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***Ramaria barenthalensis* a new record from Western Himalayas, Kashmir**

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Abstract

The genus *Ramaria* is a globally distributed genus inhabiting different substrates. In the present study samples of *Ramaria barenthalensis*, were collected during a fungal survey from Neelum Valley, Azad Jammu and Kashmir, Pakistan. Identification was carried out through combined morpho-anatomical and nuclear ribosomal DNA internal transcribed spacer (ITS) sequence analysis that confirmed its position as *Ramaria barenthalensis*. The taxon is a new record to fungi of Kashmir, Pakistan.

Keywords: Coral fungi, *Ramaria*, Mushrooms of Kashmir, nrDNA, ITS, Neelum Valley

Introduction

Ramaria species are cosmopolitan in distribution and grow on living and dead hardwood, tree trunks partially decomposed organic matter and under conifers as mycorrhizal species worldwide (Dorjey et al. 2016, Marr and Stuntz 1973, Kuo 2009). They are common fungal species associated with conifers and known as coral fungi due to colored, beautiful and much branched sporocarps. They grow in diversity of habitats and form a mycelial mat in soil beneath the sporocarps (Kumar and Gautam (2017). The genus *Ramaria* comprises more than 200 species distributed worldwide (Ghosh et al. 2021). *Ramaria flava* and *R. stricta* are common edible coralloid fungi (Krupodorova and Sevindik 2020). They have bright to orange yellow basidiocarps with rusty brown basidiospores (Humpert et al. 2001). *Ramaria stricta* (Pers.) Quel, commonly known as the strict-branch coral (Braeuer et al. 2018) is cosmopolitan in distribution and grows under deciduous and coniferous forests (Kuo, 2009). Molecular tools have been used in the identification and differentiation of ectomycorrhizal symbionts (Gardes and Bruns 1993). *Ramaria* species have been identified through scanning electron microscopy and molecular techniques by Martin et al. (2020). An internal transcribed spacer restriction length polymorphism was observed among *Ramaria* species and used as a diagnostic tool to characterize those (Nouhra et al. 2005). The region of Jammu and Kashmir is enrich with macro fungal diversity. *Ramaria apiculata* (Fr.), *Ramaria aurea* (Schaeff.) Quel, *Ramaria flavobrunnescens* var *aurea* (Fr.) Donk, and *Ramaria formosa* (Pers.) Quel. have been reported from Jammu and Kashmir, India (Sharma et al. 2022). *Ramaria apiculata*, *R. flava*, *R. flaccida*, *R. flavescens*, *R. formosa*, *R. flavescensoides*, and *R. subaurantica* have been reported previously from Pakistan (Nasim et al. 2008, Hanif et al. 2019). *Ramaria stricta* was reported previously from Azad Jammu and Kashmir based on morphological characters (Gardezi 2005). In the present study, a new collection of *Ramaria barenthalensis* in

Neelum Valley, AJK, Western Himalayas is reported. Morphological and molecular characteristics were studied to increase the knowledge about its distribution and phylogenetic.

Materials and Methods

Study area

The study sites lies in Neelum Valley, Azad Jammu, and Kashmir (AJK), Pakistan. The area is mostly hilly, and covered with dense forest of conifers. The average annual rainfall is 98 mm. The average snowfall in winter is 48cm (AJK Metrology Department, 2019). The vegetation of the study area consists of *Pinus wallichiana* A.B Jacks., *Abies pendrow* (Royle ex D. Don) Royle, *Cedrus deodara* (Roxb.) G. Don, *Aesculus indica* (Wall. ex Cambess.) Hook. and shrubs, *Viburnum grandiflorum* (Wall. ex DC.), *Indegofera heterentha* (Wall.) and *Betula jacquemontii* Spach.

Sample collection

Samples were collected from selected sites in Neelum valley AJK during 2019–20, through consecutive field surveys (Fig. 3). The Samples were cleaned and packed in to separate bags to avoid from spore contamination after proper tagging.

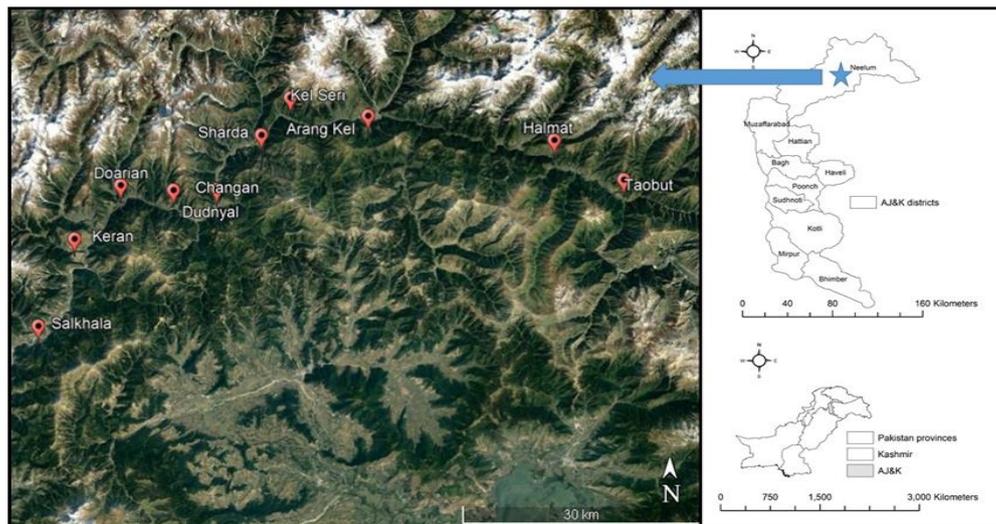


Figure 1 Map of Study area and collection sites (Neelum valley AJK)

Morpho-anatomical Characterization

Photography and tagging of fruiting bodies were carried out during the field survey. Macro-morphological features including size, shape, and color of basidiomata, were recorded. After cleaning, the fruiting bodies were dried and preserved for further studies. Slides were mounted in 5% KOH (w/v) and 1% Congo red to study micro-morphological features, size, shape, and color of basidiospores and basidia, using a light microscope (MX4300H, Japan) at 100X magnification. For basidiospores and other structures 50 individuals were measured. Names of fungi with authorities were retrieved from Index Fungorum (<http://www.indexfungorum.org>) (Fungorum I 2008).

DNA Sequencing and Phylogenetic Tree

DNA extraction was performed from 100 mg of dried sample by using a modified 2% CTAB buffer (Bruns 1991). Sample was disrupted by using high-speed shaking for 1min using a tissue lyser and incubated at 65C° for 1 hour in 350 ml CTAB buffer with 5 µg proteinase K (Sigma-Aldrich) and 15 µg RNase. After the incubation the sample was centrifuged and the aqueous layer was mixed in a new tube with phenol, chloroform and isoamyle alcohol (25:24:1). The previous step was repeated mixing of supernatant with chloroform (1:1) to clear the supernatant which was then incubated at a temperature of 4 C° for 15 in 2-propanol (3:2). The tube was again centrifuges after incubation (10m × 9200 g) and the aqueous layer was discarded. The DNA pallet was washed with Ethanol and air dried and stored at -20 C° Burn and White (1990). The DNA extraction was confirmed by gel electrophoresis using agarose gel (1%) for 30 min at 70V (Voytas 2000). Nuclear rDNA amplification of the ITS region was carried out using universal primer sequences (ITS1/ITS4), ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Gardes and Bruns 1991). Further analysis was carried out using the molecular tool BioEdit ver. 7.2.5 (Hall 1999). Most similar sequences were retrieved from NCBI (<https://www.ncbi.nlm.nih.gov/guide/>) using BLAST for the construction of a phylogenetic tree. Multiple sequence alignment was performed online by using MUSCLE <https://www.ebi.ac.uk/Tools/msa/muscle>). The final phylogram was constructed in RAxML-HPC2 using the XSEDE tool (8.2.10) with 1000 bootstrap replicates to obtain the best bipartition results. Figtree ver. 1.4.2. software was used for tree visualization and initial setting of the phylogram (Rambaut 2012). The ribosomal (rDNA) ITS sequence was deposited to GenBank, and accession number (ON209680) was obtained.

Results

Ramaria barenthalensis Franchi & M. Marchetti, Riv. Micol.61: 199 (2019)

Taxonomy

GenBank Accession No ON209680

Ecology: Under mix conifers forest, sometimes associated with *Abies pendrow* and *Betula jacquemontii* Spach, alone, scattered, and gregarious, summer and fall, common in the temperate zone of AJK. **Basidiomata:** 5-12 cm high, 4-9 cm wide with a well-developed branched mycelium. **Branches:** Vertically oriented, furcated, elongated to flattened, smooth, light brown when young and dark brown at maturity. **Base:** Variable, sometimes reduced, and well-developed, whitish to brown. **Flesh:** Whitish to light brown and soft. **Odor** is not distinctive and tastes good. **Basidiospores** [50/5/1], 6.5-9 x 3-4.6 µm, elliptical to ovoid, roughened. **Basidia:** sterigmate, 45-59 x 7.5-9 µm, **Cheilocystidia:** 46-62 x 7-8.5µm elongated. Clamp connections are present. Trama hyphae are thick-walled with clamps. Hyphae in basal tomentum are smooth, with a tapering base (Fig 1, A-J).

Material Examined: Basidiomata of *Ramaria barenthalensis* were collected from a mixed coniferous forest in decayed hardwood, during late summer, in Dawarian, Neelum Valley, Azad Jammu and Kashmir (AJK), Pakistan, 1973 a.s.l. Tariq Saiff Ullah, 21-08-2020, TS-40.

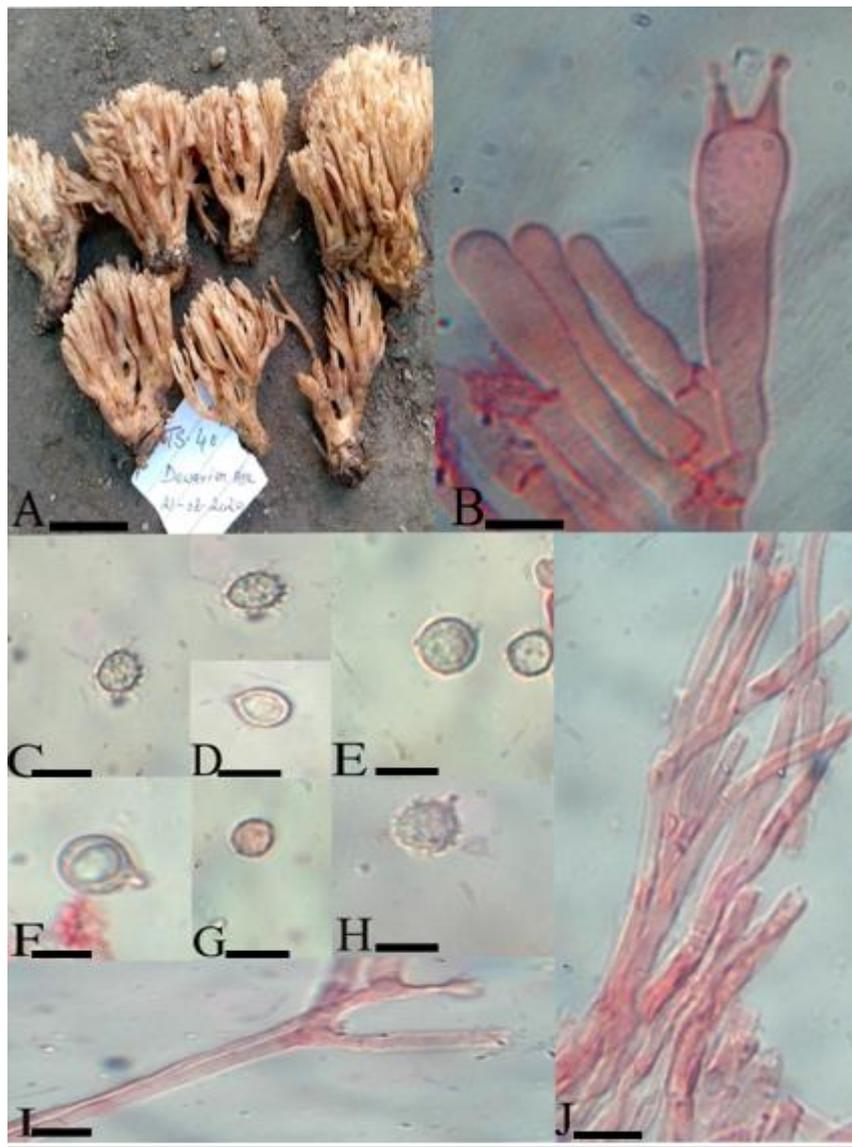
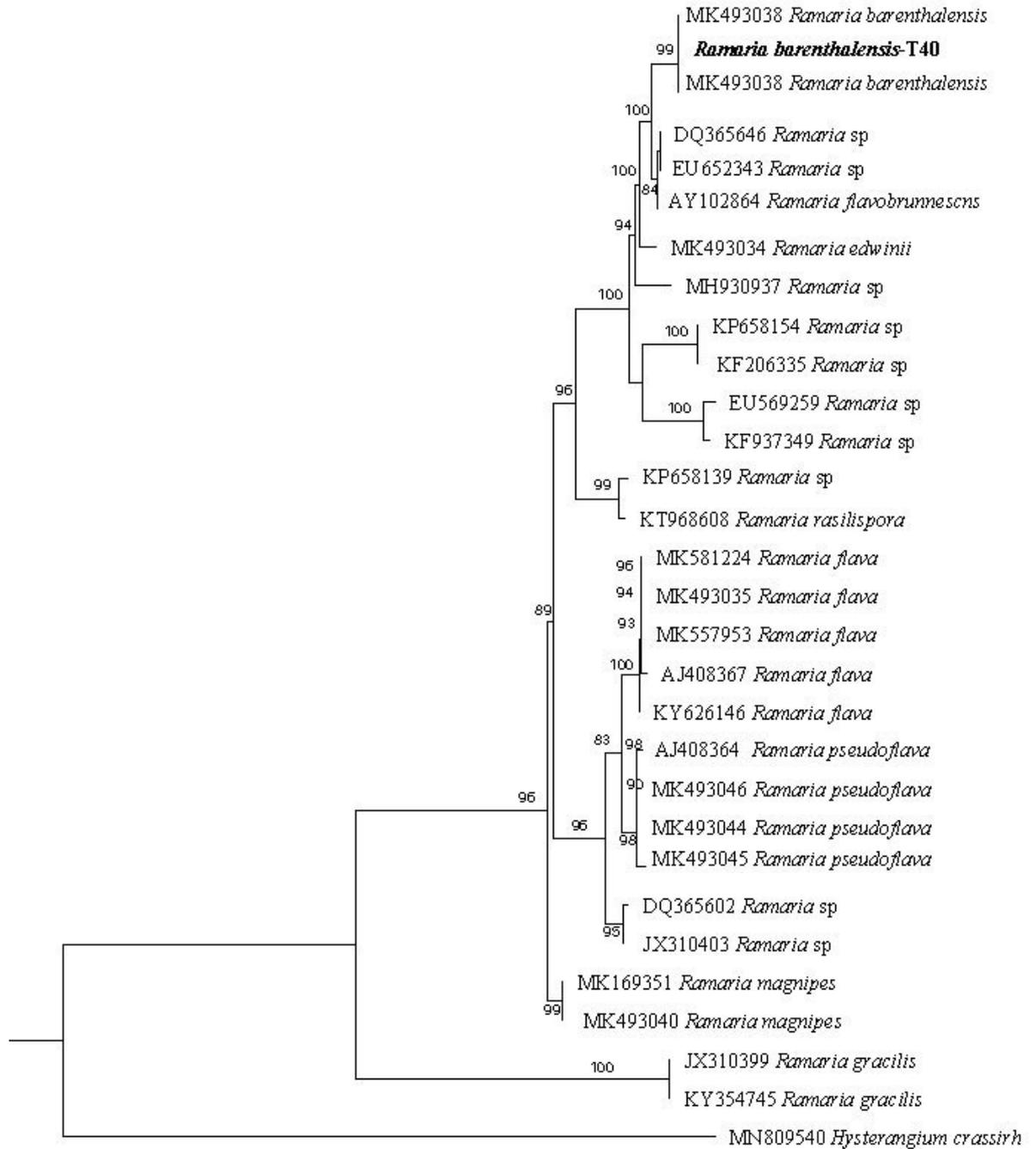


Fig 1. A. Fruiting bodies of *Ramaria barenthalensis* TS-40 B. Basidia and Basidiole. C-H Basidiospores I. Clamp connections J. Trama hyphae. Scale bars: A = 3.6 cm, B = 12 μ m, C-H = 6.7 μ m, I = 13 μ m, J = 12.8 μ m.

Phylogeny

The sequence of nrDNA of *Ramaria barenthalensis* T40, yielded a fragment of 702 base pairs. The initial blast revealed 99.33% similarity with *Ramaria barenthalensis* MK493039.1, 98% query coverage, and (0) E value. The final phylogram was constructed using the most similar sequences retrieved from GenBank. The MUSCLE tool was used for sequence alignment. The final alignment sequence was trimmed from ends at the conserved motifs. The final file data set included 702 bp from which 421 remained conserved, 256 were variable, 167 were simple information sites, and 86 were singleton sites. *Hysterangium crassirh* was chosen as an out-group taxon. The final nucleotide sequence generated in the study is submitted to GenBank database (Accession number ON209680).



0.08
Fig 2. Phylogenetic relationship of *Ramaria barenthalensis* (ON209680) based on nrDNA (ITS) generated with RAxML with 1000 bootstrap iterations. The letters in bold refer to sequence generated during this study.

Discussion

In the present study samples of *Ramaria barenthalensis* were studied by combined morpho-molecular approach. The species is distinctive in terms of morphological and anatomical features from the other described species of the genus *Ramaria*. We characterized *Ramaria barenthalensis* by basidiomata, 5-12 cm high, 4-9 cm wide with a well-developed branched, furcated, elongated to flattened and smooth mycelium with basidiospores 6.5-9 x 3-4.6 μm , elliptical to ovoid and roughened. Comparisons in morphoanatomical characteristics with other described species of the genus *Ramaria* are also given (Table 2). Our study is consistent with the original specimen description (Franchi and Marchetti 2019). In the phylogram, the specimen lies in the same clade with a strong association with *Ramaria barenthalensis* MK493038 and MK493039. The closest species was *Ramaria edwinii* (MK493034) in the phylogenetic tree. The sample, *Ramaria barenthalensis* T-40, has similar morphological features with slight differences in basidiomata to most of the species of *Ramaria* described earlier (Agerer et al. 1996). In previous studies, *Ramaria apiculata*, *R. flava*, *R. flaccida*, *R. flavescens*, *R. formosa*, *R. flavescentoides* *Ramaria mairei*, and *R. subaurantica* have been reported from the regions of Jammu and Kashmir, India and Pakistan based on morphological study (Nasim et al. 2008, Sharma et al. 2015, Hanif et al. 2019). Morphological or anatomical features alone are insufficient to identify a mushroom species. Many ambiguities are found in submitted sequences to online databases due to contaminated samples. Fifteen species of *Ramaria* were identified and characterized by Martin et al. (2020) using combined morphological and molecular tools. *Ramaria aurea* is differentiated from the other species by basidiospores up to 8-15 \times 3-6 μm (Ahmed 1972). *Ramaria stricta* was reported previously from Azad Jammu and Kashmir based on morphological characters (Gerdezi, 2005). In conclusion, *R. barenthalensis* is recorded as a new coralloid fungus to the state of Jammu and Kashmir, based on morphoanatomical and molecular characterization. This fungal species could be used as a source of food and medicines after biochemical characterization.

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Tab 1. Strain and NCBI GenBank accession numbers used in the construction of phylogenetic tree

Species	Strain	Location	Substrate	GenBank Accession Number
Ramaria sp	SD125.4	USA	Fir forest	DQ365646
Ramaria sp	OSC 81622	USA	Fir forest	EU652343
<i>R. flavobrunnesens</i>	AY102864	USA	Fir forest	AY102864
<i>R. barenthalensis</i>	AMB17386	Spain	mixed forest	MK493039
<i>R. barenthalensis</i>	AMB17381	Spain	<i>Abies</i> and <i>Picea</i> forest	MK493038
<i>R. barenthalensis</i>	T40	Kashmir	Dead hardwood of conifers	ON209680
<i>R. edwinii</i>	ALV11173	Spain	<i>Abies</i> and <i>Picea</i>	MK493034
Ramaria sp.	RAM2	Srinagar J&K	<i>Abies</i> forest	MH930937
Ramaria sp.	OSC 73311	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	KP658154
Ramaria sp.	OSC 144044	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	KF206335
Ramaria sp.	MHM312	Mexico	NA	EU569259
Ramaria sp.	NVE 367	Amazonia	NA	KF937356
Ramaria sp.	OSC 115803	USA	NA	KP658139
Ramaria sp.	DB215-08/5	USA	NA	KT968608
<i>R. flava</i>	AMB n. 17484	Italy	<i>Abies</i> and <i>Picea</i>	MK581224
<i>R. flava</i>	AMB 17393	Italy	<i>Abies</i> and <i>Picea</i>	MK493035
<i>R. flava</i>	AMB n. 17481	Italy	Mix forest type	MK557953
<i>R. flava</i>	MA-Fungi 48072	Spain	<i>Abies</i> and <i>Picea</i>	AJ408367
<i>R. flava</i>	ZT Myc 55613	Italy	NA	KY626146
<i>R. pseudoflava</i>	AMB 17390	Italy	NA	MK493045
<i>R. pseudoflava</i>	AMB 17392	Italy	NA	MK493046
<i>R. pseudoflava</i>	AMB 17391	Italy	NA	MK493044
<i>R. flava</i>	MA-Fungi 48061	Spain	<i>Abies</i> and <i>Picea</i>	AJ408364
Ramaria sp	OSC 61837	USA	Mix forest	DQ365602
Ramaria sp	OSC 134657	USA	Mix forest	JX310403
<i>R. magnipes</i>	WTU-F-063057	USA	Mix forest	MK169351
<i>R. magnipes</i>	WTU 063057	Italy	Fir forest	MK493040
<i>R. gracilis</i>	OSC 134659	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	JX310399
<i>R. gracilis</i>	OSC 112168	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	KY354745
<i>Hysterangium crassirahis</i>	OSC 4860	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	MN809540

	<i>Ramaria abietina</i>	<i>R. subaurantiaca</i>	<i>R. stricta</i>	<i>R. flava</i>	<i>R. flaccida</i>	<i>R. flavescens</i>	<i>R. apiculata</i>	<i>R. formosa</i>	<i>R. barenthalensis</i>
Basidiomata	Basidiomata up to 12 cm high, 4.5-8 cm in width, erect, branched, tips pointed with glabrous surface	Basidiomata have densely crowded branches and sometimes with a fused base. Orange to brownish	Mature Fruit bodies branched, 4-12 cm high, 4-8 cm wide. Branches with tapered and pointed ends, mostly erect and slender.	Basidiomata 10.5 -20 cm in length with a width of 7-15 cm having numerous densely crowded branches	Mycelium, multi branched, slender and less or more week.	Basidiomata branched and large up to 18.8 cm; clamps are present.	Basidiomata much branched 4-9 cm tall, fan shaped, rounded, tips	Basidiomat a up to 20 cm high and multi branched mycelium.	5-12 cm high, 4-9 cm wide with a well-developed branched mycelium, Vertically oriented, furcated, elongated to flattened, smooth
Color	Light pink to pinkish buff, pale yellow to light brown tips, darkened when bruised.	A persistent light brown to light yellow color from early stage of development to final stage of maturity.	Surface glabrous, pinkish-buff, tips of branches pale-yellow, buff brown when bruised.	Brown to light yellow more dark to ochraceous with age.	tips of branches tan to gold, no color change on bruising	Branches yellowish-brown when young, pale to pinkish brown at maturity.	Branches Brown-light to yellow brown	Branches Light brown. The flesh is white to yellowish.	Light brown when young and dark brown at maturity. Clamp connections present
odor	Slightly aromatic and bitter		Aromatic	Pleasant	Pleasant	Not known	Not known	pleasant	Distinctive
Stipe	Sometime less developed and reduced. Up to 1-2 cm in length, 0.5-1.5 cm wide, color changes to light-brown on bruising.	1-2.5 cm in length, 0.5-1.5 cm in width, sometimes absent. When present, pallid and tomentose with a tough context, light-brown on bruising.	Reduced and sometime developed up to 2 cm, flesh whitish to light brown	Stipe, 50 80 mm long with a width of 40-50 mm. Base of stipe, whitish, pale-yellow to reddish brown on bruising	Medium stipe 1.5-2 cm high and whitish	Stipe large, 2.5-6.6 cm with a width of 1.5 2 cm, whitish.	Stipe large, 1.5- 4 cm with a width of 1-1.5 cm, whitish.	Stipe large, 2- 4.5 cm with a width of 1.5-18 cm, light brown-whitish.	Stipe variable in length and width, sometimes reduced to well-developed. Flesh whitish to brown
Spores	6.5-9.5 µm in diameter, ellipsoidal, slightly warted.	Spores 7-8.5 µm, ellipsoidal	Elliptical, rough, rusty-yellowish 7.5-10.5 x 3.5-4.5 µm, clamp connections present	pale elliptical and roughened, 10.5-17.5 x 4-6.5 µm.	Pale elliptical less to more roughened, 9-14.5 x 3-4.5 µm	Thick walled, minute and rough outer surface, cyanophilous warts, ellipsoidal, tips squared, 7-10x3.5-5 µm	less to more roughened, with cyanophilous warts; ellipsoidal with a squared tip; 7-10x4-5 µm	slightly roughened, with cyanophilo us warts; ellipsoidal and squared tip; 7.5-9x3-3.5 µm	6.5-9 x 3-4.6 µm, elliptical to ovoid, roughened.
Edibility	Edible	Edible	Edible on choice	Edible	Edible on choice sometimes laxative	Edible	Not known	Not known	Edible when young
Taste	Pleasant.	Taste mild to pleasant.	bitter	Taste mild to pleasant	Pleasant.	Pleasant.	Taste mild to pleasant.	Bitter/mild	Pleasant.
Ecology	Solitary and gregarious, on hardwood and tree trunks of conifers.	Solitary and in small groups on hardwood and logs, soil and decomposed organic matter	Growing in groups, under conifers and mixed vegetation, on dead wood and on ground.	Solitary and gregarious. Fruiting season varies from late summer to autumn.	Grow on the ground. In mixed forest and hardwoods. Late autumn.	Solitary to gregarious, on decomposed hardwood in association with rotten conifer needles.	Gregarious, on soil with decomposed organic matter and conifer needles.	Gregarious, on soil with decomposed organic matter and conifer needles.	Gregarious and sometime alone, on fallen tree turns of coniferous and other mixed vegetation during late summer
References	(Hanif et al. 2006; Sultan et al. 1997; Peterson, 1969).	(Sultan et al. 1997;Peterson, 1989)	(Sultan et al. 1997, Peterson and Olexia, 1967; Peterson, 1969)	(Sultan et al. 1997; Peterson, 1969)	(Sultan et al. 1997)	(Sultan et al. 1997)	(Sultan et al. 1997)	(Kuo 2005)	Present study
Basidiomata of different specie <i>Ramaria</i>									

Table 2. Morphoanatomical features of different *Ramaria* species

