Root microbiome along an altitude gradient of the lithophytic *Ramonda heldreichii*, an endemo-relict species of Mount Olympus

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1   **Abstract**

2   We investigated the root microbiome of an endemo-relict plant species of Mount Olympus,

3   *Ramonda (Jankaea) heldreichii* (Boiss.) C.B.Clarke, at various altitudes (400m-1200m asl),

4   through amplicon sequencing. Microbial communities (prokaryotes, fungi, protists) revealed

5   the significant impact of roots on the tight rhizosphere (TR) that were less diverse and less

6   altitude-impacted compared with the loose rhizosphere (LR). Prokaryotic α-diversity was

7   highly affected by root, whereas that of fungi was comparatively more sensitive to altitude.

8   The TR-associated taxonomic groups, included well equipped taxa for tolerating biotic and

9   abiotic stresses (drought/metal tolerance, microcystin degradation, psychrotolerance, chitin

10   degradation), while the LR-associated taxa mainly included microorganisms with

11   chemolithoautotrophic potential. Relative abundances of the N-cycling and greenhouse gas

12   associated *Nitrosopheraceae*, were increased with altitude. We explored the root microbiome

13   of the multi-million year old Jankaea species, and took a step towards understanding mountain

14   microbiology, climate change, and bioprospecting, straight from the steep inclines of Mount

15   Olympus.

16   **Keywords:** Mountain ecosystem; Mount Olympus; Microbial diversity; Altitude; Jankaea

17

18 **Main text**

19 Mount Olympus, Greece, is a pristine mountain environment retaining endemic plant species

20 with interesting ecological profiles and climate adaptation modes (Zindros et al., 2020). Here,

21 the Mount Olympus endemic *Ramonda heldreichii* (Boiss.) C.B.Clarke (previously known as

22 *Jankaea heldreichii* Boiss.; hereafter named Jankaea; Figure 1A) of the *Gesneriaceae* plants

23 (Turland et al., 2013), was studied for altitude-related rhizospheric microbial diversity

24 differences. *Gesneriaceae* are lithophytic, growing on oligotrophic damp cracks/crevices of

25 limestone rocks. They have branched and superficial root systems, and tolerate large

26 temperature, humidity, and irradiation fluctuations (Legardón and García-Plazaola, 2023).

27 Jankaea is considered an endemo-relict species (extant throughout most Cenozoic era and

28 associated climate shifts) and a resurrection plant, with unique desiccation/freeze tolerance

29 mechanisms, allowing it to recover from as low as 10% relative water content and very low

30 temperatures (Stevanović and Glišić, 1997). For this reason it has a broad altitude-wise

31 distribution, between 100 m and 2400 m asl, at the Olympus Mountain. It is, therefore, an ideal

32 plant for studying the root harboured microbial diversity under altitude/temperature/drought

33 gradients.

34

35 Jankaea rhizosphere-soil samples were collected from three altitudes/locations of Mnt.

36 Olympos (1.low altitude: Litochoro ~400m; 2.middle altitude: Agios Dionysios ~ 800m; 3.high

37 altitude: Prionia ~1200m) in late June 2023. Soil shaken off the root was considered as loosely

38 associated (LR), whereas soil not possible to shake off, but instead was washed off the root

39 system with sterile distilled water, was considered as tightly attached (TR). Samples were

40 DNA-extracted and analysed for microbial phylogenetic marker diversity (16S rRNA gene,

41 fungal ITS2, and the eukaryotic 18S rRNA gene focusing on protists) *via* amplicon sequencing

42   using standard in-house methods (SI). For protists, low sequence read numbers of some

43   samples led to the reporting only of statistical tests with sufficient power. The sequencing data

44   are available at the National Center for Biotechnology Information (NCBI) with the accession

45   number PRJNA1144380.

46

47   Analysis of the α-diversity revealed distinct patterns between TR and LR samples, with the TR

48   having reduced index values compared with the LR in nearly all cases (Figure 1B-D). Two-

49   way ANOVA showed that the environment type (TR *vs* LR) was the primary factor affecting

50   α-diversity. Location/altitude affected the fungal and eukaryotic α-diversity to a greater extent

51   than that of prokaryotes.

52

53   In β-diversity analysis, the environment type (TR *vs* LR) was the most important factor for

54   prokaryotic communities (R^2 of 28% out of the model total 42.4%, with P 0.001; Figure S1).

55   For fungi, location/altitude was most important (R^2 of 24.6% out of 51.29%, P 0.001) with the

56   environment type being significant, yet, of less importance. Principal coordinates analysis

57   (PCoA) indicated that LR were more different than the TR between locations/altitudes for

58   bacteria and fungi (Figure 2 B&E *vs* A&D). This was further backed by the performed β-

59   dispersion permutation tests (Figure 2 and Figure S2) that showed significant dispersion

60   differences of the sample dissimilarities from the location centroids in the case of the TR

61   samples and non-significant in the case of the SR samples, suggesting that the TR associated

62   significant PERMANOVA differences are probably dispersion artifacts.

63

64   Principal response curves performance for prokaryotes indicated TR-associated taxa,

65   including: *Steroidobacter*, reported to degrade toxic compounds and steroids, and tolerate

66 metals/metaloids (Fahrbach et al., 2008; Gong et al., 2016; Rahman et al., 2023);

67 *Phytohabitans*, with isolates from orchid plants (Inahashi et al., 2010); *Lysinimonas* tolerating

68 psychrophilic alpine environments (Donhauser et al., 2020); and the *Niastella*, containing

69 chitin degraders isolated from Korean ginseng soil (Weon et al., 2006) (Figure S3 A&B). LR-

70 associated taxa included several chemolithoautotophs (Clark et al., 2021) like *Nitrospira*,

71 *Chloroflexi,* and Planctomycetes, along with *Myxococci*, encompassing competitive biocontrol

72 agents (Bull et al., 2002). Comparison between the locations for LR revealed the significant (P

73 ≤ 0.05) increase along altitude increase (connected with temperature decrease) in the ammonia

74 oxidizing archaeal (AOA) *Nitrososphaeraceae* family (Figure S4A; Table S3). Despite a rough

75 census with previous results where low temperatures positively affected AOA in constructed

76 wetlands and sediments (along with an observed intensification by 90 % of N_2O production)

77 (Zeng et al., 2014; Jiang et al., 2023), other studies showed soil-specific and pH related

78 responses (Gubry-Rangin et al., 2017). However, any temperature change seems to affect the

79 balance of these nitrogen cycling guilds and greenhouse gas emitters. *Vicinamibacterales* and

80 *Microlunatus* as principal microbial groups in LR, were significantly ($P \leq 0.05$) affected by

81 location/altitude (Figure S3B&C; Table S3), possibly involved actively in nutrient flux from

82 rock to soil as they are known for their phosphate associated metabolic activity (Wu et al.,

83 2021; Zhao et al., 2024). *Gaiellales* had higher abundance ($P \leq 0.001$) at higher altitudes

84 (2.middle/3.high) in comparison with the lower altitude (1.low) in LR (Figure S4). Similar

85 observations were recorded by Zhang et al. (2022) under their altitudinal study performed in

86 China.

87

88 Fungi, on the other hand, included mostly Ascomycetes in TR, and to a lesser extend

89 Basidiomycetes (Figure S5). *Agaricomycetes*, *Dothideomycetes*, *Singerocomus* and

90 *Tomentella* were among the most abundant classes in both LR and TR. *Tomentella* were

91 previously found in high relative abundances at *Ramonda* (close relatives of *Jankaea*) plant

92 rhizosphere (Lozo et al., 2023). The differential responses of *Singerocomus* and *Tomentella*

93 towards altitude in LR (increase *vs* decrease respectively) probably suggests ectomycorrhizal

94 shifts (Figure S6 A&B; Table S3) towards functional redundancy-based homeostasis regarding

95 carbon fluxes. In both prokaryotes and fungi, the microbial community distances between TR

96 and LR were lower at the 1.low samples compared with the 2.middle/3.high (Figure S3A/S6A).

97

98 Core microbiome analysis of TR soils revealed *Planctomycetes*, *Myxococci*, *Chloroflexi*,

99 *Proteobacteria*, and *Actinobacteria* as dominant and prevalent (≥ 0.1 % relative abundance and

100 $\geq 90\%$ prevalence; Figure 2C). Fungi were dominated by Ascomycetes (Figure 2F), and protists

101 by *Cercozoa* (Figure S7).

102

103 Jankaea rhizosphere soil microbiome analysis revealed that TR dwellers were largely atypical

104 and well equipped for biotic/abiotic stresses. Furthermore, a microbially-driven nutrient flux

105 enhancement or maintenance, compared with those of temperate soils, including autotrophs

106 and mixotrophs, was implied. Notably, when compared with closely affiliate *Ramonda* plant

107 species (Lozo et al., 2023), the overall root-microbiome profile shows remarkable similarities.

108 Jankea and the other species of the genus *Ramonda* (*R. serbica*, *R. nathaliae*, and *R. myconi*),

109 are the European representatives of the tropical-subtropical family *Gesneriaeceae* and are

110 among the few flowering desiccation tolerant plants of the Northern Hemisphere. In the sparse

111 existing literature, *Ramonda* rhizospheres (Praeg et al., 2019) were identified as sources of

112 plant growth promoting microbial isolates that protected wheat plants from drought stress.

113 Overall, our findings show a plant-specific and trait-wise unique Jankaea microbiome,

114 designed to support growth under harsh climatic and nutritional conditions. This is an

115 exploratory study to investigate microbiota of the resurrection plant *R. heldreichii* at various

116 altitudes, as a first step towards gaining insights about this highly interesting angiosperm

117 residing at the steep inclines of Mount Olympus.

118

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Legends to Figures

Figure 1 (A) Photo of *R. heldreichii* (Jankaea) at Mnt. Olympus. Boxplots (left panels) and interaction plots (showing the sample means per group; right panels) summarize the Shannon index values of the various environment types (LR *vs* TR for the loose soil and plant root tightly associated respectively) and locations (low, middle, high elevation) for the datasets of prokaryotes (B), fungi (C), and eukaryotes (D). Two-way ANOVA model variance and its partitioning is provided for each dataset above its corresponding interaction plot with the model *P*-values being indicated by the stars (* for $P \le 0.05$; ** for $P \le 0.01$; *** for $P \le 0.001$)

Figure 2 Principal coordinates analysis (PCoA) scatter plots for Bray-Curtis dissimilarities. Soil samples under the influence of three different locations (low, middle & high elevation) indicating the significant effect of roots on soil microbial community structure. Tightly attached root soil samples (A & D) have less different structures than loosely associated soils (B & E) for both the prokaryotic (A & B) and fungal (D & E) markers as also depicted by PERMANOVA and β-dispersion analysis (above each panel). Core microbiomes of the TR samples: heatmaps showing the various detection threshold levels in the x-axis (minimum relative abundances in samples) and colour-coded according to the taxon prevalence among samples for the corresponding detection threshold, for prokaryotes (C) and fungi (F).