Journal of Limnology

Journal of Limnology 2026; 85:2248
doi:10.4081/jlimnol.2026.2248

Evidence for an additional population of the cave-dwelling squat lobster *Munidopsis polymorpha* in Lanzarote (Canary Islands): implications for management and conservation

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Abstract

The endangered anchialine squat lobster *Munidopsis polymorpha* is restricted to northern Lanzarote. To understand genetic isolation and conservation risks, we genotyped 26 individuals at eight microsatellite loci from four sites in La Corona lava tube and Charcos de Luis. Lava tube sites shared most alleles and were effectively undifferentiated, whereas Charcos de Luis harbored nine private alleles, the highest allelic richness, and clear differentiation from the lava tube complex. We detected no Hardy-Weinberg deviations among testable locus x site combinations and no linkage disequilibrium. These results are consistent with two candidate management units: La Corona lava tube and Charcos de Luis. We outline genomic priorities to guide future conservation plans. To account for our reduced sampling size, we screened for null alleles and repeated the analyses on a reduced set of loci passing conservative quality filters, obtaining congruent patterns of differentiation.

Key words: biodiversity conservation; anchialine; groundwater-dependent ecosystems; subterranean biology.

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Introduction

Anchialine ecosystems comprise coastal groundwater habitats connected to fresh and marine environments. These transitional ecosystems contain limnological habitats (e.g., ponds, pools, and flooded caves) that remain chemically and physically connected to the marine environment (Mejía-Ortiz *et al*., 2022). Such ecosystems host exceptional subterranean biodiversity on Lanzarote, with 38 described endemic species, many representing isolated crustacean and annelid lineages (Martínez *et al*., 2016). These systems have long been used by people for diverse purposes, such as tourism, salt production, fish preservation, and groundwater source for various infrastructures. In addition, some perched aquifers in the island's highlands have been tapped for drinking water and irrigation (Martínez *et al*., 2016).

Such multiple uses impose layered anthropogenic pressures on narrowly distributed anchialine species, whereas conservation plans are further complicated by our limited knowledge of their biology, population structure, and evolutionary history. The creation of protected areas in northern Lanzarote, such as the Natural Monument of La Corona or the Special Area of Conservation of Los Jameos (Natura 2000 network), has aided conservation, but protected-area status alone does not always safeguard subterranean habitats (Mammola *et al*., 2024). A pragmatic approach is to focus effort on an umbrella species whose distribution and habitat overlap with those of other anchialine exclusive taxa. On Lanzarote, the endemic squat lobster *Munidopsis polymorpha* Koelbel, 1892 ("jameíto" or "grillo blanco") is a natural flagship species. This species is the only known obligate subterranean squat lobster and an iconic example of anchialine fauna with deep-sea affinities, as well as a symbol of the island's natural heritage (Iliffe *et al*., 2025).

Most known records of *M. polymorpha* concentrate along La

Corona lava tube, the only extensive cave system that allows human access to the island's coastal aquifer through three localities: Cueva de los Lagos, further inland, Jameos del Agua, and Túnel de la Atlántida - partially extending below the seafloor. Populations appear denser in the illuminated lake at Jameos del Agua and at the entrance pools of Túnel de la Atlántida (Wilkens *et al*., 1990). Along the dark passages, only wandering individuals are observed. Additionally, a high number of individuals have been consistently observed in Montaña de Arena, an accumulation of sand 700 m from Túnel de la Atlántida entrance. In contrast, isolated records exist from Charcos de Luis, an anchialine pool complex farther north (Wilkens *et al*., 1993), and in Salinas de los Agujeros, a saltworks facility southwest of the cave where the species was recorded only once in the 1980s (Martínez *et al*., 2016). Despite continuous efforts over the last 20 years, no additional specimens have been recorded from Salinas de los Agujeros and only three individuals were recovered from Charcos de Luis, despite the multiple visits during the conservation plan of the species.

Previous population genetic work genotyped eight microsatellite loci in 76 individuals from Jameos del Agua and 45 from Túnel de la Atlántida and reported extremely low variability, with an effective population size of ca. 50 individuals (Cabezas *et al*., 2008, 2012). Those studies, however, excluded deeper sections of Túnel de la Atlántida, Cueva de los Lagos, Charcos de Luis, and Salinas de los Agujeros (Martínez *et al*., 2016). Unlike the three sites within La Corona lava tube, Charcos de Luis consists of a series of shallow anchialine pools located several kilometres to the north, on the opposite side of the Malpaís de la Corona lava field (Wilkens *et al*., 2009). Geological mapping indicates that these pools are hydrologically disconnected from the lava-tube aquifer by thick, lava flows (Tomasi *et al*., 2023). Thus, Charcos de Luis represents an isolated groundwater habitat rather than an extension of the lava-tube system, suggesting the presence of distinct stygobitic populations (González *et al*., 2017). A broader geographic sampling is needed to capture the full extent of genetic diversity across the species' range and to provide a more robust basis for defining conservation units.

Here we present the first microsatellite data for two additional localities - Cueva de los Lagos and Charcos de Luis, discuss their implications for the conservation of *M. polymorpha*, and outline priorities for follow-up genomic work using state-of-the-art methods. Our limited sample size reflects two unavoidable constraints. First, collection permits for this critically endangered species restrict sampling to a small number of individuals. Second, *M. polymorpha* is exceptionally elusive outside La Corona lava tube, making collection difficult at Charcos de Luis. Despite these limitations, documenting the currently available data is important, as it provides the first genetic evidence from previously unsampled sites and forms a necessary baseline for future genomic-scale studies.

Methods

We collected leg tissue from 26 individuals *Munidopsis polymorpha* across three sites within the La Corona lava tube: Cueva de los Lagos (WGS84 coordinates: 29.157°N, 13.439°W; n=7), Jameos del Agua (29.157°N, 13.431°W; n=11), the first ca. 150 m of Túnel de la Atlántida (29.157°N, 13.430°W; n=5); and Charcos de Luis (29.202°N, 13.423°W; n=3), outside the cave complex (Fig. 1 A-D). All collections were done during April 2017. Our sampling size is limited both by the unavailability of animals in Charcos de Luis, as well as the number of individuals allowed by our permits, which are granted in the Exp. 2017/2306 from the Government of the Canary Islands. Specimens from the flooded lava tube sites were captured by SCUBA cave divers using hand nets; Charcos de Luis individuals were collected by hand. Tissue was preserved in ethanol and shipped to AllGenetics (www.allgenetics.eu) for DNA extraction and genotyping. Total DNA was isolated with the NZY Tissue gDNA kit (NZYTech) and resuspended in 100 µL. A no-template control was included in every extraction batch to monitor contamination.

Individuals were genotyped at eight species-specific microsatellite loci (Cabezas *et al*., 2009; *Tab. S1*). We used the three-primer "tailed-primer" method (Schuelke, 2000), in which each PCR contained a locus-specific forward primer, a locus-specific reverse primer carrying a 5′ tail, and a fluorescently labeled oligonucleotide matching that tail (M13: GGA AAC AGC TAT GAC CAT, labeled with HEX; CAG: CAG TCG GGC GTC ATC, labeled with FAM). PCR reactions contained 1 µL DNA, 6.25 µL Type-it Microsatellite PCR Kit (Qiagen), 4 µL PCR-grade water, and 1.25 µL primer mix (forward, tailed reverse, and the HEX-M13 or FAM-CAG oligo; *Tabs. S2* and *S3*). Thermal cycling was: 95°C 5 min; 30 × (95°C 30 s, 56°C 90 s, 72°C 30 s); 8 ×(95°C 30 s, 52 °C 90 s, 72 °C 30 s); final extension 68 °C 30 min. Every PCR set included a negative control. Amplicons were sized by capillary electrophoresis and delivered as 87 .fsa chromatogram files in total. Alleles were called in Geneious v10.2.3. Allele scoring followed a stringent procedure to minimize artefacts. Chromatograms were independently reviewed by two researchers, and discrepancies were resolved by joint inspection. Peaks affected by stutter, poor signal-to-noise ratio, or unclear morphology were excluded from scoring.

For summary statistics, we combined the two alleles per locus into a single diploid genotype and built a 'genind' object using the R package 'adegenet' v1.3-1 (Jombart and Ahmed, 2011). Populations were defined by locality. Using the function 'basic.stats' in the 'hierfstat' R package (v0.5-11; Goudet and Jombart, 2022), we calculated average of the heterozygosity (Ho), expected heterozygosity (He), and inbreeding index (F_{IS}). Ho is the observed fraction of heterozygotes (i.e. diploid individuals that carry different versions or *alleles* of the same genetic marker, or *locus;* in microsatellites, a heterozygote typically shows two fragment sizes at that locus) observed across loci and reflects the in-site genetic diversity; He is the number of heterozygotes expected under Hardy-Weinberg equilibrium (i.e. baseline for a randomly mating population with no selection, migration, mutation, or drift) from the site allelic frequencies. F_{IS} summarizes the departure between He and Ho as F_{IS}=(He-Ho)/He. Positive values indicate a heterozygote deficit due to e.g. inbreeding, hidden structure or null alleles that mask true heterozygotes as homozygotes; negative values indicate an excess, due to e.g. recent mixing of separate groups, mate selection, or small sampling size. Allelic richness (AR) was estimated with the function 'allelic.richness' in the 'hierfstat' R package, rarefied to the smallest sample size so values are comparable among sites. We also reported the total number of distinct alleles per locality across loci. Genetic differentiation between-sites was quantified using the Weir and Cockerham pairwise fixation index F_{ST} calculated with the 'pairwise.WCfst' function in 'hierfstat'. We set

1,000 bootstrap replicates over loci to derive 95% confidence intervals (CIs). F_{ST}=0 is interpreted as negligible differentiation, F_{ST}=0.05-0.15 as low-moderate, F_{ST}=0.15-0.25 strong, and F_{ST} >0.25 very strong. However, given small and uneven sample sizes, we treated these metrics as qualitative indicators and interpreted them cautiously in the *Results* chapter.

As part of the genotyping quality control, we computed per site x locus call rate and tested Hardy-Weinberg equilibrium (HWE) with exact tests (10,000 Monte-Carlo replicates; HardyWeinberg R package, Graffelman, 2015), adjusting p-values within sites by Benjamini–Hochberg (BH). We screened for linkage disequilibrium (LD) within sites using Monte-Carlo χ^2 tests across unordered genotype contingency tables (5,000 replicates) with BH correction. Locus–site combinations with less than 5 genotypes, monomorphic data, or sparse tables were treated as unassesable. Full quality controls results are in *Tab. S3*. Although these microsatellite markers were previously validated for *M. polymorpha* by Cabezas et al. (2009), we reassessed null alleles for the present dataset using the 'null.all' function in 'adegenet' v1.3-1 (Jombart and Ahmed, 2011) and inspected their correspondence with HWE deviations. Three loci showed elevated null-allele frequencies, so we repeated all analyses on a conservative, five-loci filtered dataset. The results were qualitatively identical to those from the full dataset. We also conducted exploratory PCA and DAPC multivariate analyses to visually show the difference in allelic composition amongst the individuals of each locality (see *Supplementary Analyses* for all those additional methods).

Results

Quality control revealed high call rates overall (site means: Lagos = 100%, Túnel = 97.5%, Jameos = 75%, Charcos = 83.3%) and no Hardy-Weinberg deviations among the few testable locus x site combinations (all tested p=1.00; BH-q = 1.00). No linkage disequilibrium pairs were significant after BH within sites, although many were unassessable, thus, linkage disequilibrium is interpreted as a low-power check (*Tab. S3*). These results indicate that the markers behaved reliably and provided no evidence of demographic or genotyping artefacts, although statistical power was limited.

We found 13-14 shared alleles among individuals in La Corona lava tube, with only one or two private alleles within lava tube sites: Túnel de la Atlántida (Mp-8, allele 209; Mp-7, allele 323), Cueva de los Lagos (Mp-1: allele 395) and Jameos del Agua (Mp-2: allele 257) (Fig. 1E). In contrast, Charcos de Luis showed nine private alleles (Mp-8, allele 217; Mp-4, alleles 204 and 212; Mp-3, alleles 294, 282, and 302; Mp-6, alleles 241 and 245; Mp-7, allele 309), even if from only three individuals. Allelic richness was also highest at Charcos de Luis despite its very small sample size (Fig. 1F). Heterozygosity was moderate at Charcos and lower at the lava tube sites. F_{IS} values were small in magnitude; positive at Charcos de Luis (0.154) and Jameos del Agua (0.086), negative at Cueva de los Lagos (−0.074) and Túnel de la Atlántida (−0.180). Pairwise F_{ST} suggested a differentiation

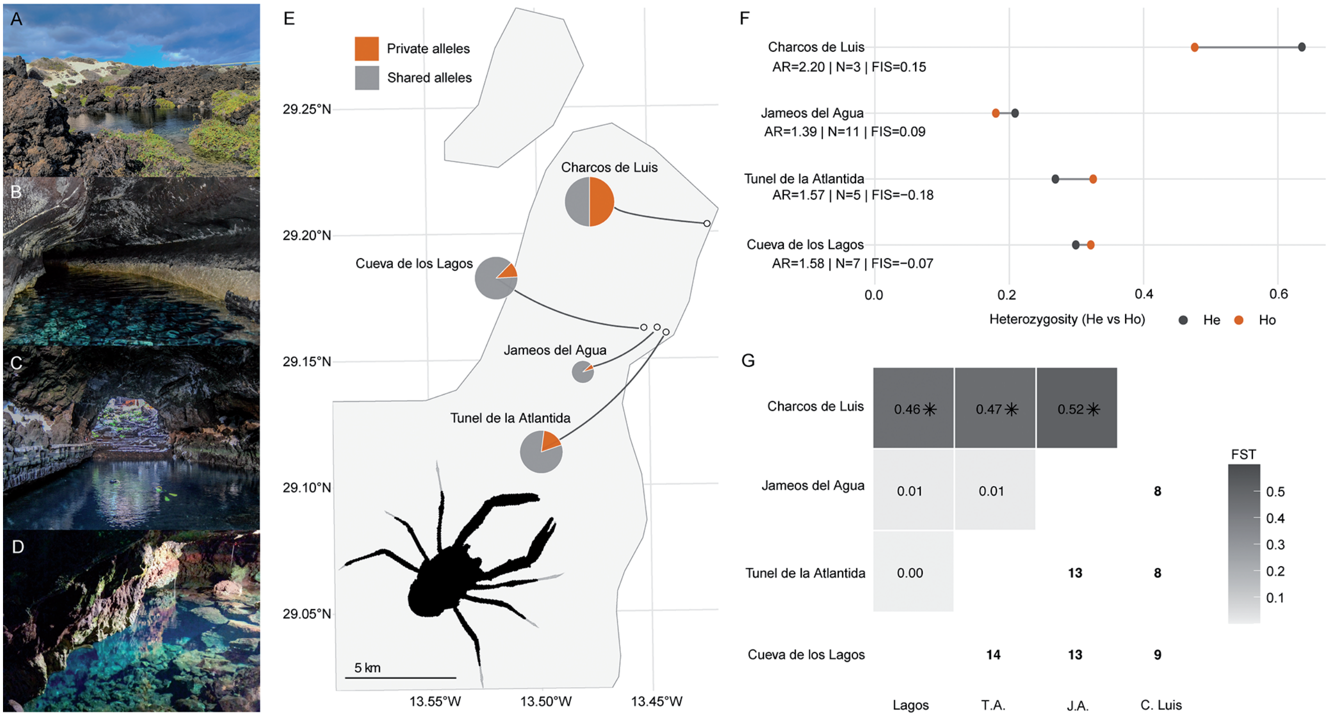

Fig. 1. Summary of the genetic diversity in *Munidopsis polymorpha* across sampled localities. A) Charcos de Luis. B) Cueva de los Lagos. C) Jameos del Agua. D) Túnel de la Atlántida. E) Map of northeast coast of Lanzarote with per-locality pies showing allele composition (orange, private alleles; grey, shared alleles among sites); pie radius scales with the total number of distinct alleles observed at each site. F) Within-site genetic diversity; dumbbells connect expected heterozygosity (He, grey) to observed heterozygosity (Ho, orange) per locality, whose differences indicate FIS; AR, allelic richness, N, sample size; FIS, inbreeding coefficient. G) Pairwise comparison matrix amongst localities; upper triangle, Weir & Cockerham fixation index (FST, greyscale); asterisks mark pairs whose bootstrap 95% CI excludes zero; lower triangle, number of shared alleles between localities.

between Charcos de Luis and the lava tube sites (Tab. 1; Fig. 1G). In contrast, pairwise comparisons among Lagos, Jameos, and Túnel de la Atlántida were strongly centred on zero. Both PCA and DAPC consistently separate individuals from Charcos de Luis from those collected within La Corona lava-tube sites, whereas no internal structure is visible among the cave localities (*Supplementary Analyses, Fig. S1*). Given the small and uneven sample sizes (n=3-11), these results should be interpreted qualitatively rather than quantitatively. Nonetheless, all metrics consistently support a marked differentiation between Charcos de Luis and the lava-tube localities.

Discussion

Our results fit the expectations for two candidate management units (sensu Palsbøll *et al*., 2007), pending genomic confirmation: i) La Corona lava tube (Cueva de los Lagos, Túnel de la Atlántida, Jameos del Agua) and ii) Charcos de Luis. Because our dataset is based on small and uneven sample sizes, this classification must remain provisional; however, the signal of differentiation is repeated across all analyses, although its magnitude must be interpreted carefully given sample size. This decision follows the precautionary principle and relies on the best available scientific evidence available today. Yet, given the critically endangered status of the species, our findings underline the urgency of denser sampling and production of genomic data. Collecting additional individuals at Charcos de Luis remains exceptionally difficult. During 18 targeted visits in 2024, only three encounters were recorded, and in all cases the animals retreated into narrow fissures before they could be captured. This practical constraint explains the persistent small sample size from this site. We did not use traps as they may compromise other endemic fauna.

Within the lava tube, the high proportion of shared alleles among Cueva de los Lagos, Túnel de la Atlántida, and Jameos del Agua is compatible with a single genetic population subdivided into local subpopulations. This pattern aligns with previous work grouping Túnel de la Atlántida and Jameos del Agua into the same population (Cabezas *et al*., 2012). By contrast, Charcos de Luis exhibits a high proportion of private alleles despite our small sample (n=3), supporting at least partial genetic isolation, and potentially representing a second population. Even with modest sample sizes, our analyses consistently recover two populations; expanding sampling with more specimens, more localities and genome-scale markers will refine their boundaries, reveal finer structure, and yield sharper estimates of connectivity and effective size.

We propose two non-exclusive mechanisms to explain this pattern. Firstly, limited hydrological connectivity along Malpaís de la Corona might restrict northward dispersal from La Corona lava tube, promoting the isolation of Charcos de Luis (Tomasi *et al*., 2023). Second, the distribution of trophic resources and the behaviour of *M. polymorpha* may promote the aggregation and permanence of individuals where food resources are consistently available (e.g. Los Jameos del Agua, entrance of Túnel de la Atlántida). In those cave sections, persistent resources can intensify local intraspecific competition and space use amongst individuals of *M. polymorpha*, potentially limiting successful arrivals from elsewhere and, over time, reducing genetic diversity in those patches. Because these sites occur within the cave system, they are isolated from the arrival of external predators and competitors, further favouring the persistence of resident individuals (but see Brito *et al*. 2009). This is consistent with previously inferred low effective sizes (Cabezas *et al*., 2012). Charcos de Luis is also comparatively rich in resources but, unlike the cave sites, there are predators like fish such as the Madeira goby *Mauligobius maderensis* (Valenciennes, 1837) and the European eel *Anguilla anguilla* (Linnaeus, 1758) (Martínez *et al*., 2016), and competitors (e.g., the crab *Xantho* sp. and the shrimp *Palaemon* sp.), which may hinder persistence of immigrants and contribute to its distinct allele composition due to the constant arrival of new individuals from different areas. These mechanisms remain hypothetical and require targeted ecological and hydrological studies.

The importance of a reference genome in the future conservation efforts of *Munidopsis polymorpha*

A microsatellite snapshot hints at two management units, but taking robust conservation decisions will largely benefit from the availability of genomic data. A chromosome-level reference genome for *M. polymorpha* would: i) yield thousands of single nucleotide polymorphisms (SNPs) to estimate effective population size through time, detect bottlenecks, and quantify fine scale gene flow among sites; ii) allow to test for local adaptation and assess inbreeding risk via runs of homozygosity and deleterious load; iii) anchor functional hypothesis (e.g., RNA-seq) to compare gene expression under key stressors and identi-

Tab. 1. Site-level genetic summary. Full quality control and pairwise results are provided in Tab. S3.

Locality	n	AR	Ho/He	FIS	Private alleles	Call rate mean	HWE tested	LD sig pairs n
Cueva de los Lagos	7	1.58	0.32/0.30	-0.07	2	100	3	0
Túnel de la Atlántida	5	1.57	0.33/0.27	-0.18	3	97.5	3	0
Jameos del Agua	11	1.39	0.18/0.21	0.09	1	75	2	0
Charcos de Luis	3	2.2	0.48/0.63	0.15	9	83.3	0	0

n, individuals genotyped; AR, allelic richness rarefied to the smallest site sample size (n=3); Ho/He, observed/expected heterozygosity (means across loci); FIS, inbreeding coefficient (Weir & Cockerham); private alleles, alleles unique to a site across all loci; call rate mean, mean % of individuals successfully genotyped per locus; HWE tested, number of locus × site combinations with ≥5 genotypes that were tested for Hardy-Weinberg equilibrium; LD sig pairs n, number of within-site locus pairs significant for linkage disequilibrium after Benjamini-Hochberg correction (q<0.05); FST vs Charcos (95% CI), Weir-Cockerham FST between each lava tube site and Charcos de Luis with 95% CIs from 1,000 bootstrap replicates over loci ("–" for Charcos). Many HWE/LD contrasts were unassessable due to small sample sizes.

fy genomic and morphological traits linked to adaptation to subterranean environments; and v) implement low-impact monitoring programs by enabling compact SNP panels and specific eDNA assays for routine, non-invasive surveys. A high-quality genome would also become a scaffold for other squat lobsters, allowing for comparative genomic work that might illuminate the similarities and differences between cave and deep-sea squat lobsters.

The importance of Charcos de Luis and other potential anchialine pool complexes in conservation

Our findings reinforce the conservation value of anchialine pools within karst and volcanic systems. Such pools can subsidize the coastal aquifer with organic matter, via both *in situ* production and marine inputs, thereby providing additional food resources for fauna otherwise confined to nutrient-poor groundwater (Pohlman, 2011). This role is exemplified at Charcos de Luis, where four out of the 22 recorded species are otherwise known from La Corona lava tube: the squat lobster *Munidopsis polymorpha,* the amphipod *Parhyale multispinosa* Stock, 1987*,* the mysid shrimp *Heteromysis cotti* Calman, 1932*,* and the calanoid copepod *Stygocyclopia balearica* Jaume & Boxshall, 1995 (Wilkens *et al*., 1993). Charcos de Luis management unit includes up to three pools, but due to their hydrological connectivity, it is considered a single management unit. It should cover a central area including the pools and a buffer area of at least 50 meters radius.

Despite this biological importance, anchialine pools, such as Charcos de Luis, are highly exposed to anthropogenic impacts. Human access is unrestricted and litter is common (Martínez *et a*l. 2016), indicating recurrent disturbance by passers-by. These conditions pose obvious threats to the fragile, groundwater-dependent populations that utilize these pond-like habitats. This situation would be improved by implementing conservation measures framed in a management plan, such as access management and physical protection to the anchialine pools (e.g., by the installation and management of low-impact barriers), monitoring of abiotic and biotic parameters (both compositional, structural and functional biotic variables and limnological parameters) and deeper research of the system. Furthermore, immediate housekeeping (trash removal) is highly desired. This might be combined with site-based designation of protection figures that safeguards both hydrological and ecological integrity of those pools.

Data sharing

The per-individual allele table and raw electropherograms are available at github.com/amartinezgarcia/Munidopsis_microsatelites

Acknowledgements

This study was carried out within the project “Elaboración del Plan de Manejo de las especies amenazadas *Munidopsis polymorpha* y *Morlockia ondinae*,” co-funded by the Government of the Canary Islands and the European Regional Development Fund (ERDF–FEDER) through the Canary Islands Operational Programme 2014–2020. We thank the Cabildo de Lanzarote and the Centros de Arte, Cultura y Turismo (CACT) for their support. We are especially grateful to the staff at Jameos del Agua, particularly Suso Fontes and Alexis Guadalupe, for facilitating access and fieldwork at the site.

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Online supplementary material:

Supplementary Analyses

Fig. S1. Exploratory multivariate analyses of microsatellite genotypes in Munidopsis polymorpha.

Tab. S1. Summary of microsatellite genotypes for Munidopsis polymorpha.

Tab. S2. Summary of the primers used for the amplification of the eight microsatellites used in the study of Munidopsis polymorpha.

Tab. S3. Genotyping quality control by site x locus.

Tab. S4. Site-level genetic summary, comparing the full 8-loci dataset with the filtered 5-loci dataset.

Received: 7 October 2025; Accepted: 6 December 2025.

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Citation: Martínez A, García-Gómez G, García-Herrero A, *et al.* Evidence for an additional population of the cave-dwelling squat lobster Munidopsis polymorpha in Lanzarote (Canary Islands): implications for management and conservation. *J Limnol 2026;85:2248*.

Contributions: Rosa Fernández, Leopoldo Moro: share contribution. Alejandro Martínez, Guillermo García-Gómez, Alvaro García-Herrero, Enrique Domínguez, Carola D. Jorge, Joaquín Vierna, Neus Mari-Mena, Antón Vizcaíno, Elena Mateo, David Brankovits, Ester M. Eckert, Diego Fontaneto: conceptualization, methodology, formal analysis, Writing – original draft, review and editing.