

SEKCIJA 1: FILOGENIJA
SESSION 1: PHYLOGENY

Retroelement phylogenomics and the root of eukaryotes

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The phylogeny of Eukaryota was the subject of important changes during the last few years. In its most recent form, the eukaryotic tree comprises 6 to 8 supergroups. It has been proposed that the root of the eukaryotic tree lies between unikonts and bikonts. Indeed, the division of eukaryotes into unikonts and bikonts on the basis of limited data, such as cell morphology, cilia structure, a few gene fusions and multigene analyses, is becoming a new biological dogma.

A very important eukaryotic genome component, retroelements, can shed a new light on finding the root of eukaryotes. Prokaryotic retroelements include retrons and retrointrons (group II introns). The direct progenitors of eukaryotic retroelements are mobile self-splicing retrointrons. They are simple and encode a single reverse transcriptase protein, whereas eukaryotic retroelements always encode multiple separate proteins. Eukaryotic retroelements are divided into a number of very large groups, such as non-LTR retrotransposons (contain 5 large groups), LTR retrotransposons (contain huge Metaviridae, Pseudoviridae, Retroviridae and BEL groups), DIRS retroelements and penelope retroelements.

The analysis of retroelement repertoires in all publicly available eukaryotic genomes (>130 nearly completed) shows extremely complex picture and a big problem of the proposed bikont/unikont dichotomy. The ancestral retroelement state inferred for the LCA of eukaryotes is extremely rich, not comparable to many eukaryotic supergroups. So, where is the problem? Problem is in the artificial bikont/unikont division of eukaryotes, especially in the most problematic Excavata supergroup. Since the transition from prokaryotes to eukaryotes (after genome fusion) was a gradual process, the eukaryotic retroelements did not originated in a single step («big bang» evolution) but through a slow and gradual process of evolution by acquisition of diverse host proteins.

Indeed, the gradual evolution of eukaryotic retroelements shows that very rare non-LTR retrotransposons of diplomonads and parabasalids correspond to the very ancestral eukaryotic retroelements. Later, in Euglenozoa, a first diversification of non-LTR retrotransposons can be observed. The DIRS group of retroelements also originated within the Euglenozoa. Even more important is that the DIRS retroelements were progenitors of the LTR retrotransposons, and the later have enormously reshaped most of eukaryotic genomes. When looking on the majority of eukaryotes from the euglenozoan perspective a huge explosion of retroelement lineages, diversity and copy numbers can be seen.

The phylogenomics of eukaryotic retroelements supports the Neoaercozoa hypothesis, since the position of diplomonads/parabasalids is at the root of the eukaryotic tree, the second supergroup that follows Neoaercozoa is Euglenozoa. The rest of the eukaryotes conforms to the unikont/bikont division. So, the eukaryotic retroelements partially support the latest multigene phylogenies of eukaryotes, with much better resolution of the Euglenozoa position in the eukaryotic tree.

High intraspecific diversity in ectomycorrhizal genus *Hydnium*

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Introduction

Hydnium species are common ectomycorrhizal fungi found in temperate forests under different deciduous and coniferous tree partners and in different soil conditions. Taxonomically the genus was first proposed by Linnaeus in 1737. Since then many species were included or excluded from the genus but still morphological differences between species cited to occur in Europe are not always clear. Several morphological species were recognised so far but no large-scale molecular evidence exists to confirm their exact number and taxonomic position. In current literature 4 species were described based on morphological and limited molecular data (Maas Geesteranus, 1975; Ostrow and Beenken, 2004). Our contribution deals with molecular diversity and phylogenetic relationships within the genus *Hydnium* based on rDNA ITS sequence analysis and a possible presence of cryptic species or common intraspecific diversity within the analysed nuclear regions.

Material and methods

Fungal material

33 specimens from the genus *Hydnium* were analysed (*H. albidum*, *H. repandum*, *H. umbilicatum* and *H. rufescens* complex). The fruitbodies were either freshly collected from various localities in years 1999 – 2002 and stored in the herbarium SFI (LJU) or were obtained from the herbarium MA-Fungi (Madrid, Spain). Eventhough *H. umbilicatum* has been cited to occur in Slovenia no reference material was available. The two exiccates included were kindly sent by Lorelei L. Norvell from the Pacific Northwest Mycology Service. Specimens with fully developed basidiomata and spores were used to ensure positive identification after morphological characters.

DNA extraction, amplification, sequencing and cloning

Twenty milligrams of the hymenium from fresh or dried material were used for the DNA extraction following standard protocols (Doyle & Doyle, 1990). For any method DNA was resuspended in pre-warmed, sterile milli-Q water to the approximate final concentration 100 ng/μl. Primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990) were used for PCR amplification of the ITS region, including 5.8 S rDNA, following the PCR protocol in Kraigher *et al.* (1995). Previous to sequencing the amplification products were cleaned using the E.Z.N.A. Clean kit and used for direct sequencing. When only weak PCR products were obtained, previous to sequence them, the products were cleaned from the gel using QIAquick Gel and cloned with pGEM®-T Easy Vector Systems.

Phylogenetic analyses

SEQAPP software for multiple sequences was used to search for the best alignment. Using the programme PAUP *Version 4.0b10 different phylogenetic analyses were performed. The alignment was analysed using maximum likelihood criterion (Felsenstein, 1981). In phylogenetic analyses sequences of *Clavulina cinerea* was used as outgroup.

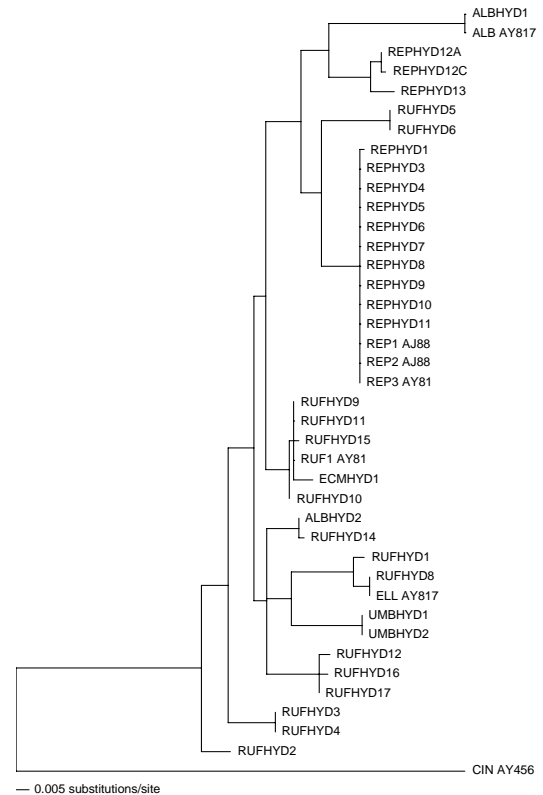
Results and discussion

The molecular data from previous study of *Hydnum* spp. (Agerer *et al.*, 1996) suggested a possible variation within the nuclear rDNA of *H. rufescens*. In our study mainly *H. rufescens* shows intraspecific variation and forms distinct clades within maximum likelihood phylogenetic tree, but also samples of *H. repandum* showed two distinct clades (REPHYD12, 13, origin from Spain, compared to other samples).

The distance between *H. umbilicatum* collections and nearest *H. rufescens* clade is similar to or shorter than distances between other clades of *H. rufescens*. Regarding the phylogenetic analysis the position of *H. umbilicatum* as a stable species is doubtful although it represents a good example of how little molecular variation can be observed in samples from geographically distant locations. *H. rufescens* shows highest polymorphism of ITS regions with one clade corresponding to *H. ellipsosporum* (Ostrow and Beenken, 2004). Based on the high molecular diversity of presumably homogeneous rDNA region in *H. rufescens* this species seems to be a species in a process of intensive speciation, not correlated to the geographical distances between different clades obtained. An important role in this process could play the ectomycorrhizal partner or some other, non-measured characteristics of the origins of the collections included in the study.

There are several possible explanations for a high molecular diversity in *H. rufescens*. Harrington and Rizzo (1999) reported a high importance of niche in determining the development and maintenance of fungal species which is not necessarily correlated to geographical distances. *H. rufescens* is a common species in Europe growing next to different ectomycorrhizal partners which could lead to a possible diversification at the molecular level.

Figure 1 (right): Phylogenetic tree for selected *Hydnum* collections, maximum likelihood tree (site variation with equal rates from all sites).



Acknowledgements

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References

- Agerer R., Kraigher H. and Javornik B. (1996). *Nova Hedwigia* 63(1-2), 183-194.
- Doyle J.J. and Doyle J.L. (1990). *Phytochemical Bulletin* 19, 11-15.
- Felsenstein J. (1981). *Journal of Molecular Evolution* 17, 368-376.
- Gardes M. and Bruns T.D. (1993). *Molecular Ecology* 2, 113-118.
- Harrington T.C. and Rizzo D.M. (1999). *Defining species in the fungi. Structure&Dynamics of Fungal Populations*. Kluwer Pr., Dordrecht, pp. 43-71.
- Kraigher H., Javornik B. and Agerer, R. (1995). *Mycorrhiza* 5 (3), 175-180.
- Maas Geesteranus R.A. (1975). *Verh. der Koninklijke Nederlandse Akademie van Wetenschappen, afd. Natuurkunde, Tweede reeks* 65, 1-127.
- Ostrow H. and Beenken L. (2004). *Zeitschrift fuer mykologie* 70/2, 137-156.
- White T.J., Bruns T., Lee S. and Taylor J. (1990). *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols. A Guide to Methods and Applications*. San Diego, Academic Press, pp. 315-322.

Phylogenetic analysis of disease resistance gene analogs from hop *Humulus lupulus* L.

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Introduction

The cultivated hop species are susceptible to many different pathogens. In Slovenia, the most devastating are downy mildew *Pseudoperonospora humuli*, powdery mildew *Sphaeroteca humuli* and fungus *Verticillium* spp. Plants use different defense mechanisms, and some of them rely on gene-for-gene interactions. Plant disease resistance genes (*R* genes) encode proteins that confer resistance to different pathogens including viruses, bacteria, nematodes and fungi. Most of the isolated *R* genes code for NBS-LRR proteins with characteristic nucleotide binding domain (NBS) and a leucine rich domain (LRR). NBS-LRR protein class is divided into two subclasses (TIR-NBS-LRR and CC-NBS-LRR) based on the amino terminal end of the NBS domain. The TIR-NBS-LRR and CC-NBS-LRR subclass differ in aminoacid motif organization, evolution and signal transduction pathways. The phylogenetic analysis, based on 92 different resistance gene analogs (RGAs) from hop, including resistance genes and RGA sequences from other plant species was carried out in order to establish the constitution of RGA hop sequences.

Material and methods

Ninety-two resistance gene analog sequences from hop cultivar Wye Target and hop line 2/1 (representing a wild accession) were previously isolated by using degenerative primers based on conserved NBS region from other plant taxa and newly design primers based on isolated hop sequences (data not shown). A phylogenetic tree was constructed, based on 92 unique aminoacid NBS sequences with open reading frame from hop along with the NBS domain of 10 *R* genes, 13 RGA sequences from different plant species and myosin heavy chain from thale-cress. A *Pto* gene coding for protein kinase served as an outgroup. The aminoacid sequence alignment using ClustalX (Thompson *et al.*, 1997) produced similarity matrix and a phylogenetic tree based on the Neighbor-joining method was constructed in program MEGA3 (Kumar *et al.*, 2004).

Results and discussion

The phylogenetic tree was constructed with the deduced aminoacid RGA hop sequences, since the similarity at the nucleotide level to *R* genes or *R* gene-like sequences from other plant species (GenBank) was not detected. On the phylogenetic tree produced from 92 different RGA hop sequences together with 10 *R* genes and 13 RGA sequences from other plant species, myosin heavy chain from thale cress and *Pto* gene which served as an outgroup, two major branches were identified (figure 1). One is combining sequences that belong to CC-NBS-LRR (60 hop RGAs) and the other to TIR-NBS-LRR subclass (30 hop RGAs) of NBS-LRR protein class of *R* genes. Another group combining only two sequences (one from Wye Target and the other from line 2/1) is placed separately with a long genetic distance from two major classes. The aminoacid motifs of those two sequences show that they belong to TIR-NBS-LRR subclass thus revealing high genetic diversity of TIR-NBS-LRR RGAs in hop. In dicot genomes the number of CC-NBS-LRR sequences is estimated as one third of all *R* genes or *R* gene-like sequences. In the *Arabidopsis thaliana* genome there are 50 CC-NBS-LRR *R* gene sequences out of all 150 *R* genes (Meyers *et al.*, 1999), although the percentage may vary according to different plant species. The ratio between CC-NBS-LRR and TIR-NBS-LRR hop sequences is 2:1. The separation of hop RGA sequences to CC-NBS-LRR and TIR-NBS-LRR subclass is supported by specific structural amino motifs as confirmed by MEME analysis (Bailey

and Elkan, 1994), that appear to have functional significance (Young, 2000). Most of the pseudogene sequences are placed within CC-NBS-LRR subclass (data not shown) thus supporting a degenerative state and hypothesis of CC-NBS-LRR sequences being common ancestors to both types of NBS-LRR genes, while TIR-NBS-LRR are evolving with more recent diversification. The phylogenetic analysis revealed that some RGA hop sequences are more similar to RGA sequences from other plant taxa than to other hop sequences, which is consistent to the hypothesis of gene duplication and further gene diversification in the evolution of NBS-LRR resistance genes (Pan *et al.*, 2000). The highest similarity of hop deduced aminoacid RGA sequences was found with *R* genes isolated from *Solanaceae* family, sharing more than 47 % of aminoacid sequence with tomato *I2* gene against fungus *Fusarium axysporium* and potato *R3a* gene conferring resistance to late blight *Phytophthora infestans*. The separation of *R* genes to CNL or TNL protein class is of great importance at studying the expression of resistance, since proteins of CNL and TNL class are involved in different signal transduction pathways.

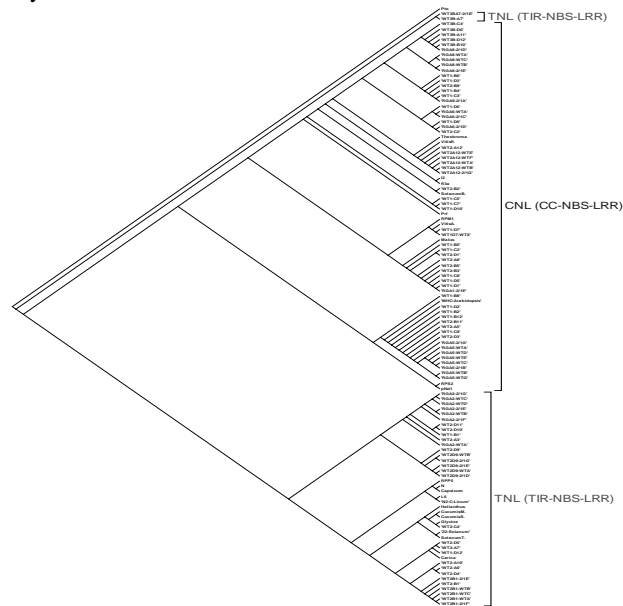


Figure 1: Phylogenetic relationship of RGA hop sequences with *R* genes or RGAs from other plant species

References

- Bailey T.L., Elkan C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings of the second international conference on intelligent systems for molecular biology. Menlo Park, California. AAAI Press.:28-36
- Kumar S., Tamura K., Nei M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5: 150-163.
- Meyers B.C. Dickerman A.W., Michelmore R.W., Sivaramakrishnan S., Sobral B.W., Young N.D. (1999). Plant Disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant Journal* 20: 317-322.
- Pan Q., Wendel J., Fluhr R. (2000). Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *Journal of Molecular Evolution* 50: 203-213.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24:4876-4882
- Young N.D. (2000). The genetic architecture of resistance. *Current Opinion in Plant Biology* 3: 285-290.

Determination of *Epilobium* species by ATR-IR spectroscopy

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Introduction

Genus *Epilobium*, in Europe represented by 26 species (Raven 1980, Snogerup 1983), is considered as taxonomically difficult due to the inconspicuous distinguishing characters and high frequency of hybridisation among almost all pairs of species. Related genus *Chamerion*, comprising three species in European flora, is often included in genus *Epilobium*. *Epilobium* and *Chamerion* species are used in traditional medicine as a treatment for benign prostate adenomas and the associated problems. Also for this reason many chemotaxonomic studies, mostly on flavonoids were made on genera *Epilobium* and *Chamerion*. Using LC-UV and thermospray LC-MS for flavonoids analyses, differences among genera *Epilobium* and *Chamerion* were evident, but there was not enough information for reliable discrimination on species level (Ducrey 1995). In contrast, FT-IR spectroscopy was already presented as rapid, effective and accurate method for pharmaceutical research, used for determination of fungal (Haag & al. 1996), plant (Belton & al. 1995, Liu & al 2006) and bacterial (Miguel Gomez & al. 2003) samples. The aim of our study was to analyse the differences among ATR-IR spectra of *Epilobium* species and find out if method is appropriate for determination of *Epilobium* specimens.

Materials and methods

70 herbarium specimens belonging to 13 *Epilobium* and 2 *Chamerion* species collected in Slovenia were analysed. Measurements were made with spectrometer FTIR Nexus (Nicolet) with Smart DuraSamplIR Diamond ATR Accessory for attenuated total reflectance (ATR) IR technique. All measurements were made on adaxial leaf side of dry leaves between leaf veins and some of them on finely crumbled leaves. Spectra that were superficially different from common spectra of one specimen were excluded from analyses. First derivative and average of all measured spectra on one specimen were calculated. SPSS 13.0 was used for statistical analyses.

Results and discussion

In almost all published papers (Belton & al. 1995, Liu & al 2006, Haag & al. 1996) samples were grounded or homogenised prior to scanning IR spectra. Our preliminary experiment showed that homogenisation is avoidable part of sample preparation, and measurements made on whole leaves also gave good results. The results of discriminant analysis of spectra using "leave one out" method provide 90% likelihood of correct classification (Tab. 1). The result is comparable to outcome published for bacteria (Miguel Gomez & al. 2003) and actinomycetes (Haag & al. 1996). Missdeterminations were mostly within sections of genus *Epilobium* and there were no missdetermination among genera. Better result, 95% likelihood of correct classification of individual spectra (Fig. 1) indicates that spectra are not only species, but also specimen characteristic.

Conclusions

The present study demonstrates that FT-IR can be used for determination of very closely related *Epilobium* species with relatively high probability, even without preliminary homogenization of samples. Rapid measurements of FT-IR spectra on dried plant material

enable us to make good standardized collection of spectra based on determined specimens from research area. The method could be then used for quality control of *Epilobium* an other similar drugs, which consist of particles of crumbled plant material.

Table 1: The correct classification of samples based on the results of discriminant analysis using "leave one out" method. Left columns show results where average spectra were calculated for every specimen. Right columns show results for all spectra individually.

	average spectra of a specimen		all spectra (8-12 per specimen)	
	no.	%	no.	%
<i>C. angustifolium</i>	-	-	21	100
<i>C. dodonaei</i>	-	-	8	100
<i>E. montanum</i>	5	100	43	100
<i>E. collinum</i>	8	100	65	97
<i>E. parviflorum</i>	5	83	70	97
<i>E. hirsutum</i>	2	50	39	100
<i>E. tetragonum</i>	3	100	26	100
<i>E. lamyi</i>	3	100	19	95
<i>E. obscurum</i>	2	67	23	100
<i>E. palustre</i>	3	75	26	83
<i>E. anagallidifolium</i>	4	100	24	100
<i>E. alsinifolium</i>	5	100	29	81
<i>E. alpestre</i>	5	83	47	100
<i>E. roseum</i>	7	88	65	93
<i>E. ciliatum</i>	8	100	56	89
average		91		95

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References

- Belton P. S., Kemsley E. K., McCann M. C., Ttofis S., Wilson R. H. and Delgadillo I. 1995. *Food Chemistry* 54(4), 437-441.
- Ducrey B., Wolfender J. L., Marston A. and Hostettmann K. (1995). *Phytochemistry* 38 (1), 129-137.
- Haag H., Gremlich H.-U., Bergmann R. and Snglier J.-J. (1996). *Jurnal of Microbiological Metods* 27, 157-163.
- Liu H. X., Sun S. Q., Lv G. H. and Chan K. K. C. 2006. *Spectrochimica Acta Part A-Molecular and Biomolecular Spectroscopy* 64 (2), 321-326.
- Miguel Gomez, M. A., Bratos Perez M. A., Martin Gil F. J., Duenas Diez A., Martin Rodriguez J. F., Gutierrez Rodriguez P., Orduna Domingo A. and Rodriguez Torres A. (2003). *Journal of Mocabiological Methods* 55, 121-131.
- Raven P. H. 1980. *Epilobium* L. V: Flora Europaea 2. Tutin T. G., Heywood V. H., Burge N. A., Moore D. M., Valentine D. H., Walters S. M. and Webb D. A. (ed.), CUP, Cambridge: 308-311.
- Snogerup S. 1982. *Willdenowia* 14: 227-229.