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Molecular Ecology of Hypogeous Mycorrhizal Fungi: *Rhizopogon roseolus* (Basidiomycotina)

By

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Summary

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In order to sort out previous difficulties in the taxonomic classification of *Rhizopogon* in Europe, particularly *R. roseolus*, and to establish the RFLP data base for identification of root-tip samples, we examined 52 basidiomes of this species, as well as 7 from other *Rhizopogon* species using molecular tools. The polymerase chain reaction (PCR) was used to amplify the ribosomal internal transcribed spacers (ITS1 and ITS2) and 5.8 S DNA from basidiomes. The cluster analysis of patterns obtained by ITS-RFLP with five endonucleases (Cfo I, Dde I, Hinf I, Mbo I and Taq I) showed a high degree of variation among isolates of *R. roseolus*, even from those forming part of the same collection. However, it was not possible to relate the different patterns with taxa proposed by earlier authors. °

Introduction

In Europe, MARTÍN 1996 reported 21 species of *Rhizopogon*, distributed over three sections. *Rhizopogon roseolus* (Corda) Th. M. Fr. is the species most common in Europe. Basidiomes are semihypogeous on sandy or calcareous soil in association with *Abies* spp., *Picea* spp, *Pinus* spp. and also under *Quercus* spp. Corda's picture of *Splachnomyces roseolus* (CORDA 1831) is the holotype of *R. roseolus* and it shows basidiomes with vinaceous peridium and white gleba. As mentioned by MARTÍN 1996, the absence of yellow colours in the basidiomes shown in this picture caused many authors to misinterpret this taxon. Some early

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authors, created new taxa mainly based on peridium colour and its changes during development. GROSS & al. 1980 discussed the possibility for differentiation of the *R. roseolus* complex referring to the spore volume. After examining 1458 herbarium samples belonging to the *R. roseolus* complex, one of us (MARTÍN 1996) concluded that it is not possible to separate different taxa either by spore volume or by peridium colour.

The use of molecular techniques in ectomycorrhizal fungi provides new information in taxa where, as in *Rhizopogon*, morphological classifications are in conflict, ambiguous or missing (HENRION & al. 1994, FISCHER 1995, LANFRANCO & al. 1995). The main purpose of the present work is to map the intraspecific polymorphism in the internal transcribed spacer regions (ITS1 and ITS2) of rDNA of *R. roseolus* to check whether this species can be separated into different taxa as proposed by earlier authors. In a previous study (MARTÍN & al. 1998) we have shown that restriction fragment length polymorphism of the ITS rDNA is useful for taxonomic studies in *Rhizopogon* and it may be a good tool to recognize the species in the root tips. In this study, the RFLP patterns of the ITS regions, including the 5.8 S rDNA of *R. roseolus* are compared with those of *R. aurantiacus* , *R. luteolus*, *R. rocabrunae* and *R. villosulus*.

Materials and Methods

Material.- Fifty-nine basidiomes belonging to 15 *Rhizopogon* collections were studied. Table 1 shows the complete list of collections including the number of basidiomes from which we isolated DNA. Herbaria are abbreviated according to HOLMGREN & al. 1990. Personal collections of Mario Sarasini (Italy), received as a gift, are designated as "Sar". The nine basidiomes from *R. roseolus* BCC-MPM 1898 were collected growing joined together in a compact group and the thirty six from Sweden, identified as *R. roseolus*, were collected around the same tree. From this last collection, we did not save any herbarium material because the basidiomes were very small (5-10 mm diameter) and, after the morphological study, all the basidiome was used to isolate the DNA. Methods of collection and study of morphological and anatomical features are essentially those of SMITH 1964, SMITH & ZELLER 1966 and MARTIN 1996. For specimens of *R. roseolus* spore volume was calculated according to GROSS & SCHMITT 1974 as $V= 0.5x\ w^2x\ l$ (volume of rotation elipsoid, where w= spore width and l= spore length) and are indicated in Table 2 with the letters A to D as mentioned in MARTIN 1996: (A) volume inferior to 22.5μm^3, (B) volume between 22.6 and 37.5μm^3 , (C) volume 37.6 to 52.6 μm^3 and (D) volume superior to 52.6 μm^3.

DNA isolation, DNA amplification and RFLP analyses.- The molecular methods were done as mentioned in MARTÍN & al. 1998. Negative (ultrapure water) and positive (Telephora sp. extractions) controls were performed for each amplification. Amplified ITS-PCR products were digested separately with six restriction enzymes (Cfo I, Dde I, Hae III, Hinf I, Mbo I, Taq I), under the conditions recommended by the manufacturer (Promega Corp., Madison, WI, USA), except from the Swedish *R. roseolus* collections (ROSRHI-13 to ROSRHI-35) where only data were obtained with Cfo I, Hinf I, Mbo I and Taq I.

RFLP data processing.- Gels were read and fragment size data from Polaroid® photos was processed using Adobe Photoshop® software, and analysed in the Taxotron® software system, especially designed for RFLP data processing (GRIMONT 1998). Fragments were considered identical when they differed by not more that 4.5-5.5 % between 75 and 450 bp. To combine the results from all the samples and the restriction enzymes Cfo I, Dde I, Hinf I, Mbo I and Taq I a distance matrix of the band-sharing indices was constructed. From this the average similarity was calculated, and the resulting values were then subjected to single-linkage cluster analysis. Futher details of the

analysis of RFLP data are given by KÅRÉN & al. 1997.

Results

Amplifications.- The ITS region was successfully amplified, using the ITS 1 and ITS4 primers, from all the samples, except for one *R.roseolus* specimen (BCC-MPM 1898-4) and one *R. luteolus* (BCC-MPM 2505). Gel electrophoresis of undigested PCR products revealed a band of approximately 970 bp in *R. aurantiacus*, 895 bp in *R. luteolus*, 940 bp in *R. rocabrunae* and 725 bp in *R. villosulus*. In specimens of *R.roseolus* in which only one band was obtained, the size was 700-750 bp. Three specimens of *R. roseolus* (BCC-MPM 1898-3, -7 and -8) consistentely displayed twin bands (700 bp and 750 bp), and were not used for RFLP analysis. No PCR products were obtained in any of the control reactions omitting template DNA.

Table 1. Isolates used in this study. Collection names according to MARTÍN 1996.

Collection	Geographical area	Probable mycorrhizal host	Basidiomes
R.aurantiacus A.H. Smith, BCC-MPM 1560	Sweden, Uppsala	*Pinus sylvestris*	1
R. luteolus Fr. & Nordholm, BCC-MPM 2504	Spain, Barcelona,	*Pinus* sp.	1
R. luteolus Fr. & Nordholm, BCC-MPM 2505	Spain, Pontevedra	*Pinus* sp.	1
R. luteolus Fr. & Nordholm, Sar-520	Spain, Girona	*P. radiata, Quercus* sp.	1
R.rocabrunae M.P. Martín, BCC-MPM 1995	Spain, Barcelona	*P. pinaster*	2
R. roseolus (Corda) Th. M. Fr., BCC-MPM 1511	Spain, Mallorca	*P. sylvestris*	1
R. roseolus (Corda) Th. M. Fr., BCC-MPM 1898	Spain, LLeida	*P. sylvestris*	9
R. roseolus (Corda) Th. M. Fr., Sar-108	Italy, Appiano Gentile	*P. sylvestris*	1
R. roseolus (Corda) Th. M. Fr., Sar-286	Italy, S. Vicenzo	*P. pinaster*	1
R. roseolus (Corda) Th. M. Fr., Sar-451	Italy, Marina di Vecchiano	*P. pinaster*	1
R. roseolus (Corda) Th. M. Fr., Sar-463	Italy, Bibione Pineda	*P. pinaster*	1
R. roseolus (Corda) Th. M. Fr., Sar-521	Spain, Girona	*P. pinaster, Quercus ilex*	1
R. roseolus (Corda) Th. M. Fr., Sar-610	Italy, Fondo	*P. sylvestris, Picea* sp. *Larix* sp.	1
R. roseolus (Corda) Th. M. Fr. , (Rosendal)*	Sweden, Uppsala	*P. sylvestris*	36
R. villosulus Zeller & Dodge, HOLOTYPUS, NY	USA, Oregon	*Pseudotsuga menziesii*	1

Material is not conserved in herbarium.

Table 2. List of basidiomes included in the RFLP analyses. The code refers to the abreviation used for each basidiome as appears in the text and figures. Spore volume to *R. roseolus* basidiomes codified as mentioned in the text, according to MARTÍN 1996. Fragment lengths (base-pairs) after endonuclease digestion with Cfo I, Hinf I and Mbo I.

Code	Specimen	Spore Volume	Cfo I			Hinf I						Mbo I			
AURRHI-1	BCC-MPM 1560	-	478	372	125	317	231	160	130			363	258		
LUTRHI-1	BCC-MPM 2504	-	484	367		325	230	163	135			367	261		
LUTRHI-3	Sar 520	-	487	370		326	239	165	133			370	262		
ROCRHI-1	BCC-MPM 1995y	-	383	239	228	341	249	158	130			308	258	109	71
ROCRHI-2	BCC-MPM 1995o	-	383	239	228	341	248	158	131			308	255	107	70
ROSRHI-1	BCC-MPM 1511	D	368	174	137	234	213	149	118			251	158	126	117
ROSRHI-2	BCC-MPM 1898-1	B	371	171	139	724						250	235	158	
ROSRHI-3	BCC-MPM 1898-2	B	745			730						247	234	158	
ROSRHI-4	BCC-MPM 1898-5	B	370	171		235	210	132	117			249	232	158	
ROSRHI-5	BCC-MPM 1898-6	B	366	165	134	231	208	132	117			245	230	157	
ROSRHI-6	BCC-MPM 1898-9	C	367	169	134	732						249	234	157	
ROSRHI-7	Sar108	C	372	175	145	236	210	146	117	90	67	246	238	159	
ROSRHI-8	Sar286	C	373	173	139	329	206	145	116	88	64	250	240	160	
ROSRHI-9	Sar451	B	374	173	139	231	207	145	116	87	64	251	241	159	
ROSRHI-10	Sar463	C	376	171	136	232	208	145	116	90	66	251	243	160	
ROSRHI-11	Sar521	B	371	169	144	236	208	132	115	88	64	251	236	157	
ROSRHI-12	Sar610	C	370	172	137	289	210	116	88			249	158	143	98
ROSRHI-13	Rosendal-C1	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-14	Rosendal-C2	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-15	Rosendal-C3	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-16	Rosendal-C4	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-17	Rosendal-C6	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-18	Rosendal-C7	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-19	Rosendal-C8	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-20	Rosendal-C10	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-21	Rosendal-C11	C	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-22	Rosendal-D2	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-23	Rosendal-D4	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-24	Rosendal-D7B	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-25	Rosendal-D9	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-26	Rosendal-D10	B	201	133	100	237	207	133	116			255	239	161	58
ROSRHI-27	Rosendal-E2	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-28	Rosendal-J2	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-29	Rosendal-J3	C	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-30	Rosendal-K1	C	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-31	Rosendal-K2	B	201	133	100	237	207	133	116			255	239	161	58
ROSRHI-32	Rosendal-K3	B	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-33	Rosendal-K4	C	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-34	Rosendal-K8	C	377	172	133	237	207	133	116			255	239	161	58
ROSRHI-35	Rosendal-K9	B	344	260		400	360	75				360	320		
VILRHI-1	(Holotypus)	-	371	353		242	210	137	132			257	242	160	

RFLP analyses.- The restriction enzyme Hae III did not cut the ITS region in any of the samples. Samples of DNA digested with Cfo I, Dde I, Hinf I, Mbo I and Taq I showed in general good resolution with characteristic patterns. Table 2 shows the band sizes obtained for each basidiome isolate and the five enzymes used in the UPGMA cluster analysis.

R. roseolus shows high polymorphism; different RFLP patterns were obtained, even among basidiomes from the same collections. The two *R. luteolus* specimens did not show polymorphism when cutting with the five enzymes. Moreover, *R. aurantiacus* showed patterns similar to those of *R. luteolus* with all the enzymes tested, except Taq I where the pattern was clearly different from those of *R. luteolus* as well as from the other *Rhizopogon* species. The two basidiomes of *R.*

rocabrunae, both belonging to the same collection, did not show intraspecific polymorphism, but the patterns were different from the other *Rhizopogon* analysed, mainly with Cfo I, Mbo I and Taq I. The patterns of *R. villosulus* were in accordance with those mentioned in MARTÍN & al. 1998.

Table 3. List of basidiomes included in the RFLP analyses. The code refers to the abreviation used for each basidiome as appears in the text and figures. Fragment lengths (base-pairs) after endonuclease digestion with Dde I and Taq I.

Code	Dde I				Taq I						
AURRHI-1	534	203	137	90	258	205	145	108	92	66	
LUTRHI-1	520	171	134		399	162	95	81			
LUTRHI-3	518	174	134		401	164	93	82			
ROCRHI-1	496	164	140	89	149	139	131	112	102	94	80
ROCRHI-2	491	164	139	89	149	140	132	114	104	96	82
ROSRHI-1	702				286	104	89	85	64		
ROSRHI-2	524	186			288	107	89	81			
ROSRHI-3	522	190			291	108	88	82			
ROSRHI-4	535	184			288	106	86	80			
ROSRHI-5	396	184			288	104	86	83			
ROSRHI-6	527	184			290	109	84	79			
ROSRHI-7	496	187			263	88					
ROSRHI-8	440	187			281	88					
ROSRHI-9	440	182			281	88					
ROSRHI-10	446	182			285	90					
ROSRHI-11	546	185			281	87					
ROSRHI-12	387	185			283	91					
VILRHI-1	295	190	151	91	295	193	104	77	50		

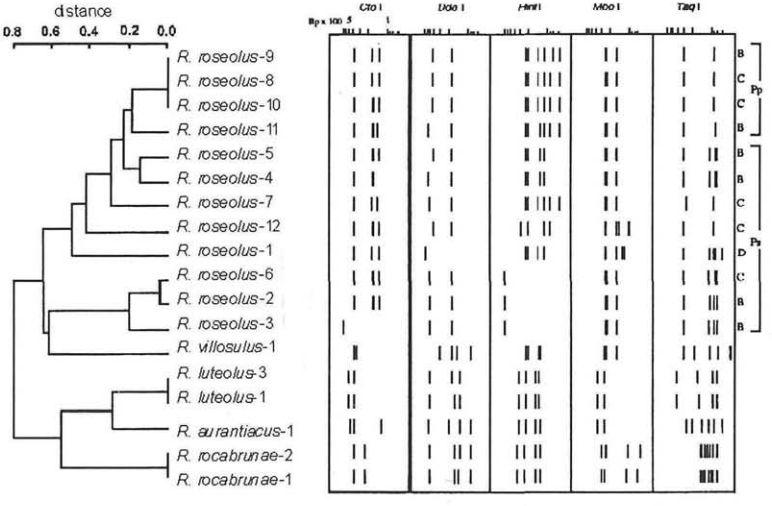

Fig. 1. Phenogram of the UPGMA cluster analysis of the RFLP patterns obtained with the restriction enzymes Cfo I, Dde I, Hinf I, Mbo I and Taq I. The spore volume of *R. roseolus* is indicated according to GROSS & al. 1980 and MARTÍN 1996. Mycorrhizal host: *Pinus pinaster* (Pp) and *Pinus sylvestris* (Ps).

Discussion

This study represents the first attempt to relate the taxa mentioned by early authors, in the *Rhizopogon roseolus* complex, with the polymorphism of the ITS rDNA using RFLP analysis.

Basidiomes of *R.roseolus* show a high polymorphism compared with data obtained for *R.villosulus* in MARTIN & al. 1998. The taxonomic conclusions for *R. roseolus* complex in GROSS & SCHMITT 1974 are not supported by this analysis since it is not possible to correlate the RFLP patterns with the spore volume: different basidiomes of the same sample, with the same spore volume, cluster in different branches (BCC-MPM 1898: ROSRH-2, -3, -4, -5). The relationship between *R. villosulus* and *R. roseolus* is not totally resolved. The UPGMA analysis seems to show an insufficient resolution since the pattern of the holotype of *R. villosulus* clusters among those of *R.roseolus.* The presence of *R.villosulus* between *R. roseolus* was unexpected due to the differences in both the morphological characters (e.g. duplex peridium vs. simple peridium) as the geographical origin (USA vs. Europe) and the mycorrhizal host (*Pseudotsuga menziesii vs. Pinus spp.*). The *R. roseolus* basidiomes are split out into two main groups, one of them formed by three basidiomes from collection BCC-MPM 1898 and the *R. villosulus* collection and the other with the rest of the basidiomes. No relation was observed in this separation due to the spore volume, since basidiomes from BCC-MPM 1898 with the same spore volume (B) were in both groups. This is in agreement with our previous results (MARTÍN 1996). Nor are the two main groups in the phenogram discrete with respect to the geographical origin or mycorrhizal host. This is in agreement with MOLINA & al. 1992 and ALVAREZ & al. 1993, who state that *R. roseolus* does not seem to be very specific regarding its mycorrhizal host. However, three basidiomes collected in three different regions of Italy under *Pinus pinaster* (ROSRHI-8, -9 and -10) cluster together with a high similarity coefficient (r=1). Moreover, these patterns were very similar (r=0.9111) to those obtained from a sample collected in Spain probably under the same mycorrhizal host (ROSRHI-11).

With respect to the other taxa included in this study, while the morphological characters allow a separation of *R. aurantiacus* and *R. luteolus* (MARTÍN 1996), the RFLP patterns are similar. The number of basidiomes of *R. rocabrunae* included in this study was certainly not enough to assess, whetherthis species is really monomorphic in its ITS rDNA; however it shows that RFLP may be a good tool to identifyherbarium or badly preserved collections: the fresh young fruitbodies of *R. rocabrunae* show the peridium covered by numerous isodiametric squamules giving an aspect of berries of the strawberry tree (*Arbutus*), whereas mature basidiomes take the colour of burnt umber, but in herbarium samples the squamules are inconspicous and probably this species has been misididentified with *R. roseolus.*

In conclusion, RFLP-analyses in *R. roseolus* has revealed a high level of polymorphism in the ITS rDNA not related with morphological characters stated by early authors. To get a complete picture of the genetic variability in this taxon more specimens must be analysed, using molecular characters. This will allow us to clarify the relation with the mycorrhizal host and to maximizestability of the

classification of the species in the genus *Rhizopogon*. RFLP reveals only a very small part of the possible nucleotide variation in the ITS by using restriction enzymes.Our current works aimed at sequencing some of these European basidiomes will allow comparison with the *Rhizopogon* data base being assembled by Lisa Grubisha at Oregon State University.

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