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New record of *Russula juniperina* (Russulaceae, Basidiomycota) from Turkey evidenced by morphological characters and phylogenetic analysis

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Abstract

Russula is one of the most speciose genera among the agarics, but phylogenetic relationships among species and subgeneric groupings are poorly understood. Among them *Russula juniperina* is reported for the first time from Turkey based on morphology and molecular phylogeny. Comprehensive description of the morphological characters, macro and microphotographs of this poorly known species are provided, and comparison with phenetically similar and phylogenetically related species are discussed. The pileipellis structure in this species indicates its placement within the cuprea/juniperina clade of sect. *Russula* subsect. *Maculatina.* Our phylogenetic analysis shows that *R. juniperina* is phylogenetically closely related to, but distinct from *R. adulterina* and *R. cuprea* based on the ITS dataset. The lowest sequence divergence among in-group taxa was observed between *R. juniperina* and *R. adulterina* 0.006%, the next was between *R. juniperina* and *R. cuprea* 0.015%. The highest sequence divergence with in-group taxa was found between *R. badia* and *R. font-queri*, 0.134%. Thus, it was determined that genetically, *R. juniperina* was closer to *R. adulterina* than to *R. cuprea*.

Key words – new record – phylogeny – rare species – taxonomy – Turkish mycobiota

Introduction

The genus *Russula* Pers. was first named by Persoon (1796), in his work on northern European species. Since that time, many researchers, both experts and amateurs, have worked on this genus, particularly in Europe, USA and Africa (Romagnesi 1996, Sarnari 1998, Miller & Buyck 2002, Sarnari 2005). Recently, new taxa belonging to this genus have been reported from Asia, principally from India and China (Wang et al. 2009, Li et al. 2011, 2012, 2013a, 2013b, 2015a, 2015b, 2018, Dutta et al. 2015, Zhao et al. 2015, Zhang et al. 2017, Paloi et al. 2018, Song et al. 2018). As a result of this nearly three centuries of study, more than 750 taxa of *Russula* have been identified, and this number continues to increase with new studies (Kirk et al. 2008, Buyck & Atri 2011, Paloi et al. 2018).

Some *Russula* taxa can be easily distinguished from one another. However, biotic and abiotic factors cause great colour variation, and the large number of morphologically very similar species

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and also the difficulty of determining some microscopic structures make the identification of species difficult, conducting to frequent misidentification. This and similar situations are now being solved by detailed morphological and molecular studies (Adamčík et al. 2016, Çolak & Işıloğlu 2016).

The aim of this study was to determine, based on both morphological and molecular data, the taxonomic position of *Russula juniperina*, which according to some sources is a synonym of *R. cuprea* (Bon 1988, www.indexfungorum.org) and also to compare it with phenetically similar and phylogenetically close species. *R. juniperina* is reported for the first time from Turkey by this study.

Materials & Methods

Morphological studies

The *Russula* specimens used in the study were collected during field trips in autumn 2014 from Afyonkarahisar province in inner west region of Turkey. Firstly, photographs were taken and the colour changes by the application of various chemicals of the specimens are noted (Largent et al. 1977, Galli 1996, Socha et al. 2011). In addition, the morphological and ecological features were recorded along with the date and numbers. The macroscopical description and images of the basidioma were obtained by observation of fresh and dried specimens. For microscopical analyses, the dried materials were rehydrated in 5% KOH, and subsequently stained with Melzer's solution. The following abbreviations are used in the descriptions: Lm for the average length of all the measured basidiospores, Wm for the average width of all the measured basidiospores, Q for the quotient of length and width of all the measured basidiospores, and Qm for the average of all calculated Q values for all basidiospores measured. At least thirty mature basidiospores from basidioma were measured. The cuticle structure was examined by taking sections from the skin of the cap. In determining the parts of the cuticle, Congo Red were used (Largent et al. 1977, Galli 1996). Identification of the samples was conducted according to Galli (1996), Sarnari (1998) and García (2011). The dried samples are kept at the fungarium of Süleyman Demirel University (GUL), Isparta. The names of taxon and author are quoted according to MycoBank (www.mycobank.org).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from 1 mg of dried specimen using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo research, Irvine, CA, USA). Universal primers ITS5m/ITS4 were used for the ITS region amplification (White et al. 1990). All PCR products were sequenced in Sanger DNA sequencing service (Source Bioscience, Berlin, Germany), with the same primers used in the PCR reactions. Sequence assembly and editing were carried out using ClustalX (Thompson et al. 1997) and MEGA 7 (Kumar et al. 2016). The newly generated sequences are deposited in GenBank (www.ncbi.nlm.nih.gov) under the accession numbers (MH999871).

Sequence alignment and phylogenetic analyses

The sequences obtained in this study were combined with published *Russula* ITS rDNA sequences selected from GenBank and Unite (http://unite.ut.ee) databases on the basis of the greatest similarity based on BLAST search, outcomes of recent phylogenetic studies focused on *Russula* (Adamčík et al. 2016). The sequence accession numbers in the analysis are provided in Table 1. All sequence data were performed using the G-INS-I option implemented in the MAFFT version 7.110 (Katoh & Standley 2013). In addition, final alignments were manually corrected via MEGA 7 (Kumar et al. 2016). In both Maximum Likelihood (ML) and Bayesian Inference (BI) analyses, *Russula delica* (KX094989) was used as the outgroup taxon.

Phylogenetic tree inference was performed for the ITS dataset by both ML and BI methods. For the ML analysis, the best-fit models of sequence evolution were determined as Bayesian

information criterion (BIC) and Akaike information criterion (AICc) with the help of MEGA version 7. Bootstrap analysis with one-thousand replicates was used to test the statistical support of the branches. ML analysis was performed based on the K2+G (Kimura 2-parameter) model (Kumar et al. 2016) where the initial tree for the heuristic search was obtained by applying the Neighbour-Joining and BioNJ method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G= 0.1989)). BI was calculated using Markov Chain Monte Carlo (MCMC) methods with MrBayes version 3.2.6 (Ronquist et al. 2012). The general time reversible (GTR) model was employed with gamma-distributed substitution rates. Markov chains were run for 10^7 generations, saving a tree every 1000^{th} generation, with two runs per analysis. The initial 25% trees recovered were excluded as a burn-in, and a 50% majority consensus tree of the remaining trees was then used to calculate the posterior probabilities (PP) of the group. Only Bayesian posterior probability (BPP) values ≥0.95 and Maximum likelihood bootstrap (MLB) values of ≥60 were reported in the resulting tree (Fig. 1). Branch lengths were estimated as mean values over the sampled trees.

Table 1 Specimens used in molecular phylogenetic studies and their GenBank and Unite accession numbers for ITS. Accession number in boldface indicate newly generated sequence.

Taxon	**nrITS**	**Geographic origin**	**References**
R. adulterina	AY061651	Europe	Miller & Buyck 2002
R. aurantioflammans	KU928167	–	Adamčík et al. 2016
R. badia	MG679813	Czech Republic	Unpublished
R. cuprea	KT934010	Germany	Looney et al. 2016
R. cuprea	UDB002420	United Kingdom	Unpublished
R. cuprea	KU886592	Germany	Adamčík et al. 2016
R. cuprea	KU886591	Sweden	Adamčík et al. 2016
R. decipiens	KU928140	Spain	Adamčík et al. 2016
R. decolorans	AF418637	Germany	Eberhardt 2002
R. decolorans	UDB000332	Germany	Unpublished
R. delica	KX094989	China	Unpublished
R. dryadicola	KU928141	Italy	Adamčík et al. 2016
R. firmula	KJ867372	China	Cao et al. 2014
R. font-queri	KU949378	–	Adamčík et al. 2016
R. globispora	KU928144	Germany	Adamčík et al. 2016
R. heilongjiangensis	MG719932	China	Li et al. 2018
R. integra	MG687326	Czech Republic	Unpublished
R. intermedia	UDB019766	Estonia	Unpublished
R. juniperina	**MH999871**	**Turkey**	**Present paper**
R. juniperina	KU886596	Spain	Adamčík et al. 2016
R. juniperina	MF716847	Spain	Unpublished
R. juniperina	KU928148	Germany	Adamčík et al. 2016
R. juniperina	KU928149	–	Adamčík et al. 2016
R. maculata	KU928156	Germany	Adamčík et al. 2016
R. nympharum	KU928159	France	Adamčík et al. 2016
R. quercilicis	KY613996	Spain	Caboň et al. 2017
R. rubra	KU928161	Sweden	Adamčík et al. 2016
R. rutila	KY582688	Slovakia	Caboň et al. 2017

Table 1 Continued.

Taxon	nrITS	Geographic origin	References
R. seperina	MG687341	Czech Republic	Unpublished
R. veternosa	KU928165	Belgium	Adamčík et al. 2016
R. vinososordida	KX813545	USA	Bazzicalupo et al. 2017

The phylogenetic distances were computed using the Kimura-2-parameter (K2P) method which is used to calculate sequence divergences between and within species and is expressed as numbers of base substitutions per site (Kimura 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 23 selected nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 337 positions in the final dataset. Evolutionary divergence analyses based on the ITS sequence set were calculated using MEGA v.7.0.25 (Kumar et al. 2016).

Results

Molecular phylogeny

Taking the ITS data set as a base, our phylogenetic analysis contains a total of 31 sequences (including 27 from GenBank and 3 from Unite). The final dataset included the species examined (*R. juniperina*) and the alignment contained 948 nucleotide sites (including gaps), of which 613 characters are constant, 153 parsimony-uninformative and 182 parsimony-informative. The resulting phylogram with the highest log likelihood value (-1473.63) is represented in the present manuscript. We selected the topology resulting from the first iteration to present here (Fig. 1, *-InL* = 1443.834). The phylogram obtained using Bayesian (MCMC) analyses showing the Bayesian posterior probability (BPP) displayed similar topology to the phylogram obtained using Maximum likelihood (ML) analyses in MEGA7; therefore, only the ML phylogenetic tree with both Bayesian posterior probability (BPP) and Maximum likelihood bootstrap (MLB) values has been indicated in Fig. 1. Accession numbers of newly generated nrITS sequence, as well as other GenBank and Unite sequences used for conducting the phylogenetic analysis, are presented in Fig 1.

According to our molecular analysis results (Fig. 1) with one sequenced collection of *R. juniperina* from Turkey and four from Germany and Spain, it grouped monophyletically with *R. adulterina* and *R. cuprea* as a separate lineage within the *Russula* subsect. *Maculatinae*, forming a well-supported branch (MLB=100%, BPP=1.0). Also, it was determined that genetically, *R. juniperina* was closer to *R. adulterina* than to *R. cuprea*.

The genetic divergence matrix of the 22 taxa studied depending on ITS sequences was estimated according to the KP2 formula (Table 2). The lowest sequence divergence within in-group taxa, 0.006%, was between *R. juniperina* and *R. adulterina*, the next was 0.015% between *R. juniperina* and *R. cuprea* in the ITS dataset. The highest sequence divergence within in-group taxa, 0.134%, was between *R. badia* and *R. font-queri*. This also confirms that, as the phylogenetic tree shows, these taxa are members of different species (Fig. 1).

Taxonomy

Russula juniperina Ubaldi, Micologia Italiana 14 (3): 25 (1985) Figs 2–5

Facesoffungi number: FoF 06142

Description – Pileus 5–8 cm in diam., at first convex, later flattening, surface smooth, sticky when wet, colour bright blood-red, occasionally orange-red, centre generally yellowish-cream, sometimes with whitish areas on the surface. Peeling 1/3. Lamellae crowded, sometimes forked, widening towards the edge of the cap. Colour at first white-ivory cream at first, later creamish-yellow. Stipe 3.5–6 × 1–2 cm, white, interior spongy, cylindrical, clavate in form. Taste definitely

acrid. Odour odourless or with a very weak fruity-geranium smell. Macrochemical reactions: $FeSO_4$ light pinkish-orange, Guaiac quickly gives a positive result. KOH and ammoniac nil. Spore-print golden to dark yellow in colour.

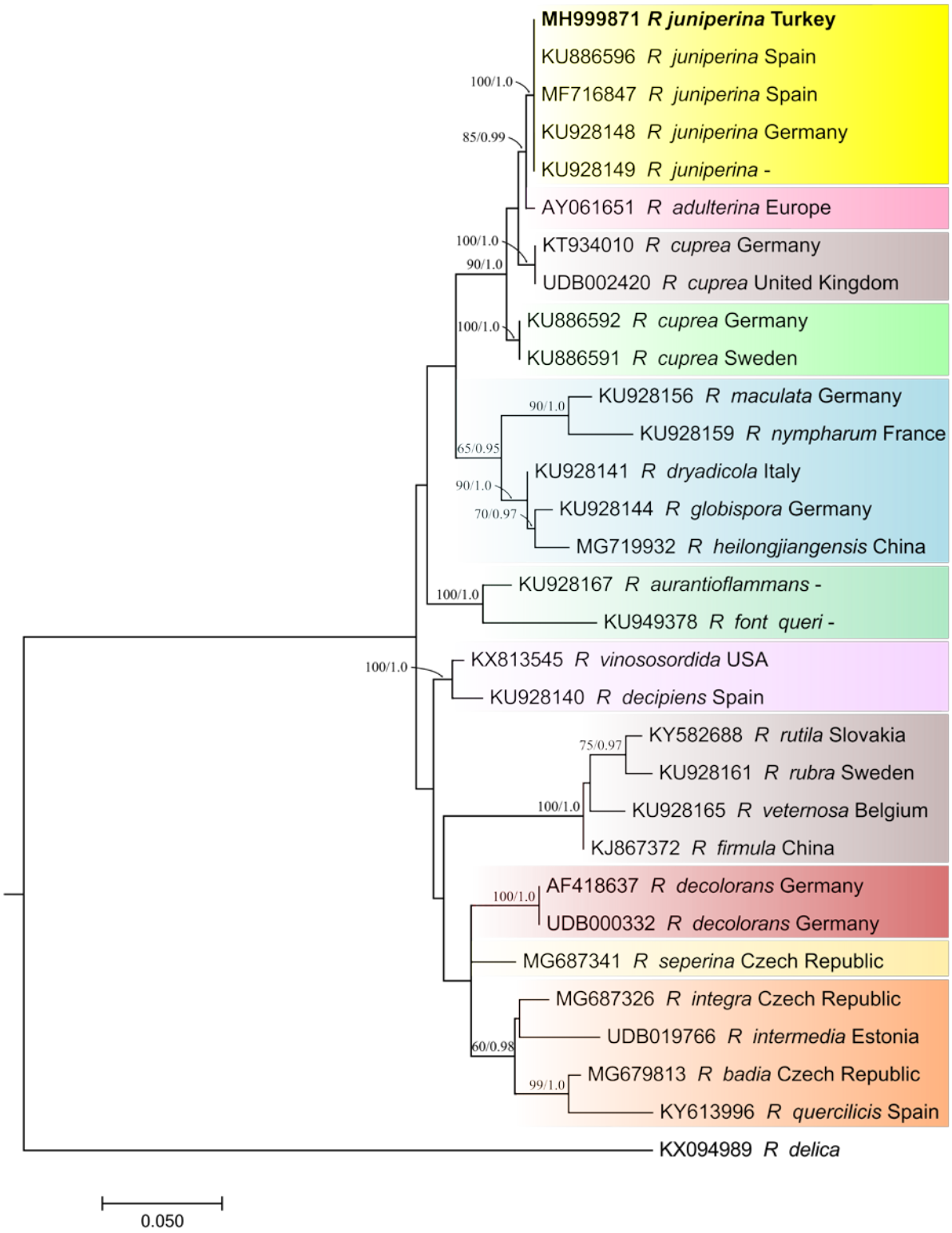

Fig. 1 – Phylogenetic relationships of Turkey collection of *Russula juniperina* with other closely related species based on Maximum likelihood analysis of ITS-rDNA sequences. Statistical support values of Maximum likelihood bootstrap values (≥60%) and Bayesian posterior probabilities (≥0.95) are shown at each node. The new sequence obtained in this work are marked with bold color. The analysis involved 31 sequences, with *Russula delica* as the out-group.

Table 2 Genetic divergence matrix among *Russula* taxa based on ITS sequences data calculated using the formula of Kimura-2 parameter method.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	-																					
2	0.006	-																				
3	0.015	0.015	-																			
4	0.073	0.073	0.069	-																		
5	0.077	0.076	0.073	0.031	-																	
6	0.048	0.048	0.051	0.045	0.059	-																
7	0.059	0.059	0.062	0.055	0.070	0.009	-															
8	0.066	0.066	0.070	0.055	0.070	0.015	0.018	-														
9	0.070	0.069	0.066	0.090	0.097	0.071	0.075	0.083	-													
10	0.088	0.088	0.084	0.093	0.101	0.089	0.093	0.093	0.048	-												
11	0.051	0.051	0.048	0.062	0.077	0.055	0.059	0.067	0.052	0.077	-											
12	0.065	0.065	0.061	0.069	0.080	0.055	0.059	0.066	0.066	0.091	0.025	-										
13	0.066	0.066	0.058	0.081	0.088	0.062	0.066	0.074	0.081	0.092	0.052	0.052	-									
14	0.070	0.069	0.066	0.077	0.084	0.066	0.070	0.078	0.077	0.088	0.056	0.062	0.015	-								
15	0.081	0.081	0.073	0.089	0.088	0.070	0.074	0.082	0.073	0.092	0.059	0.059	0.032	0.035	-							
16	0.062	0.062	0.055	0.073	0.073	0.052	0.055	0.062	0.070	0.080	0.049	0.048	0.022	0.025	0.015	-						
17	0.045	0.044	0.041	0.065	0.084	0.055	0.059	0.066	0.066	0.091	0.028	0.048	0.056	0.059	0.063	0.052	-					
18	0.085	0.084	0.080	0.092	0.117	0.077	0.081	0.089	0.081	0.108	0.052	0.059	0.071	0.083	0.075	0.071	0.052	-				
19	0.055	0.062	0.066	0.077	0.084	0.055	0.066	0.074	0.073	0.108	0.052	0.059	0.075	0.071	0.067	0.064	0.052	0.059	-			
20	0.089	0.089	0.093	0.062	0.085	0.045	0.049	0.063	0.094	0.132	0.074	0.073	0.089	0.093	0.081	0.085	0.077	0.081	0.044	-		
21	0.091	0.099	0.107	0.095	0.107	0.088	0.100	0.108	0.112	0.134	0.077	0.073	0.101	0.106	0.093	0.089	0.077	0.066	0.048	0.076	-	
22	0.093	0.100	0.105	0.105	0.113	0.081	0.093	0.101	0.101	0.130	0.070	0.077	0.103	0.108	0.087	0.091	0.074	0.067	0.049	0.077	0.028	-

1- *R. juniperina* (MH999871), 2- *R. adulterina* (AY061651), 3- *R. cuprea* (KT934010), 4- *R. maculata* (KU928156), 5- *R. nympharum* (KU928159), 6- *R. dryadicola* (KU928141), 7- *R. globispora* (KU928144), 8- *R. heilongjiangensis* (MG719932), 9- *R. aurantioflammans* (KU928167), 10- *R. font-queri* (KU949378), 11- *R. vinososordida* (KX813545), 12- *R. decipiens* (KU928140), 13- *R. rutila* (KY582688), 14- *R. rubra* (KU928161), 15- *R. veternosa* (KU928165), 16- *R. firmula* (KJ867372), 17- *R. decolorans* (AF418637), 18- *R. seperina* (MG687341), 19- *R. integra* (MG687326), 20- *R. intermedia* (UDB019766), 21- *R. badia* (MG679813), 22- *R. quercilicis* (KY613996).

Basidiospores 8–10.5(–11) × 7–9 μm, Lm × Wm = 9.5 × 8 μm, Q = (1.1–)1.13 × 1.2(–1.3) μm, Qm = 1.2 μm, globose, warts conical in structure extending to 0.8 μm, surface not reticulate. Basidia 50–70 × 10–14 μm, clavate, with 4 sterigmata. Hymenial cystidia 14–17.5 μm in broad, fusiform in shape, ends pointed, thin-walled. Pileipellis composed of epicutis, apical cells of pileipellis 2–4 μm broad, branched hairs, somewhat clavate, often showing diverticulae; dermatocystidia numerous, approximately 4–10 μm in broad, mostly 1-many septate, claviform, sometimes with diverticulae.

Trama composed of connective hyphae 3-6 µm diam and hyaline. Stipitipellis composed of parallel hyphae, up to 5 µm broad, caulocystidia absent. Clamp connections absent in all tissues.

Ecology – It grows in *Quercus* sp. and *Juniperus oxycedrus* L. forest in autumn. Inedible (Galli 1996).

Specimens examined – TURKEY, Afyonkarahisar Province, Sultandağı district, Dereçine village, 19 October 2014, leg. and det. Ö.F. Çolak (ÖFÇ 928 in GUL).

Fig. 2 – *Russula juniperina*. a–d Basidiocarp. e Peeling.

Discussion

Detailed descriptions of *Russula juniperina* have been provided in previous studies (Galli 1996, Sarnari 1998, Pidlich-Aigner 2008, García 2011, Mua et al. 2017). A comparative analysis of the Turkish specimen and data provided by other authors are presented in Table 3: it shows that the size of macroscopic and microscopic structures of our samples are compatible with previous findings.

In terms of biogeographic distribution, *R. juniperina* has so far been reported from the Mediterranean (Italy and Spain) and European-Siberian (Germany, Austria and Slovakia)

phytogeographic regions, and is here reported for the first time in the Irano-Turanian (Turkey: Afyonkarahisar) phytogeographic region. The factors influencing the distribution of species and the reasons why they grow in different regions according to their preferences for host or climate can be elucidated in future broader studies. According to the literature, *R. juniperina* is generally reported as occurring with some *Quercus* spp. (*Q. cerris* L., *Q. faginea* Lam., *Q. ilex* L., *Q. petraea* (Mattuschka) Liebl., *Q. pubescens* Willd., *Q. suber* L.) and *Juniperus communis* L., and rarely in mycorrhizal association with *Fagus* spp. and *Pinus sylvestris* L. (Galli 1996, Sarnari 1998, Pidlich-Aigner 2008, García 2011, Mua et al. 2017). In the present study, this species was found to occur in association with *Quercus* sp. and *Juniperus oxycedrus*.

Phylogenetically, *R. juniperina* is closely related to but distinct from *R. adulterina* and *R. cuprea* based on the ITS data (Fig. 1). The collection of *R. juniperina* from Turkey clustered with the collections of *R. juniperina* from Germany and Spain. Besides, *R. juniperina* was confirmed in both analyses with strong bootstrap support (100%) and posterior probability values (1.0). The results of ITS phylogeny of *R. juniperina* from Turkey agree with the topologies proposed by Adamčík et al. (2016) (Fig. 1).

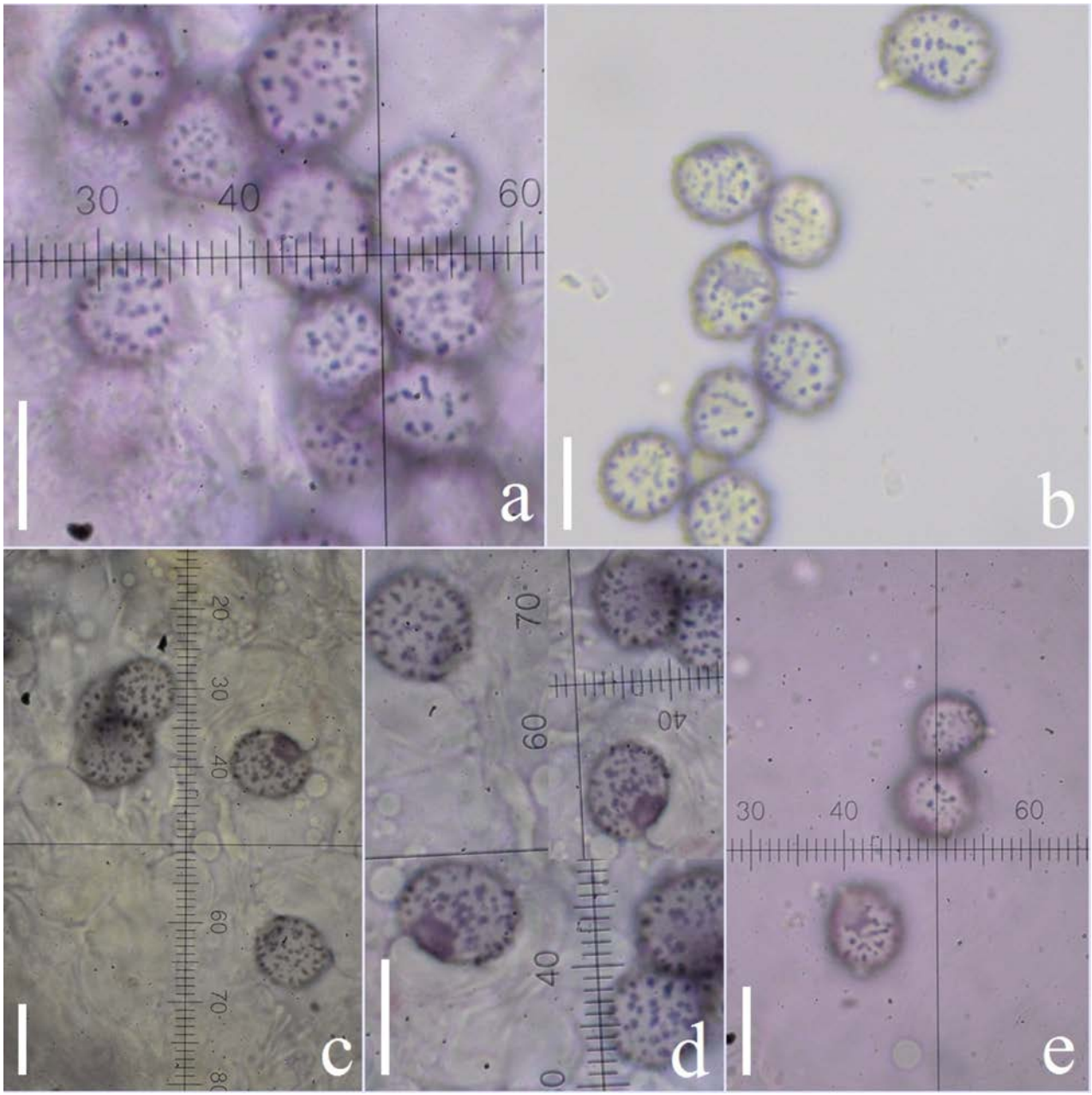

Fig. 3 – *Russula juniperina*. a–e Basidiospores. Scale bars = 10 µm.

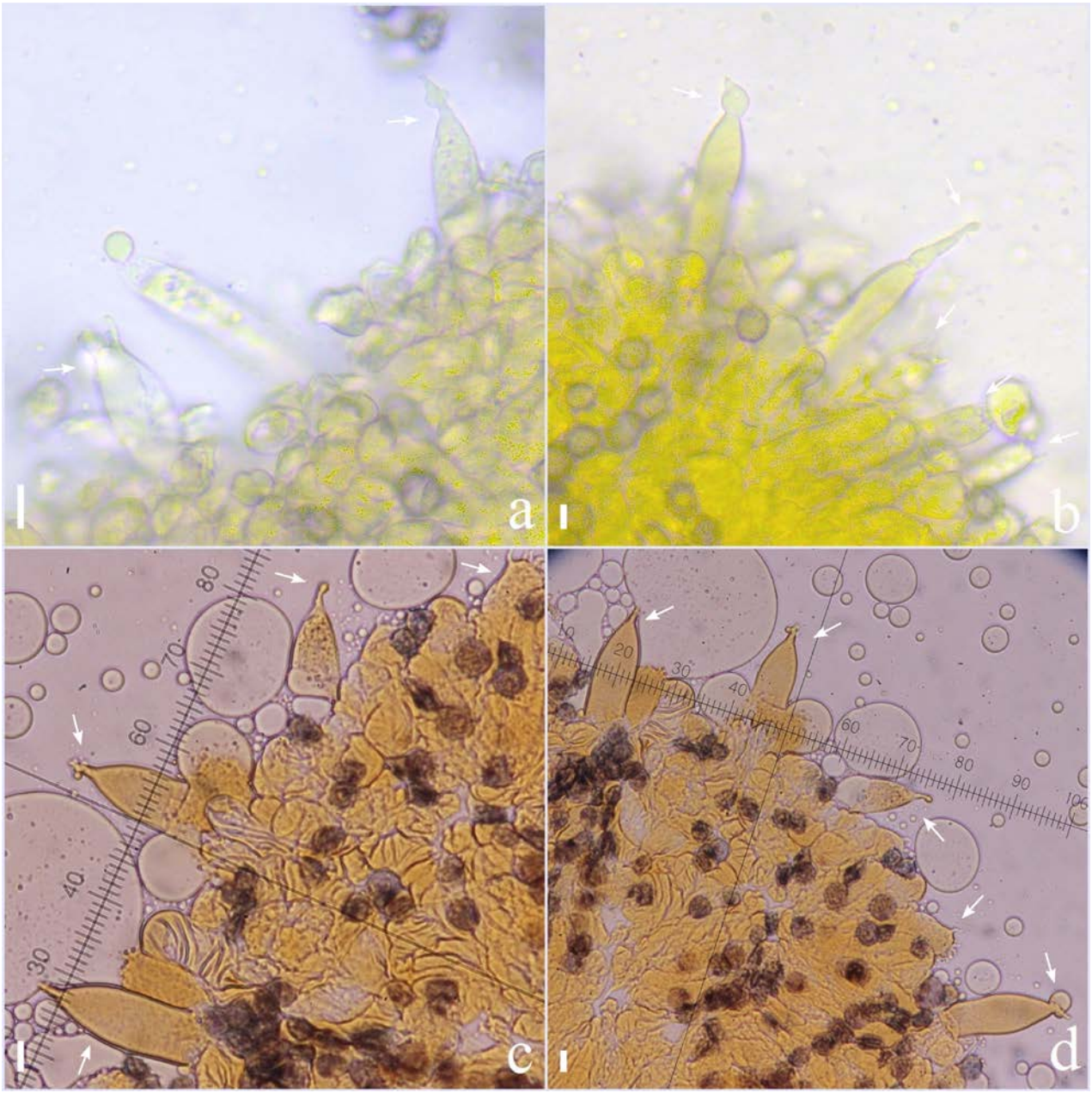

Fig. 4 – *Russula juniperina*: a–b Basidia and hymenial cystidia. c–d Hymenial cystidia. Scale bars = 10 µm.

The lowest sequence divergence among in-group taxa was observed between *R. juniperina* and *R. adulterina* 0.006%, the next was between *R. juniperina* and *R. cuprea* 0.015% in the ITS dataset. The highest sequence divergence with in-group taxa was found between *R. badia* and *R. font-queri*, 0.134%. Thus, it was determined that genetically, *R. juniperina* was closer to *R. adulterina* than to *R. cuprea*.

Ecologically, *R. adulterina* unlike *R. juniperina* forms mycorrhizae mainly with the conifers *Abies alba* Mill., *Picea abies* (L.) H. Karst. and *P. excelsa* (Lam.) Link. Also, the size of the spores (8–15 × 7.5–10 µm) and warts (1.6–2 µm) of *R. adulterina* are considerably larger than those of *R. juniperina*. In addition, the number of septa in the dermatocystidia (0–1 septa) is less than in *R. juniperina*, and there are no diverticulae in the structure (Galli 1996, Romagnesi 1996, Sarnari 1998, Kränzlin 2005, Kibby 2014).

Morphologically, *R. juniperina* is most comparable to *R. cuprea*, and in some sources (Bon 1988, www.indexfungorum.org), *R. juniperina* is even given as a synonym or a sub-taxon for *R. cuprea*. However, the cap colour of *R. cuprea* is generally dark brown-copper, violet purple or

partly yellowish green. In terms of macrochemical reactions, in fresh *R. cuprea* unlike *R. juniperina*, the application of guaiac gives a slow result. Microscopically, the spore warts of *R. juniperina* are 0.8 µm and it is shorter than those of *R. cuprea* (1.5 µm) (Galli 1996, Sarnari 1998, García 2011).

According to the current checklists and literature, 133 taxa of the *Russula* have been reported from Turkey (Sesli & Denchev 2008, Doğan & Öztürk 2015, Solak et al. 2015, Işık & Türkekul 2017, Çolak et al. 2018). With this study, the number of taxa of this genus has increased to 134 in the country.

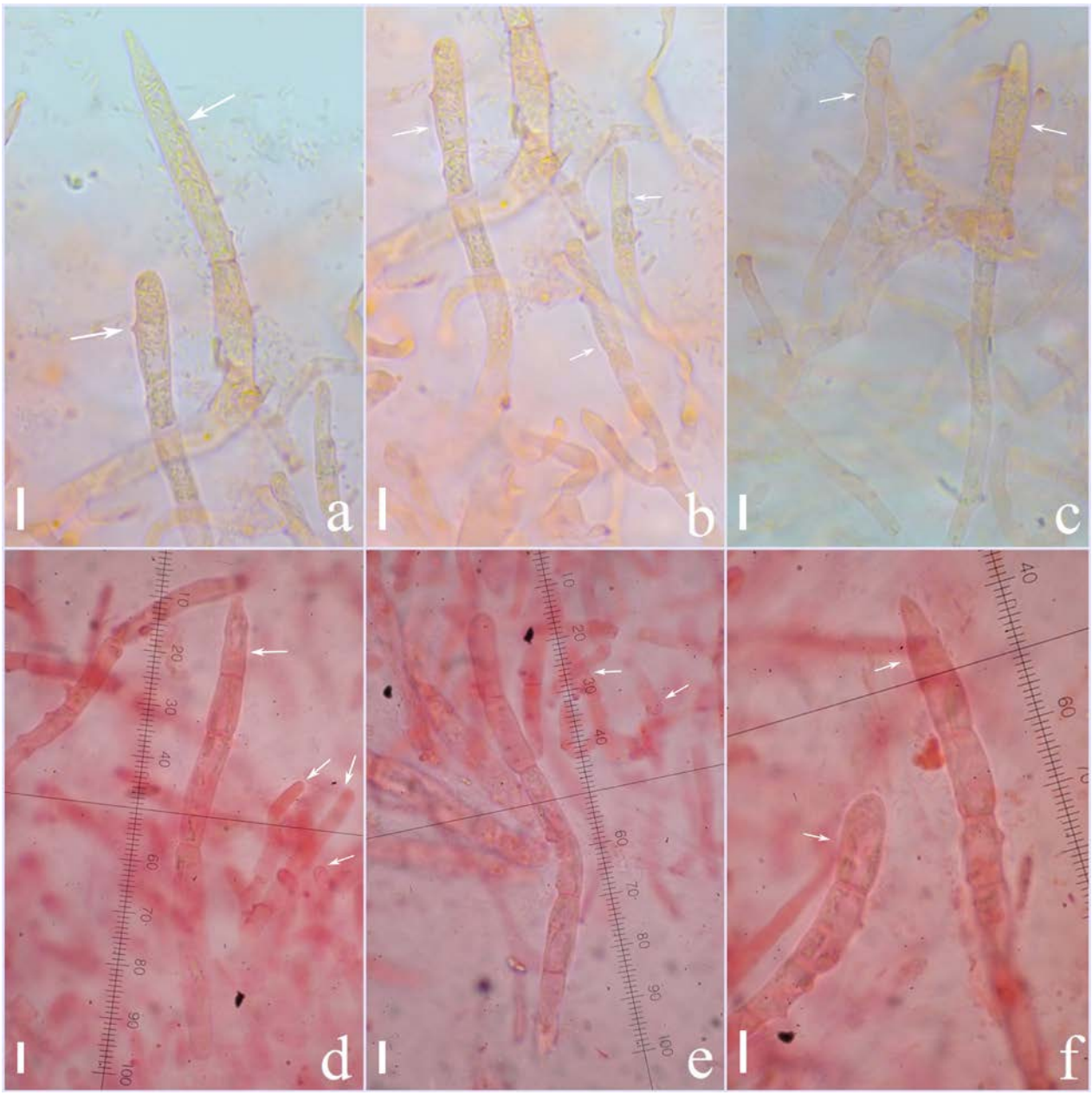

Fig. 5 – *Russula juniperina*. a–f Elements of Pileipellis. a, b, c, f Dermatocystidia with diverticulum. d and e Hyphal endings in pileipellis. Scale bars = 10 µm.

Table 3 Comparison of different characters of *R. juniperina* with earlier published works.

References	Size of cap (mm)	Size of stem (mm)	Size of spores (µm) / Size of warts (µm)	Size of basidia (µm)	Size of hymenial cystidia (µm)	Width of cuticle (µm)	
						Dermatocystidia	Other cuticular hyphae
Galli 1996	55–90	35–70 × 10–15	8–11(12) × 8–10 / 0.5–0.8	–	9–11	7–12	2–4
Sarnari 1998	55–110(130)	45–70(80) × 18–25	8–10.4(11) × 7.2–9 / 0.8	48–70 × 12–16	50–120 × (8)10–17	4–9	2–3.5(4.2)
Sarnari 1998 **	55–90	35–70 × 10–15	9–11 × 8–10 / 0.5–0.8	48–70 × 12–16	9–11	–	–
Pidlich-Aigner 2008	78–120	68–83 × 22–45	8–10.3 × 7–8.8 / 0.8	–	65–110 × 8–13	3–8.5	1.5–5
García 2011	(36)51–98(107)	35–58(73) × (11)14–33(43)	(8.3)8.5–10.5(11.3) × (7)7.5–8.8(9.3) / 0.6-0.8	45–66.3 × 12.8–16.2	62.5–117(151.9) × 9.6–16.2	(2.2)5.4–7.5(8.6)	2.3–3.5
Mua et al. 2017	60–100	40–80 × 15–25	(7.5)8–8.9–9.8(10.2) × (6.2)6.8–7.4–8.1(8.8) / 1.0	40–56 × 10–14.5	60–100 × 10–15	4–10	2–3.5
This study	50-80	35–60 × 10–20	8–10.5(–11) × 7–9 / 0.8	50–70 × 10–14	14–17.5	10	2–4

**Description of original material

Acknowledgements

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