

# The Exobasidiales: an evolutionary hypothesis<sup>1</sup>

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To gain insight in the phylogenetic relationships within the Exobasidiales, septal pore apparatus, host-parasite interactions, sori, hymenia, basidia, and nucleotide sequences from the 5' terminal domain of the nuclear large subunit rRNA gene were studied and compared.

The results of our molecular phylogenetic analyses correlate well with the morphological data and both reflect the distribution of parasites on several host groups. Thus, the Exobasidiales seem to be divided into four groups, which are distinguishable by basidial morphology and host range as follows: (i) the Exobasidiaceae parasitizing mainly Ericaceae are characterized by an abaxial orientation of the hilar appendices of the ballistospore on the elongate basidia, (ii) the Cryptobasidiaceae occurring mainly on Lauraceae sporulate inside the host tissue with elongate gastroid basidia, (iii) the Brachybasidiaceae living on monocots are characterized by elongate basidia bearing two ballistospore with adaxially oriented hilar appendices, and (iv) the Graphiolaceae occurring on palms produce chains of gastroid basidia in distinct basidiocarps. The arrangement of the four groups and the tree topology within *Exobasidium* derived from the molecular analyses essentially parallel phylogenetic host relationships, suggesting cospeciation. Based on our results, however, the radiation of *Exobasidium* on Vaccinioideae cannot be explained by cospeciation alone.

In the new system of Ustilaginomycetes (BAUER, OBERWINKLER & VÁNKY 1997), all species lacking teliospores belong either to the Microstromatales (BEGEROW, BAUER & OBERWINKLER 2001) or Exobasidiales. BAUER et al. (2001a) used the order Exobasidiales for species having local complex host-parasite interaction apparatus with the formation of interaction rings. Molecular analyses confirmed this group (BEGEROW, BAUER & OBERWINKLER 1997; BAUER et al. 2001a). Morphologically, however, the Exobasidiales possess a high degree of divergence (BAUER, BEGEROW & OBERWINKLER 1998, BAUER et al. 2001a). In addition, phylogenetic and evolutionary aspects within this group are poorly understood. For example, the G+C content of the DNA, RFLP analyses of the nuclear large subunit rRNA gene as well as molecular phylogenetic analyses of the nuclear small subunit rRNA gene show a great heterogeneity even among the *Exobasidium* species tested (BLANZ & OBERWINKLER 1983, BLANZ & DÖRING 1995, DÖRING & BLANZ 2000). Therefore, in the present study we compare morphological, ultrastructural, and molecular characters of members of the Exobasidiales, in order to estimate the phylogenetic relationship in this group. Based on the results, evolutionary strategies are discussed.

## Material and methods

Specimens, the respective characters studied, and the origin of the sequences are listed in Table 1.

For the study of sori, freehand sections through infected areas of leaves were mounted in 3% KOH and examined with light microscope using phase contrast optics.

For the study of the ballistospore or gastroid nature of *Laurobasidium lauri*, the ballistospore-fall method was used (DERX 1930): living sori were fixed inside the caps of Petri dishes containing 2% water agar and kept at room temperature. After two days the agar underneath the sori was cut out in pieces of about 15 mm in diameter, transferred to slides, and observed by light microscopy.

For TEM, except for *Exobasidium pachysporum* all specimens were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature overnight. Following six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1% osmium tetroxide in the same buffer for 1 h in the dark, washed in distilled water, and stained in 1% aqueous uranyl acetate for 1 h in the dark. After five washes in distilled water, samples were dehydrated in acetone, using 10 min changes at 25%, 50%, 70%, 95%, and 3 times in 100% acetone. Samples were embedded in Spurr's plastic and sectioned with a diamond knife. Ultrathin serial sections were mounted on formvar-coated, single-slot copper grids, stained with lead citrate at room temperature for 5 min, and washed with distilled water. They were examined using a transmission electron microscope operating at 80 kV.

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Tab. 1. Specimens and characters studied

Specimens	Hosts	Characters studied <sup>1</sup>	Sequences <sup>2</sup>	Source <sup>3</sup>
<b>1. Exobasidiales</b>				
<i>Arcticomyces warmingii</i> (Rostr.) Savile	<i>Saxifraga bryoides</i> L.	H, S	AF 487380*	R.B. 3081
<i>Botryoconis tumefaciens</i> (Winter) H. & P. Sydow	laurel	H		Rbh., Fgi. eur. 3295 (M)
<i>Brachybasidium pinangae</i> (Rac.) Gäum.	<i>Pinanga kuhlii</i> Blume	H		Sydow, Fgi. exot. exs. 455 (M)
<i>Clinoconidium bullatum</i> H. Syd.	<i>Phoebe neurophylla</i> Metz & Pittier	H, S	AF 487383*	Sydow, Fgi. exot. exs. 553 (M)
<i>Clinoconidium cf. bullatum</i>	<i>Apollonias barbujana</i> (Cav.) Bornm.	H, S	AF 487382*	R.B. 3002 <sup>4</sup>
<i>Clinoconidium</i> sp.	<i>Cinnamomum japonicum</i> Sieb.	H, S	AF 487381*	R.B. 3082 <sup>5</sup>
<i>Coniodictyum chevalieri</i> Har. & Pat.	<i>Zizyphus mucronata</i> Willd.	H, S	AF 487384*	R.B. 1000 <sup>6</sup>
<i>Dicellomyces calami</i> R. Berndt & N. D. Sharma	<i>Calami cf. rotangis</i> L.	H		R.B. 3084 <sup>7</sup>
<i>Dicellomyces scirpi</i> Raitv.	<i>Scirpus sylvaticus</i> L.	H, S	AF 487385*	R.B. 1032
<i>Drepanoconis brasiliensis</i> Schröter & P. Henn.	<i>Ocotea</i> sp.	H		Rbh., Fgi. eur. 4495 (M)
<i>Exobasidiellum graminicola</i> (Bres.) Donk	<i>Bromus inermis</i> Leysser	H		Krieger, Fgi. saxon. 664 (M)
<i>Exobasidium arescens</i> Nannf.	<i>Vaccinium myrtillus</i> L.	H, S	AF 352057 <sup>Be</sup>	R.B. 2047
<i>Exobasidium bisporum</i> Sawada ex A. Ezuka	<i>Eubotryoides grayana</i> Hara	S	AF 487386*	DSM 4454
<i>Exobasidium gracile</i> (Shirai) Sydow	<i>Camellia</i> sp.	S	AF 487387*	DSM 4441
<i>Exobasidium japonicum</i> Shirai	<i>Rhododendron lateritium</i> Planch	S	AF 487388*	DSM 4463
<i>Exobasidium karstenii</i> Sacc. & Trott.	<i>Andromeda polifolia</i> L.	H, S	AF 487389*	R.B. 2052
<i>Exobasidium myrtilli</i> Siegm.	<i>Vaccinium myrtillus</i> L.	H, S	AF 487390*	R.B. 2055
<i>Exobasidium oxycocci</i> Rostrup ex Shear	<i>Vaccinium oxycoccos</i> L.	H		F.O. 18925
<i>Exobasidium oxycocci</i> Rostrup ex Shear	<i>Vaccinium oxycoccos</i> L.	H, S	AF 487391*	R.B. 2086
<i>Exobasidium pachysporum</i> Nannf.	<i>Vaccinium uliginosum</i> L.	H, S	AF 487392*	R.B. 947
<i>Exobasidium pieridis-ovalifoliae</i> Sawada	<i>Lyonia neziki</i> Nakai & Hara	S	AF 487393*	DSM 4455
<i>Exobasidium reticulatum</i> Ito & Sawada	<i>Thea sinensis</i> L.	S	AF 487394*	DSM 4520
<i>Exobasidium rhododendri</i> (Fuck.) Cram.	<i>Rhododendron ferrugineum</i> L.	H, S	AF 009858 <sup>B</sup>	R.B. 2050
<i>Exobasidium rostrupii</i> Nannf.	<i>Vaccinium oxycoccos</i> L.	H, S	AF 009857 <sup>B</sup>	R.B. 949
<i>Exobasidium shiraianum</i> Henn.	<i>Rhododendron degranianum</i> Carr.	S	AF 487395*	DSM 4522
<i>Exobasidium</i> sp.	<i>Rhododendron</i> sp.	H		F.O. 39680
<i>Exobasidium sundstroemii</i> Nannf.	<i>Andromeda polifolia</i> L.	H, S	AF 487396*	R.B. 2051
<i>Exobasidium symploci-japonicae</i> Kusano & Tokubuchi	<i>Symplocos</i> sp.	S	AF 487397*	DSM 4523
<i>Exobasidium vaccinii</i> (Fuck.) Woronin 1	<i>Vaccinium vitis-idaea</i> L.	H, S	AF 009858 <sup>B</sup>	R.B. 945
<i>Exobasidium vaccinii</i> (Fuck.) Woronin 2	<i>Vaccinium vitis-idaea</i> L.	H, S	AF 487398*	R.B. 2073
<i>Exobasidium yoshinagai</i> Henn.	<i>Rhododendron reticulatum</i> D. Don ex G. Don	S	AF 487399*	DSM 4524
<i>Graphiola cylindrica</i> Kobayasi	palm	S	AF 487400*	JCM 8561
<i>Graphiola cylindrica</i> Kobayasi	palm	H		R.B. 3083 <sup>8</sup>
<i>Graphiola phoenicis</i> (Moug.) Poiteau	<i>Phoenix canariensis</i> Chaub.	H, S	AF 009862 <sup>B</sup>	F.O. 29350
<i>Kordyana celebensis</i> Gäum.	<i>Commelina</i> sp.	S	AF 487401*	HB 17
<i>Kordyana tradescantiae</i> (Pat.) Rac.	<i>Tradescantia</i> sp.	H, S	AF 487402*	F.O. 47147
<i>Laurobasidium lauri</i> (Geyler) Jülich	<i>Laurus azorica</i> (Seub.) Franco	H, S	AF 487403*	M.P. 2371
<i>Muribasidiospora hesperidium</i> (Maire) Kamat & Rajendren	<i>Rhus glaucescens</i> A. Rich.	H		IMI 5074
<i>Muribasidiospora indica</i> Kamat & Rajendren	<i>Rhus lancea</i> E. Mey. ex Harv. & Sond.	H, S	AF 352058 <sup>Be</sup>	F.O. 47397
<i>Proliferobasidium heliconiae</i> Cunningham	<i>Heliconiae bihai</i> L.	H		BPI 726024

Tab. 1. continued

## 2. Other Ustilaginomycetes

<i>Entyloma microsporium</i> (Unger) Schröter	<i>Ranunculus repens</i> L.	S	AF 007530 <sup>B</sup>	F.O. 37329
<i>Georgafischeria riveae</i> Thirum. & Naras.	<i>Rivea hypocrateriformis</i> Chois	S	AF 009861 <sup>B</sup>	HUV 15614
<i>Microstroma juglandis</i> (Bereng.) Sacc.	<i>Juglans regia</i> L.	S	AF 009867 <sup>B</sup>	F.O. 39211
<i>Rhaphospora nymphaeae</i> D. D. Cunn.	<i>Nymphaea alba</i> L.	S	AF 007526 <sup>B</sup>	R.B. 862
<i>Tilletia caries</i> (DC.) Tul.	<i>Triticum aestivum</i> L.	S	AJ 235308 <sup>Bo</sup>	CBS 160.85
<i>Ustilago hordei</i> (Pers.) Lagerh.	not cited	S	L 20286 <sup>BER</sup>	D.M. 11.2C

1) H = Hyphal septation, cellular host-parasite interaction and sporulation; S = Sequence

2) Origin of sequences: B = BEGEROW, BAUER & OBERWINKLER (1997), BE = BEGEROW, BAUER & OBERWINKLER (2001), BER = BERRES, SZABO & McLAUGHLIN (1995), Bo = BOEKHOUT, FELL & O'DONNELL (1995), \* = new sequences

3) BPI = US National Fungus Collections, Beltsville, USA; CBS = Culture collection of the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DSM = Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; D.M. = D. Hills, Corvallis, USA; HB = Institut für angewandte Mikrobiologie, Wien, Austria; HUV = Herbarium Ustilaginales Vánky, Tübingen, Germany; F.O. = Herbarium F. Oberwinkler, Tübingen, Germany; IMI = Herbarium of the CABI Bioscience UK Centre, Egham, England; M = Botanische Staatssammlung, München, Germany; M.P. = Herbarium M. Piepenbring, Frankfurt, Germany; R.B. = Herbarium R. Bauer, Tübingen, Germany

4) Material obtained from H. D. Frey, Tübingen, Germany

5) Material obtained from M. Kakishima, Tsukuba, Japan

6) Material obtained from J. P. van der Walt, Pretoria, South Africa

7) Material obtained from R. Berndt, Tübingen, Germany

8) Material obtained from R. J. Bandoni, Vancouver, Canada

*Exobasidium pachysporum* was prepared by high pressure freezing and freeze substitution. Infected areas of leaves were removed with a 2 mm cork borer. To remove air from intercellular spaces, samples were infiltrated with distilled water containing 6 % (v/v) (2.5 M) methanol for approximately 5 min at room temperature. Single samples were placed in an aluminium holder (one half with a hollow of 0.3 mm depth for the sample and the other a flat top) and frozen immediately in the high pressure freezer HPM 010 (Balzers Union, Lichtenstein) as described in detail by MENDGEN et al. (1991). Substitution medium (1.5 ml per specimen) consisted of 2% osmium tetroxide in acetone which had been dried over calcium chloride. Freeze substitution was performed at  $-90^{\circ}\text{C}$ ,  $-60^{\circ}\text{C}$ , and  $-30^{\circ}\text{C}$ , 8h for each step, using a Balzers freeze substitution apparatus FSU 010. The temperature was then raised to approximately  $0^{\circ}\text{C}$  over a 30 min period and samples were washed in dry acetone for another 30 min. Infiltration with an Epon/Araldite mixture (WELTER, MÜLLER & MENDGEN 1988) was performed stepwise: 30% resin in acetone at  $4^{\circ}\text{C}$  for 7 h, 70 % and 100 % resin at  $8^{\circ}\text{C}$  for 20 h each and 100% resin at  $18^{\circ}\text{C}$  for approximately 12h. Samples were then transferred to fresh medium and polymerized at  $60^{\circ}\text{C}$  for 10 h. Finally, samples were processed as for chemically fixed samples described above except that the sections were additionally stained with 1 % aqueous uranyl acetate for 1 h.

Molecular data were obtained using the same methods as described earlier (BEGEROW, BAUER & OBERWINKLER 1997).

We used the 5' terminal region of the nuclear large subunit rRNA gene to build an alignment of 534 bp. The alignment was produced using Clustal X (JEANNMOUGIN et al. 1998) and optimized visually. Because of problems in the alignment the positions 35-42 and 373-385 were excluded in the phylogenetic analyses. Phylogenetic analyses were done with PAUP® 4.0b8a (SWOFFORD 1998). Maximum parsimony was carried out in three steps. First, a heuristic search was done with 100.000 random sequence additions without branch swapping to search for the best islands. Thereafter we performed TBR branch swapping over the 17 best trees from the first step, which resulted in 104 most parsimonious trees. The consensus tree from the 104 trees with 757 steps was computed. Finally, bootstrap values were calculated for 1000 replicates with 10 random sequence additions without branch swapping in each replicate. Modeltest 3.0 (POSADA & CRANDALL 1998) was carried out to determine a model of DNA substitution that fits the data set and TIMIG was selected from the Akaike Information Criterion (base frequencies:  $\pi_A = 0.2673$ ,  $\pi_C = 0.1741$ ,  $\pi_G = 0.3026$ ,  $\pi_T = 0.2326$ ; substitution rates:  $A/C = G/T = 1.0000$ ,  $A/G = 2.4778$ ,  $A/T = C/G = 0.7648$ ,  $C/T = 6.4718$ ; gamma shape parameter = 0.7648; percentage of invariant sites = 0.3651). Neighbor-joining analysis was done using genetic distances according to the specified substitution model (see SWOFFORD et al. 1996). 10.000 replicates were used for bootstrap analysis. The alignment is available upon request.

