals. The EOO of *L. zacynthium* is 93 km², the AOO is 28 km² and the local EOO is 9,036 m² (Fig. 1).

Re-evaluation of the range of the three species in 2023 revealed no substantial change, except for a decrease in the local EOO of subpopulation Lp11, probably as a result of new recreational facilities in Porto Roxa. The EOO and AOO of *L. phitosianum* were not affected, however.

Population size

The population size of *L. korakonisicum* remained relatively stable during the monitoring period. *Limonium phitosianum* showed varying trends, with some subpopulations decreasing and others increasing in size. The population of *L. zacynthium* significantly declined in 2018, primarily because of reductions in the Lz1 subpopulation (Table 2). By 2023, the population size of all three species had decreased (Table 2). Observations of developmental stages were consistent across all studied species, with a predominance of reproductive individuals in all subpopulations and with seedlings comprising only a small proportion of individuals (Supplementary Table 2).

TABLE 2 Number of mature individuals (counted in complete censuses, except for subpopulations noted), local extent of occurrence (EOO, in m²) and plants per m² in each subpopulation and population of the three *Limomium* species in 2014–2018 and 2023.

	2014			2015			2016			2017			2018			2023		
Subpopulation	Number	Local EOO	Plants/m²	Number	Local EOO	Plants/m²	Number	Local EOO	Plants/m²	Number	Local EOO	Plants/m²	Number	Local EOO	Plants/m²	Number	Local EOO	Plants/m²
L. korakonisicum																		
	67	463.0	0.145	97	463.0	0.209	86	463.0	0.186	77	463.0	0.166	86	463.0	0.186	34	463.0	0.07
L. phitosianum																		
Lp1[1]	552	904.0	0.61	528	904.0	0.58	576	904.0	0.64	708	904.0	0.78	676	904.0	0.75	404	904.0	0.45
Lp2[1]	610	4,222.5	0.14	550	4,222.5	0.13	675	4,222.5	0.16	635	4,222.5	0.15	380	4,222.5	0.09	383	4,222.5	0.09
Lp3	341	281.0	1.20	342	281.0	1.20	440	281.0	1.60	376	281.0	1.30	261	281.0	0.93	406	281.0	1.40
Lp4	391	4,828.5	0.08	285	4,828.5	0.06	266	4,828.5	0.06	255	4,828.5	0.05	234	4,828.5	0.05	130	4,828.5	0.03
Lp5	103	51,783.0	0.002	133	51,783.0	0.003	84	51,783.0	0.002	52	51,783.0	0.001	50	51,783.0	0.001	65	51,783.0	0.001
Lp6	265	1,096.8	0.24	266	1,096.8	0.24	430	1,096.8	0.39	500	1,096.8	0.46	234	1,096.8	0.21	301	1,096.8	0.27
Lp7	299	2,320.5	0.13	212	2,320.5	0.09	138	2,320.5	0.06	260	2,320.5	0.11	250	2,320.5	0.10	68	2,320.5	0.03
Lp8[1]	866	1,237.5	0.70	594	1,237.5	0.48	990	1,237.5	0.80	804	1,237.5	0.65	656	1,237.5	0.53	215	1,237.5	0.17
Lp9[1]	962	6,590.0	0.15	791	6,590.0	0.12	923	6,590.0	0.14	659	6,590.0	0.10	725	6,590.0	0.11	922	6,590.0	0.14
Lp10	173	602.0	0.29	279	602.0	0.46	481	602.0	0.80	474	602.0	0.79	430	602.0	0.71	186	602.0	0.31
Lp11[1]	228	4,591.5	0.05	168	4,591.5	0.04	140	4,591.5	0.03	140	4,591.5	0.03	100	4,591.5	0.02	86	2,288.0	0.04
Lp12	437	4,598.0	0.095	430	4,598.0	0.09	512	4,598.0	0.11	647	4,598.0	0.14	553	4,598.0	0.12	530	4,598.0	0.12
Lp13													14	150.0	0.09	15	150.0	0.10
Lp14													70	238.1	0.29	78	238.1	0.32
Total	5,227	83,055.3		4,578	83,055.3		5,655	83,055.3		5,510	83,055.3		4,549	83,443.4		3,789	81,139.9	
L. zacynthium																		
Lz1[2]	2,109	6,590.0	0.32	2,241	6,590.0	0.34	1977	6,590.0	0.30	2,109	6,590	0.32	791	6,590.0	0.12	857	6,590.0	0.13
Lz2				252	266.0	0.95	356	266.0	1.34	248	266.0	0.93	236	266.0	0.89	228	266.0	0.86
Lz3				167	2,104.9	0.08	143	2,104.9	0.07	129	2,104.9	0.06	149	2,104.9	0.07	137	2,104.9	0.07
Lz4	2	25.0	0.08	2	25.0	0.08	2	25.0	0.08	2	25.0	0.08	2	25.0	0.08	4	25.0	0.16
Lz5	5	50.0	0.10	5	50.0	0.10	5	50.0	0.10	5	50.0	0.10	5	50.0	0.10	2	25.0	0.08
Total	2,116	6,665.0		2,667	9,035.9		2,483	9,035.9		2,493	9,035.9		1,250	9,035.9		1,228	9,010.9	

[1]Counts of mature individuals were in 20 random 5 × 5 m plots.
[2]Counts of mature individuals were in four random 5 × 5 m plots.

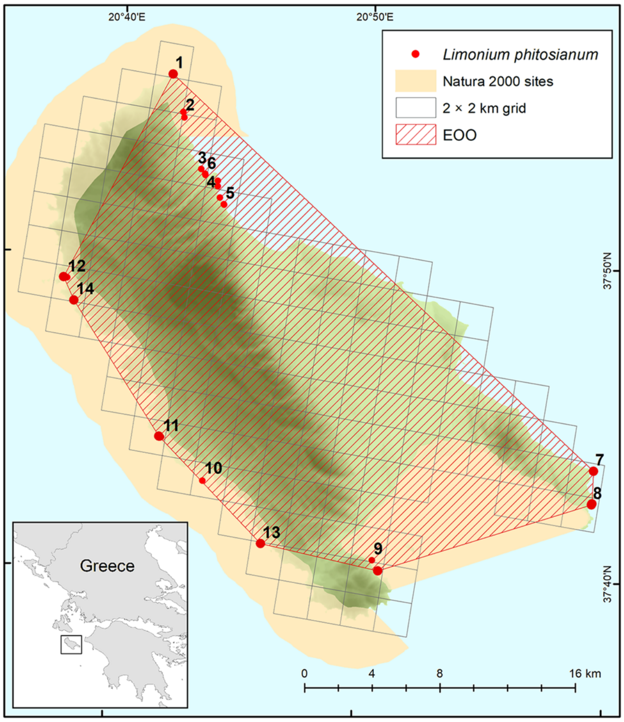

FIG. 2 Distribution of *Limonium phitosianum*, its area of occupancy (i.e. the number of occupied 2 × 2 km grid cells), and the estimated extent of occurrence (EOO). Numbers indicate subpopulations (Lp1, etc.; Table 1).

Reproductive biology

For *L. korakonisicum*, the survival rate of seedlings ranged from 25% in 2016 to 50% in 2015 and 2017 (Table 3). Pearson's correlation coefficient revealed a significant positive correlation between annual seedling survival rate and the population growth rate ($r = 0.593$, $P < 0.05$). The mean annual relative reproductive success was consistently high (69.3–76.3%). Flowering was 52.6 days on average (early August–late September). Fruiting was mid August–mid/late October (average duration 63.4 days), sometimes extending to early November (Fig. 3).

For *L. phitosianum*, seedling survival rate varied from 27.3% in 2018 to 57.5% in 2017, and relative reproductive success was consistently high (67–74.3%). The number of flowers and caryopses were significantly higher in 2017 and 2018 compared to earlier years. Flowering was 50.4 days (early August–late September) and fruiting 54.4 days (mid August–early October) on average (Fig. 3). Fruiting duration was inversely correlated ($r = -0.7098$, $P < 0.05$) with mean monthly precipitation during September–October.

For *L. zacynthium*, seedling survival rate was 35.7–66.7%. Relative reproductive success was consistently high (69.3–76.3%). Mean number of caryopses per flower and relative reproductive success were significantly higher in 2017 and 2018 compared to earlier years. The duration of flowering (56–67 days, late July–late September) and fruiting (average 56 days, late August–mid-October) was relatively stable throughout the monitoring period (Fig. 3).

Population viability analysis

For *L. korakonisicum*, population extinction risk is zero over the next 10 years, and increases to 4.2% within the next 50 years (Fig. 4). The population of *L. phitosianum* faces no extinction risk in either period (Fig. 4). However, subpopulations Lp2, Lp5 and Lp7 have increased risks of extinction over the next 50 years, of 13.3–58% (Supplementary Fig. 1). In the next 10 years, subpopulations Lz1 and Lz3 of *L. zacynthium* have a gradual reduction in size but no risk of extinction. However, within the next 50 years, the species faces a high risk of extinction (67.8%), with a substantial likelihood (89.1%) that subpopulation Lz1 will go extinct.

Threats

The threats identified for *L. korakonisicum* are: tourism development (threat code 1.3), including construction of canteens and secondary roads (4.1), resulting in the reduction of the species' habitat, and competition with other species (8.2), especially with sympatric seedlings of *L. phitosianum*.

The primary threat to *L. phitosianum* is uncontrolled tourism development (1.3), particularly through resort construction and alteration of rocky shores affecting subpopulations Lp1, Lp3, Lp4 and Lp8–11, leading to habitat degradation and loss. Additionally, trampling in highly tourist-visited areas such as Lp7, Lp8 and Lp10 poses a significant threat (6.1). An immediate threat in Porto Limnionas-Roxa (Lp11) arises from the invasive plant *Carpobrotus edulis* (8.1.2). These threats were identified across all 14 locations.

The threats to *L. zacynthium* include: tourism development (1.3), such as the construction of a canteen at Marathias (Lz1) and alteration of rocky shores in subpopulations Lz1, Lz4 and Lz5; competition with other species (8.2) observed at Lz1, where *L. zacynthium* competes with *L. phitosianum*; and trampling (6.1) occurring at Marathonisi islet (Lz2). The extinction of *L. zacynthium* from its *locus classicus* at Keri Lake beach as a result of tourism development underscores the severity of this threat to endemic littoral plant species. These threats were identified across four of the five locations.

Conservation status assessment

We assess *L. korakonisicum* as Critically Endangered based on criteria B1ab(v) + 2ab(v), with EOO < 100 km² (B1) and AOO < 10 km² (B2), and only one location (a) and a projected continuing decline (b) in number of mature individuals (v),

TABLE 3 Reproductive characteristics of the three *Limonium* species during the monitoring period; n indicates the number of randomly selected mature individuals or number of stems of tagged individuals. Different superscript letters indicate significant differences ($P < 0.05$) between years for each reproductive parameter.

	2014	n	2015	n	2016	n	2017	n	2018	n
L. korakonisicum										
Mean number of stems per individual ± SE			21.5 ± 4.8	10	29.6 ± 9.3	10	27.6 ± 6.0	10	19.8 ± 4.7	10
Mean number of flowering/fruiting stems per individual (F) ± SE			11.3 ± 3.6	10	16.9 ± 6.2	10	11.0 ± 3.0	10	10.7 ± 3.5	10
Mean number of flowers per stem ± SE			1.6 ± 0.2	20	1.4 ± 0.2	20	1.8 ± 0.3	20	1.9 ± 0.2	20
Mean number of flowers per individual ± SE			21.6 ± 6.0	10	23.1 ± 8.8	10	15.3 ± 4	10	26.9 ± 8.7	10
Mean number of fruits (caryopses) per flower ± SE			0.8 ± 0.0		0.7 ± 0.1		0.7 ± 0.01		0.8 ± 0.0	
Mean number of fruits (caryopses) per stem (S) ± SE			14.2 ± 3.1	20	19.3 ± 0.7	20	13.1 ± 2.4	20	24.0 ± 2.0	20
Fecundity (mean number of caryopses per individual) (S × F)			183.1		327.8		142.7		260.9	
Seed rain (caryopses/m^2)			38.4		60.9		22.2		48.5	
Seedling survival (%)			50.0		25.0		50.0		40.0	
Relative reproductive success (%)			75.3		72.3		69.3		76.3	
L. phitosianum										
Mean number of stems per individual ± SE	15.9 ± 4.4	60	17.2 ± 2.6	100	24.7 ± 3.3	100	31.3 ± 7.1	110	28.1 ± 6.3	100
Mean number of flowering/fruiting stems per individual (F) ± SE	11.9 ± 3.0	120	14.8 ± 1.9	200	18.1 ± 2.8	200	20.4 ± 7.0	220	22.5 ± 4.2	200
Number of flowers per stem ± SE[1]	1.8 ± 0.3[a]	60	2.8 ± 0.4[a]	100	3.4 ± 0.3[a]	100	4.7 ± 0.4[b]	110	5.0 ± 1.0[b]	100
Number of flowers per individual ± SE[1]	18.8 ± 6.7[a]	60	25.0 ± 6.7[a]	100	24.6 ± 3.9[a]	100	103.8 ± 22.8[b]	110	54.7 ± 15.4[b]	100
Mean number of fruits (caryopses) per flower ± SE	0.7 ± 0.0	60	0.7 ± 0.1	100	0.7 ± 0.0	100	0.7 ± 0.0	100	0.8 ± 0.0	100
Mean number of fruits (caryopses) per stem (S) ± SE	16.6 ± 1.4[a]	60	20.7 ± 1.5[a]	100	24.6 ± 4.2[a]	100	43.2 ± 4.4[b]	100	41.7 ± 5.5[b]	100
Fecundity (mean number of caryopses per individual) (S × F)	197.9		290.4		436.2		467.8		802.2	
Seed rain (caryopses/m^2)	1.9		2.4		4.5		4.7		6.7	
Seedling survival (%)			56.3		53.1		57.5		27.3	
Relative reproductive success (%)	67.0		71.9		68.7		74.3		74.2	
L. zacynthium										
Mean number of stems per individual ± SE			22.9 ± 5.7	30	28.22 ± 6.7	30	20.2 ± 7.2	30	22 ± 1.7	30
Mean number of flowering/fruiting stems per individual (F) ± SE			16.4 ± 6.1	30	14.2 ± 3.2	30	16.5 ± 5.0	30	17.3 ± 3.3	30
Number of flowers per stem ± SE[1]			2.3 ± 0.3	30	2.8 ± 0.5	30	2.6 ± 0.3	30	2.4 ± 0.4	30
Number of flowers per individual ± SE[1]			41.0 ± 15.7	30	38.4 ± 8.9	30	42.8 ± 10.7	30	48.3 ± 6.0	30
Mean number of fruits (caryopses) per flower ± SE			0.7 ± 0.0[a]		0.7 ± 0.1[a]		0.8 ± 0.0[b]		0.8 ± 0.0[b]	
Mean number of fruits (caryopses) per stem (S) ± SE			29.3 ± 4.2	60	24.0 ± 8.0	60	39.2 ± 7.1	60	26.3 ± 4.9	60
Fecundity (mean number of caryopses per individual) (S × F)			475.1		333.0		635.3		456.6	
Seed rain (caryopses/m^2)			131.2		85.7		164.0		56.1	
Seedling survival (%)					66.7		35.7		39.8	
Relative reproductive success (%)			70.7[a]		68.0[a]		72.5[b]		79.4[b]	

[1]Measurements taken at specific points in time.

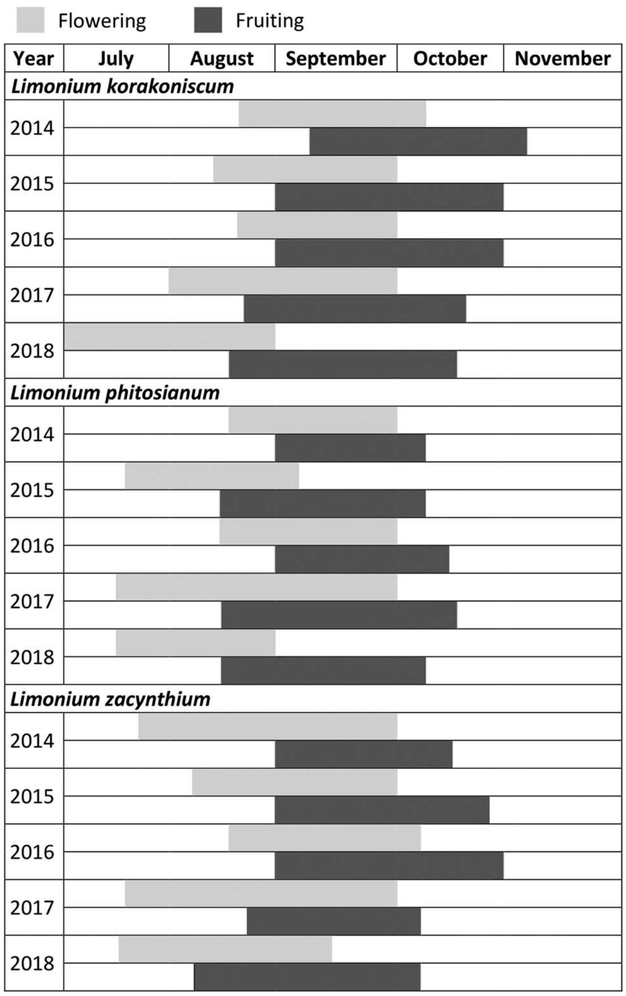

FIG. 3 Flowering and fruiting periods of the three species of *Limonium* during 2014–2018.

and C2a(ii), with < 250 mature individuals (C) and a projected continuing decline in number of mature individuals (2), and all individuals in a single subpopulation (a(ii)). We assess *L. phitosianum* as Near Threatened, as it is close to meeting the criteria for Vulnerable based on its EOO and AOO, with the exception of the number of locations, which is > 10. We assess *L. zacynthium* as Endangered based on criteria B1ab(iv,v) + B2ab(iv,v), with EOO < 100 km² (B1) and AOO < 10 km² (B2), and ≤ 5 locations (a), and a projected continuing decline in number of mature individuals (b(v)) and number of locations or subpopulations (b(iv)).

Genetic analyses

Analysis of the five microsatellite loci revealed 28 alleles (1–22 alleles/locus) in total. Ln115 and Ln152 loci were monomorphic in both species, and Ln39 is monomorphic in *L. phitosianum* but polymorphic in *L. zacynthium*, with a private allele in subpopulation Lz2. Ln68 was monomorphic in *L. zacynthium* and polymorphic in *L. phitosianum*, with a private allele in subpopulation Lp7. Ld423 was highly polymorphic, with 22 different alleles across all subpopulations. Of these, seven were present in both species, and the others were species-specific. *Limonium phitosianum* had 11 private alleles, some of which were subpopulation-specific (three in subpopulation Lp7, six in subpopulation Lp10), and two occurred in more than one subpopulation (Lp1 and Lp7; Lp7 and Lp10). *Limonium zacynthium* had four private alleles, one in subpopulation Lz1 and three in Lz2.

Mean number of alleles (Na) ranged from 1.000 to 3.200 (for subpopulationa Lp1 and Lp10, respectively), and the mean effective number of alleles (Ne) ranged from 1.000 to 2.706 (for the same subpopulations). Shannon's information index (I) ranged from 0.000 to 0.474 (for Lp1 and Lp10, respectively). Observed heterozygosity (Ho) was lowest in Lz3 and Lp1 (0.000) and highest for Lp7 (0.220), and expected heterozygosity (He) had the lowest and highest values in Lp1 and Lp7, respectively (Supplementary Table 3). The inbreeding coefficient (Fis) values were positive for all subpopulations, except for Lp1 where no value was obtained by *GENEPOP*. Fis was 1.000 for Lz3, and for the other subpopulations the following values were estimated: 0.0913 (Lz2), 0.1091 (Lp7), 0.1884 (Lp10), 0.3000 (Lz1). The mean fixation index (F_{ST}) value was 0.172 when calculated only among *L. phitosianum* subpopulations and 0.151 among *L. zacynthium* subpopulations. The overall F_{ST} calculated using the data available for both species was 0.159, and the value of gene flow (Nm) was 0.424.

Discussion

Population size and reproductive biology

In this study, we monitored all extant subpopulations of three rare *Limonium* species endemic to Zakynthos Island for 5 years. The EOO and AOO remained stable throughout the study for all three species.

There was a prevalence of mature individuals of *L. korakonisicum*, with low seedling abundance. The rocky calcareous substrate and potential competition with seedlings of the sympatric *L. phitosianum* could impede successful establishment and growth of *L. korakonisicum* seedlings, although relative reproductive success was consistently high, indicating this is not a limiting factor for the species' survival. Given a seed germination rate of up to 86% (Valli & Artelari, 2015), we suggest that seedling establishment and survival could be a bottleneck in the species' reproductive cycle. The population viability analysis indicated a 3.9% extinction risk over the next 50 years. However, deterministic models may underestimate quasi-extinction probability by not considering other factors influencing population viability (Akçakaya et al., 1999; Morris & Doak, 2002).

There were fluctuations in the seedling survival rate of *L. phitosianum*. The lowest spring rainfall during monitoring,

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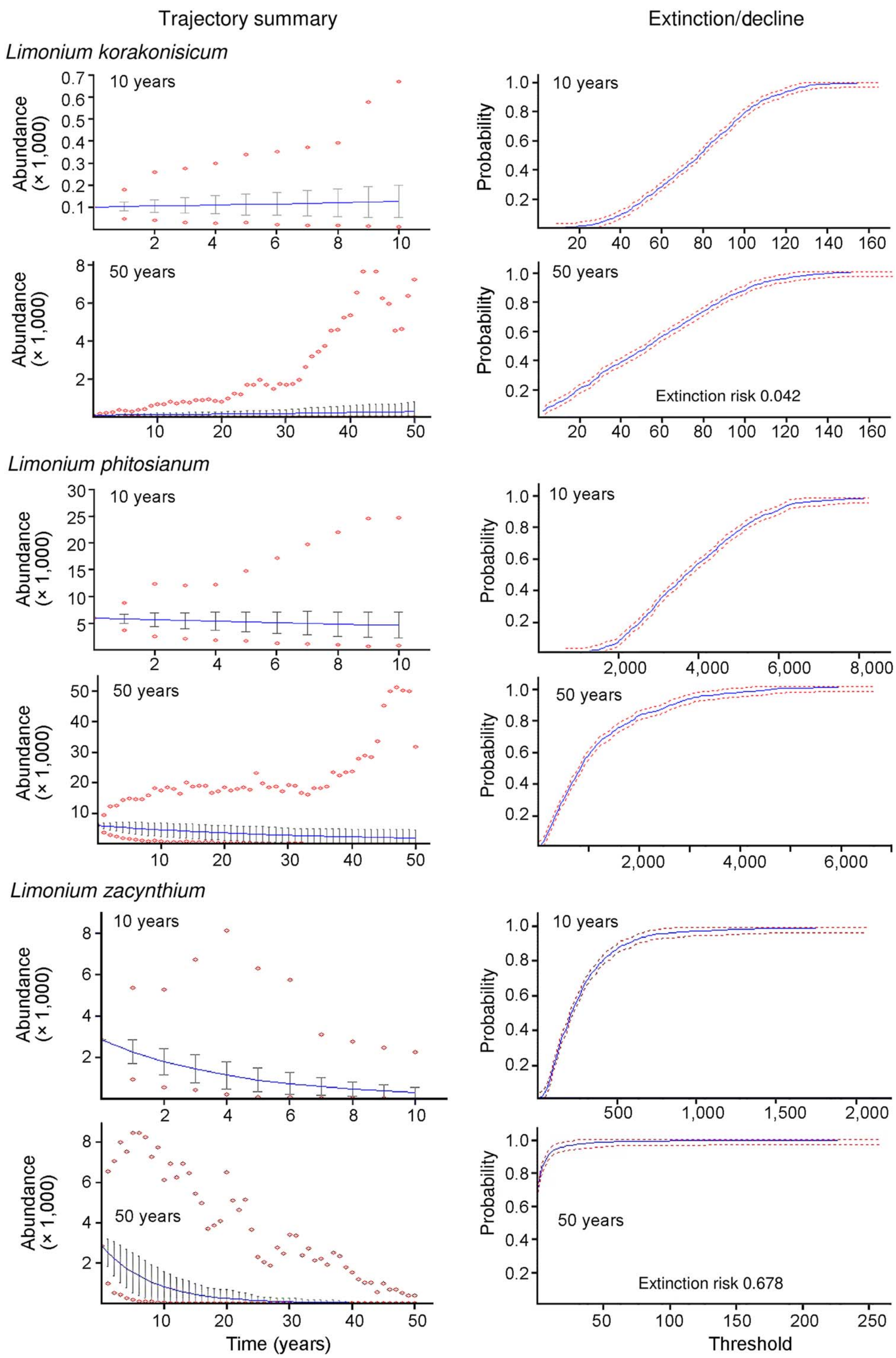

FIG. 4 Population viability analysis for the three species of *Limonium* over the next 10 years and the next 50 years from 2018. The graphs display the average number of extant populations (solid line), along with ±1 standard deviation. Dots indicate the minimum and maximum numbers of extant populations. This analysis illustrates potential population trends and variability of each species over the specified time periods.

in 2018 (14.7 mm), corresponded with the species' lowest seedling survival rate, suggesting dependence on salinity influenced by climate conditions. The negative correlation of fruiting duration with mean monthly precipitation in September–October emphasizes the importance of water availability in fruit ripening. Seed dispersal in *L. phitosianum* is triggered by water absorption, a mechanism also observed in *Limonium creticum* (Fournaraki, 2010). The high annual seed production but relatively low per cent of seedlings indicates that seedling establishment is the critical stage for this species.

The population size of *L. zacynthium* is notably small, and it halved in 2018, primarily a result of a decrease in subpopulation Lz1, where the highest number of dead individuals occurred in that year. This decline, combined with increased relative reproductive success and seed production in the previous year, could be linked to increased vegetation density, indicating the species is a weak competitor. Different developmental stages in halophytes have distinct optimal salinity thresholds (Espinar et al., 2005; Redondo-Gómez et al., 2008). The high negative correlation of seedling survival with mean monthly precipitation in the preceding October–December suggests that intense precipitation during these wet months decreases seedling survival the following year. Additional studies on seed germination and seedling growth under varying salinity conditions are necessary to investigate this.

The differences in the population sizes of the studied species in 2023 compared to 2018 may be attributed to variations in developmental stages within populations. Similar annual variations in population size have been reported in other *Limonium* species (e.g. Laguna et al., 2016; González-Orenga et al., 2021). The markedly lower population size of *L. korakonisicum* in 2023 underscores the urgent need for implementing conservation management measures for this species.

Genetic variability

The analysis of microsatellites indicated reduced genetic diversity and inbreeding and restricted gene flow within and among subpopulations of *L. phitosianum* and *L. zacynthium*. Genetic diversity is critical for maintenance of long-term fitness, adaptation and potential for survival (Frankham et al., 2002; Bouzat, 2010). Small population sizes and restricted distribution area can elevate inbreeding levels (e.g. Barrett & Kohn, 1991; Falconer & Mackay, 1996), and discrepancies between observed and expected heterozygosity can suggest potential inbreeding (Schimidt et al., 2021). In our study, expected heterozygosity was generally higher than the observed, indicating inbreeding across all subpopulations analysed except Lp1. In addition, the positive inbreeding coefficient (Fis) across all subpopulations indicates an excess of homozygous individuals (i.e. more inbreeding than randomly expected); its high value implies a high degree of inbreeding for at least subpopulations Lz1 and Lz3, and gene flow appears to be reduced among subpopulations. The value of the mean fixation index (F_{ST}) across all analysed subpopulations for both species indicates a certain degree of genetic differentiation among them (F_{ST} measures the proportion of genetic variance that is a result of differences between populations compared to within populations). Higher F_{ST} values typically reflect greater genetic differentiation and reduced gene flow among populations (Frankham et al., 2002). In this study, the observed mean F_{ST} value implies limited gene flow and restricted intercrossing (Balloux & Lugon-Moulin, 2002), contributing to genetic divergence among subpopulations. This is also the case for F_{ST} values calculated within each species separately, reinforcing the conclusion that there is significant genetic divergence and lack of genetic exchange among the subpopulations both within and among the species studied.

Conservation implications

The status of the three species of *Limonium* studied is directly related to the identified threats. The main threat is coastal development for tourism, leading to habitat degradation. Although these species occur both within and outside protected areas of the Natura 2000 Network (GR2210001, GR2210002), management within protected areas, overseen by the Management Unit of Zakynthos and Ainos National Parks and the Protected Areas of the Ionian Islands, is the most feasible option as the majority of land on Zakynthos is privately owned. We recommend installation of signage in heavily touristic areas, education of landowners about the importance of conservation, and installation of fencing at selected sites (such as Korakonisi and Marathonisi islet) as initial management measures for the conservation of the three species. These measures would curtail the effects of trampling and foster improved conditions for seedling growth. Additionally, eradication of *Carpobrotus edulis* in Porto Limnionas-Roxa (subpopulation Lp11) by manual uprooting would reduce the threat from this invasive species. The establishment of a plant micro-reserves network (Laguna et al., 2020) within the Management Unit's jurisdiction would benefit multiple species simultaneously and ensure management, protection and collective benefits for all three studied species.

Our findings align with the higher levels of inbreeding depression observed in stressful environments, such as littoral rocks, resulting in decreased seedling survival rates (Frankham et al., 2017). We recommend genetic mixing for *L. zacynthium* through the creation of safety neopopulations, as suggested by Laguna & Ferrer-Gallego (2012), using seeds sourced from all known natural subpopulations. Similarly, for *L. korakonisicum* this strategy should be employed alongside the reinforcement of the existing subpopulation, a combined approach successfully applied for other *Limonium* species (e.g. Caperta et al., 2014; Laguna et al., 2016). These in situ conservation efforts should be complemented by ex situ measures such as seed bank storage and propagation trials, to facilitate future reintroduction and/or population reinforcement (Krigas et al., 2022).

In 2024, the staff of the Management Unit for Zakynthos and Ainos National Parks and the Protected Areas of the Ionian Islands deployed informational signs across the Natura 2000 Network. These signs alert the public to the existence of rare and threatened plant species and emphasize that uprooting them is strictly prohibited. This initiative marks the beginning of the management and conservation of these species.

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Author contributions Conception and study design: A-TV, PT; data collection: A-TV; monitoring and data analysis: A-TV, PT; genetic analysis: CP, EL, VP; writing: A-TV, VP, PT; revision: all authors.

Conflicts of interest None.

Ethical standards All monitoring and experimental procedures were approved by the Hellenic Ministry of the Environment and Energy, Directorate of Forest Protection (approval no. MEE/DFP/125613/6014) and this research abided by the *Oryx* guidelines on ethical standards.

Data availability All relevant data are available in the article and supplementary material.

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