ORIGINAL ARTICLE

Exobasidium darwinii, a new Hawaiian species infecting endemic *Vaccinium reticulatum* in Haleakala National Park

Marcin Piątek · Matthias Lutz · Patti Welton

Abstract Hawaii is one of the most isolated archipelagos in the world, situated about 4,000 km from the nearest continent, and never connected with continental land masses. Two Hawaiian endemic blueberries, *Vaccinium calycinum* and *V. reticulatum*, are infected by *Exobasidium* species previously recognized as *Exobasidium vaccinii*. However, because of the high host-specificity of *Exobasidium*, it seems unlikely that the species infecting *Vaccinium calycinum* and *V. reticulatum* belongs to *Exobasidium vaccinii*, which in the current circumscription is restricted to *Vaccinium vitis-idaea*. We collected a fresh specimen of *Exobasidium* on *Vaccinium reticulatum* in Haleakala National Park, and analysed it by a combined morphological and molecular approach. The morphological and phylogenetic analyses (based on the LSU and the concatenated ITS+LSU sequence data) showed that *Exobasidium* on *Vaccinium reticulatum* belongs to an undescribed species, distinct from *Exobasidium vaccinii* and any other species. The name *Exobasidium darwinii* is proposed for this novel taxon. This species is characterized among others by the production of peculiar witches' brooms with bright red leaves on the infected branches of *Vaccinium reticulatum*. Relevant characters of *Exobasidium darwinii* are described and illustrated, additionally phylogenetic relationships of the new species are discussed.

Keywords Exobasidiomycetes · ITS · LSU · Molecular phylogeny · Ustilaginomycotina

M. Piątek (✉)
Department of Mycology, W. Szafer Institute of Botany,
Polish Academy of Sciences,
Lubicz 46,
PL-31-512 Kraków, Poland
e-mail: m.piatek@botany.pl

M. Lutz
Lehrstuhl Organismische Botanik,
Institut für Evolution und Ökologie, Universität Tübingen,
Auf der Morgenstelle 1,
72076 Tübingen, Germany
e-mail: matthias.lutz@uni-tuebingen.de

P. Welton
Haleakala National Park, National Park Service,
P.O. Box 369, Makawao, HI 96768, USA
e-mail: Patti_Welton@nps.gov

Introduction

Exobasidium Woronin is the largest genus within the Exobasidiales with 178 epithets included in the *Index Fungorum* (http://www.indexfungorum.org/Names/Names.asp). Some of these names are invalidly published, others are synonymous or represent species of other genera (e.g., *Arcticomyces* Savile, *Exobasidiellum* Donk, *Kordyana* Racib., *Laurobasidium* Jülich, *Muribasidiospora* Kamat & Rajendren). Yet the genus is poorly known and, except for Europe, there is no comprehensive study of *Exobasidium*. In the past, *Exobasidium* species were described based on supposed host specificity, but this concept was later abandoned by Burt (1915) and Savile (1959), who lumped together many species, especially within *Exobasidium vaccinii* (Fuckel) Woronin and *Exobasidium vaccinii-uliginosi* Boud. The high level of host specialization was again postulated for European *Exobasidium* species by Nannfeldt (1981) on the basis of a detailed analysis of morphological characters correlated with host plant and physiological data (the latter elaborated by Sundström 1964). His ideas were basically supported by molecular analyses of Blanz and Oberwinkler (1983), Blanz and

Döring ([1995](#)) and Begerow et al. ([2002](#)), although in this latter study support for most branches was low and obtained resolution was not fully satisfactorily for conclusions about evolutionary relationships within both *Exobasidium* and the Exobasidiales.

Located in the middle of the Pacific Ocean, Hawaii is one of the most isolated archipelagos in the world. The islands are situated about 4,000 km from the nearest continent and have never been connected with continental land masses. The indigenous flowering plants of Hawaii include 1,029 species, of which ca. 89% are endemic to the islands (Sakai et al. [2002](#)) likely harbouring many undescribed pathogenic fungi that are usually strictly correlated with host plants. Two Hawaiian endemic blueberries, *Vaccinium calycinum* Sm. and *V. reticulatum* Sm. (incl. *V. peleanum* Skottsb.), were reported as hosts for *Exobasidium* species producing peculiar witches' brooms with bright red leaves on infected branches (Gardner [1985](#)). Although the causal agent was first considered as a mycoplasma-like organism, it soon became evident that it is rather a species of *Exobasidium*, which has been referred to as *Exobasidium vaccinii* but with uncertainty whether it is native or alien on Hawaiian blueberries (Gardner [1985](#)). However, in the light of the high specificity of *Exobasidium*, usually to the level of host species, rarely to a group of closely allied species (Nannfeldt [1981](#)), it seems unlikely that the species infecting *Vaccinium calycinum* and *V. reticulatum* belongs to *Exobasidium vaccinii*. That latter species is quite a different fungus, producing localized, conspicuous and much thickened concavities on leaves of *Vaccinium vitis-idaea* L. Only occasionally it may cause deformations of all shoot-tips (Nannfeldt [1981](#)), which however are never as strong as on the Hawaiian blueberries. Therefore, we hypothesize that *Exobasidium* on *Vaccinium calycinum* and *V. reticulatum* represents in fact one (or even two) distinct species. To test this hypothesis, we collected a fresh specimen of *Exobasidium* on *Vaccinium reticulatum* in Haleakala National Park, and analysed it by a combined morphological and molecular approach. Unfortunately, the corresponding specimen of *Exobasidium* on *Vaccinium calycinum* has not hitherto been re-collected.

Materials and methods

The *Exobasidium* specimen infecting *Vaccinium reticulatum* was collected in Haleakala National Park in Hawaii on 17 December 2007. This specimen was used in subsequent morphological and phylogenetic analyses. Information on the specimens of *Exobasidium arescens* Nannf. and *E. rostrupii* Nannf. sequenced for the present study is given in Table [1](#).

Morphological analyses

The macroscopic symptoms of host infection and microscopic features of *Exobasidium* on *Vaccinium reticulatum* were studied using dried herbarium material. The terminology used in the species description and in the text follows Nannfeldt ([1981](#)). Morphological observations and measurements of basidia, basidiospores and conidia were made by standard light and phase contrast microscopy (LM) of the material mounted in 3% KOH. A Nikon Eclipse 80i light microscope was used for LM studies. Thirty basidiospores and conidia were measured from the studied collection, using NIS-Elements BR 3.0 imaging software. LM micrographs were taken with a Nikon DS-Fi1 camera. The observations of basidia were supplemented by scanning electron microscopy (SEM). For this purpose, a fragment of infected leaf was glued to the carbon tab and fixed to an aluminium stub with double-sided transparent tape. The sample was sputter-coated with carbon using a Cressington sputter-coater and viewed with a Hitachi S-4700 scanning electron microscope, at 10 kV and 9,000 nA, with a working distance of ca. 12 mm. SEM micrographs were taken in the Laboratory of Field Emission Scanning Electron Microscopy and Microanalysis at the Institute of Geological Sciences of the Jagiellonian University, Kraków (Poland).

Molecular analyses

Genomic DNA was isolated from the herbarium specimens. For methods of isolation and crushing of fungal material, DNA extraction, amplification, purification of PCR products, sequencing, and processing of the raw data, see Lutz et al. ([2004](#)). We determined base sequences of the ITS1/2 region of the nuc-rDNA including the 5.8 S rDNA (ITS) and of the 5′-end of the nuc-LSU rDNA including the domains D1/D2 (LSU). The ITS was amplified using the primer pair ITS1f and ITS4 (Gardes and Bruns [1993](#)). The LSU was amplified using the primer pair NL1 and NL4 (O'Donnell [1992](#), [1993](#)). For amplification of both regions, we adjusted the annealing temperature to 45°C. DNA sequences prepared in the course of this study were deposited in GenBank; accession numbers are given in Table [1](#), and Figs. [1](#) and [2](#).

To obtain a hypothesis on the phylogenetic position of the specimen infecting *Vaccinium reticulatum*, we followed two strategies: (1) we analysed its LSU sequence together with sequences of most *Exobasidium* species available in GenBank, including sequences of all available species growing on *Vaccinium* L. and all available species producing witches' brooms, respectively, and sequences of selected representatives of other genera of Exobasidiales (GenBank accession numbers are given in Fig. [1](#)), and (2) we analysed the concatenated ITS+LSU sequences of the specimen from Hawaii within a dataset reduced to the

Table 1 List of species used in the molecular analyses with host plants, country of origin, voucher/strain information, GenBank accession numbers, and sources

Species	Host plant, country	Voucher/strain	GenBank acc. no. (ITS/LSU)	Source
Arcticomyces warmingii (Rostr.) Savile	*Saxifraga bryoides* L., –	R. Bauer 3081/–	–/AF487380	Begerow et al. 2002
Clinoconidium bullatum Syd.	*Phoebe neurophylla* Mez & Pittier, –	M – Sydow Fungi Exot. exs. 553/–	–/AF487383	Begerow et al. 2002
Exobasidium arescens Nannf.	*Vaccinium myrtillus* L., Slovenia	Slovenia, Bled, Pokljuka, Mocila, 28 Jul. 2006, leg. M. Lutz, TUB 015031	FJ896135/FJ896136	This study
Exobasidium bisporum Sawada ex Ezuka	*Eubotryoides grayana* (Maxim.) H. Hara var. *oblongifolia* (Miq.) H. Hara, Japan	IFO H-12038/IFO 30152	AB180368/AB177596	GenBank, submitted by H. Nagao in 2004
Exobasidium bisporum Sawada ex Ezuka	*Eubotryoides grayana* (Maxim.) H. Hara var. *glabra* (Komatsu) H. Hara, Japan	IFO H-12021/IFO 9942	AB180364/AB177598	GenBank, submitted by H. Nagao in 2004
Exobasidium camelliae Shirai	*Camellia japonica* L., Japan	EC (TUK)/MAFF 238578	–/AB176712	GenBank, submitted by H. Nagao in 2004
Exobasidium cylindrosporum Ezuka	*Rhododendron macrosepalum* Maxim., Japan	TSH-B0127/MAFF 238177	–/AB176713	GenBank, submitted by H. Nagao in 2004
Exobasidium darwinii M. Piątek & M. Lutz	*Vaccinium reticulatum* Sm., Hawaii	U.S.A., Hawaii, Maui, north facing slopes of Haleakala, near Hosmers Grove, 17 Dec. 2007, leg. P. Welton, holotype: KRAM F-47124, isotype: TUB 019166	FJ896133/FJ896134	This study
Exobasidium gracile (Shirai) Syd. & P. Syd.	*Camellia sasanqua* Thunb., Japan	–/TUK-E30	–/AB177592	GenBank, submitted by H. Nagao in 2004
Exobasidium inconspicuum Nagao & Ezuka	*Vaccinium hirtum* Thunb. var. *pubescens* (Koidz.) T. Yamaz., Japan	TSH-B0080/ MAFF 238616	AB180347/ AB177556	GenBank, submitted by H. Nagao in 2004
Exobasidium japonicum Shirai	*Rhododendron indicum* (L.) Sweet, Japan	TSH-B0011/ MAFF 238176	–/AB177548	GenBank, submitted by H. Nagao in 2004
Exobasidium karstenii Sacc. & Trotter	*Andromeda polifolia* L., –	R. Bauer 2052/–	–/AF487389	Begerow et al. 2002
Exobasidium kishianum Nagao & Ezuka	*Vaccinium hirtum* Thunb. var. *pubescens* (Koidz.) T. Yamaz., Japan	TSH-B0070/MAFF 238623	AB180353/ AB177577 (both as *Exobasidium sawadae*, see text)	GenBank, submitted by H. Nagao in 2004
Exobasidium kishianum Nagao & Ezuka	*Vaccinium hirtum* Thunb. var. *pubescens* (Koidz.) T. Yamaz., Japan	NIAES 10519/MAFF 306199	–/AB178241 (as *Exobasidium sawadae*, see text)	GenBank, submitted by H. Nagao in 2004
Exobasidium miyabei Nagao, Akimoto & Kishi	*Rhododendron dauricum* L., Japan	TSH-B0075/MAFF 38594	–/AB177561	GenBank, submitted by H. Nagao in 2004
Exobasidium myrtilli Siegm.	*Vaccinium myrtillus* L., –	R. Bauer 2055/–	–/AF487390	Begerow et al. 2002
Exobasidium nobeyamense Nagao & Ezuka	*Rhododendron wadanum* Makino, Japan	TSH-B0002/MAFF 238597	–/AB177582	GenBank, submitted by H. Nagao in 2004
Exobasidium otanianum Ezuka	*Rhododendron reticulatum* D. Don ex G. Don f. *glabrescens* (Nakai & H. Hara) T. Yamaz., Japan	TSH-B0061/MAFF 238613	–/AB177593	GenBank, submitted by H. Nagao in 2004
Exobasidium oxycocci Rostr. ex Shear	*Vaccinium oxycoccos* L., –	R. Bauer 2086/–	–/AF487391	Begerow et al. 2002
Exobasidium pachysporum Nannf.	*Vaccinium uliginosum* L., Japan	–/MAFF 238621	AB180352/AB177573	GenBank, submitted by H. Nagao in 2004

Table 1 (continued)

| Species	Host plant, country	Voucher/strain	GenBank acc. no. (ITS/LSU)	Source
Exobasidium pentasporium Shirai	*Rhododendron obtusum* (Lindl.) Planch. var. *kaempferi* (Planch.) E.H. Wilson, Japan	–/MAFF 238600	–/AB177581	GenBank, submitted by H. Nagao in 2004
Exobasidium reticulatum S. Ito & Sawada	*Thea sinensis* L., –	–/DSM 4520	–/AF487394	Begerow et al. 2002
Exobasidium rhododendri (Fuckel) C.E. Cramer	*Rhododendron ferrugineum* L., –	R. Bauer 2050/–	–/AF009856	Begerow et al. 1998
Exobasidium rostrupii Nannf.	*Vaccinium oxycoccos* L., Poland	Poland, Kotlina Sandomierska basin, Stary Jawornik, near Tarnów, 11 Jul. 2006, leg. J. Piątek & M. Piątek, KRAM F-47454, TUB 019165	FJ896132/ FJ896137	This study
Exobasidium shiraianum Henn.	*Rhododendron degronianum* Carrière, Japan	TSH-B0023/MAFF 238602	–/AB177549	GenBank, submitted by H. Nagao in 2004
Exobasidium sundstroemii Nannf.	*Andromeda polifolia* L., –	R. Bauer 2051/–	–/AF487396	Begerow et al. 2002
Exobasidium symploci-japonicae Kusano & Tokubuchi	*Symplocos lucida* (Thunb.) Siebold & Zucc., Japan	NIAES 20521/MAFF 238811	–/AB178255	GenBank, submitted by H. Nagao in 2004
Exobasidium vaccinii (Fuckel) Woronin	*Vaccinium vitis-idaea* L., –	R. Bauer 945/–	–/AF009858	Begerow et al. 1998
Exobasidium vaccinii (Fuckel) Woronin	*Vaccinium vitis-idaea* L., Japan	TSH-B0120/MAFF 238668	AB180362/AB177560	GenBank, submitted by H. Nagao in 2004
Exobasidium woronichinii Nagao	*Rhododendron brachycarpum* D. Don ex G. Don, Japan	TSH-B0081/MAFF 238617	–/AB177557	GenBank, submitted by H. Nagao in 2004
Graphiola phoenicis (Moug. ex Fr.) Poit.	*Phoenix canariensis* Chabaud, –	F. Oberwinkler 29350/–	–/AF009862	Begerow et al. 1998
Kordyana tradescantiae (Pat.) Racib.	*Tradescantia* L. sp., –	F. Oberwinkler 47147/–	–/AF487402	Begerow et al. 2002
Laurobasidium lauri (Geyl.) Jülich	*Laurus azorica* (Seub.) Franco, –	M. Piepenbring 2371/–	–/AF487403	Begerow et al. 2002
Muribasidiospora indica Kamat & Rajendren	*Rhus mysorensis* G. Don (published as *Rhus lancea* L.f.), –	F. Oberwinkler 47397/–	–/AF352058	Begerow et al. 2001
Tilletiopsis pallescens Gokhale	*Vaccinium* L. sp., Japan	–/MAFF 410168	–/AB178262	GenBank, submitted by H. Nagao in 2004
Tilletiopsis pallescens Gokhale	*Rhododendron obtusum* (Lindl.) Planch. var. *kaempferi* (Planch.) E.H. Wilson, Japan	–/MAFF 410169	–/AB178263	GenBank, submitted by H. Nagao in 2004

DSM Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; *EC TUK* unknown abbreviation; *IFO* Culture Collection, Institute for Fermentation, Osaka, Japan; *IFO H* Herbarium, Institute for Fermentation, Osaka, Japan; *F. Oberwinkler* Herbarium F. Oberwinkler, Tübingen, Germany; *KRAM F* Mycological Herbarium of W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków, Poland; *M* Herbarium of Botanische Staatssammlung München, München, Germany; *MAFF* Microorganisms Section of the NIAS (National Institute of Agrobiological Sciences) Genebank, Tsukuba, Japan; *M. Piepenbring* Herbarium M. Piepenbring, Frankfurt, Germany; *NIAES* Mycological Herbarium, National Institute of Agro-Environmental Sciences, Tsukuba, Japan; *R. Bauer* Herbarium R. Bauer, Tübingen, Germany; *TSH-B* Mycological Herbarium of Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Japan

closest relatives according to the results of the LSU analyses (GenBank accession numbers are given in Fig. 2). The species and sequences used in phylogenetic analyses are listed in Table 1.

Sequences were aligned for both datasets with MAFFT 6.611 (Katoh et al. 2002, 2005; Katoh and Toh 2008) using the L-INS-i option. Both alignments [length: 559 bp (LSU), 1,106 bp (ITS+LSU); variable sites: 185 (LSU), 163 (ITS+LSU)] were used throughout their length. We avoided both manipulation of the alignment by hand and manual exclusion of any positions as recommended by Giribet and Wheeler (1999) and Gatesy et al. (1993), respectively.
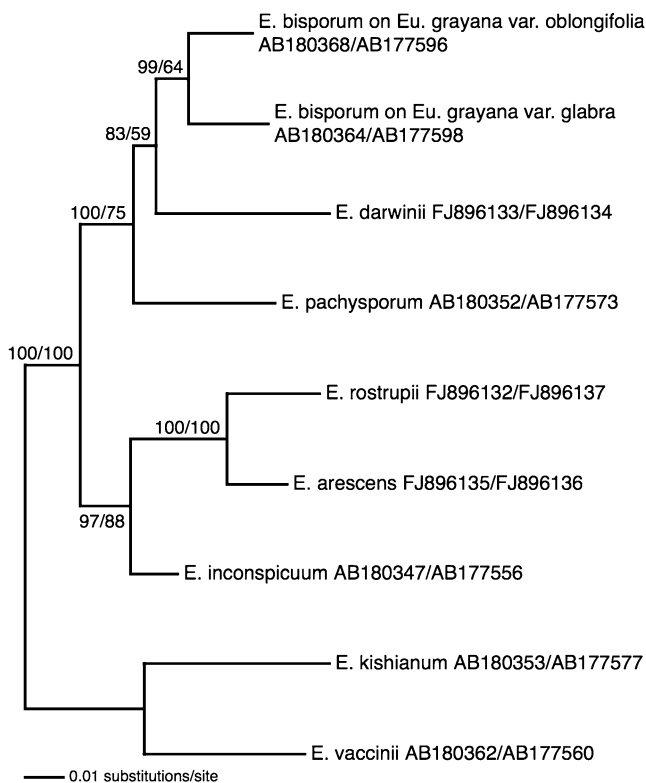
Fig. 1 Bayesian inference of phylogenetic relationships within the sampled Exobasidiales: Markov chain Monte Carlo analysis of an alignment of LSU base sequences using the GTR+I+G model of DNA substitution with gamma distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model. A 70% majority-rule consensus tree computed from 18,000 trees that were sampled after the process had reached stationarity is shown. The topology was rooted with *Tilletiopsis pallescens* (AB178241, AB178262, AB178263). Numbers on branches before slashes are estimates for *a posteriori* probabilities, numbers on branches after slashes are percentage bootstrap values of 1,000 replicates. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. E. = *Exobasidium*, T. = *Tilletiopsis*
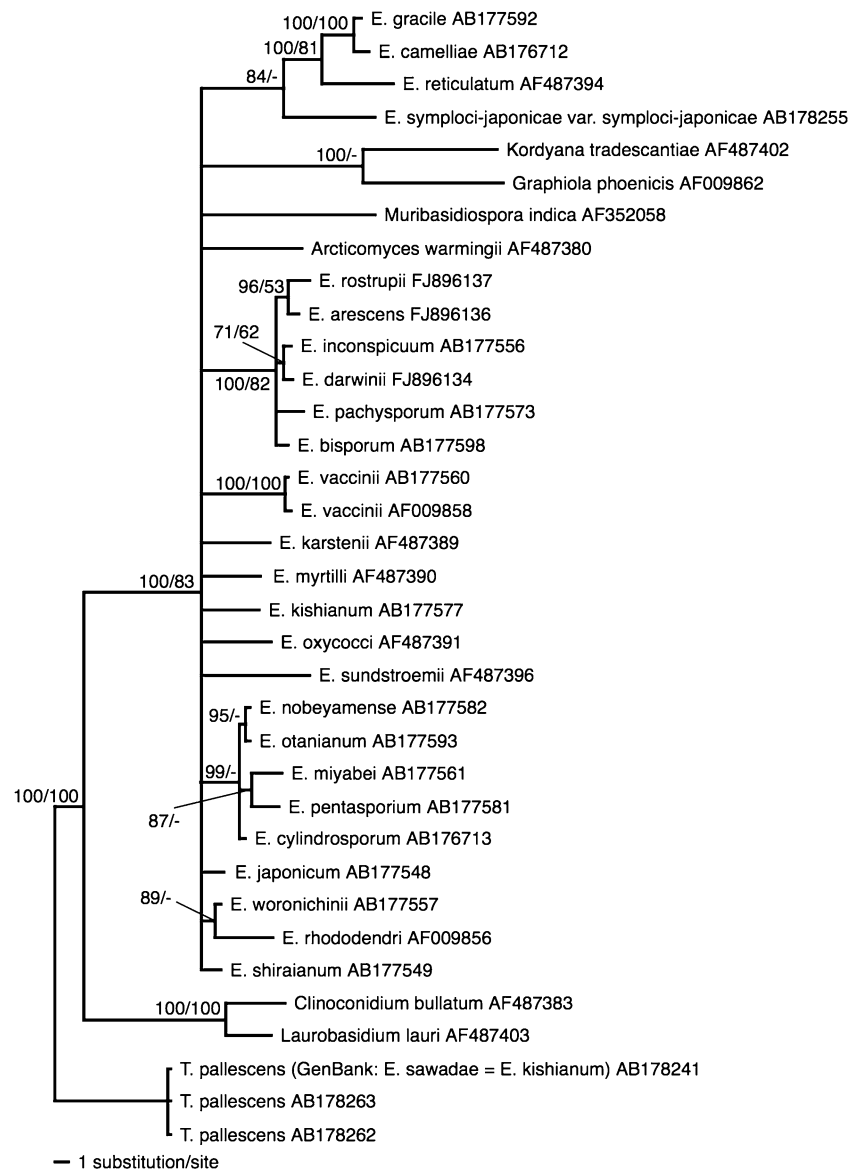
For phylogenetic analyses, we used both neighbour-joining analysis and a Bayesian approach. For neighbour-joining analysis, the data were first analysed with Modeltest 3.7 (Posada and Crandall 1998) to find the most appropriate model of DNA substitution. For the LSU dataset, the hierarchical likelihood ratio test proposed the SYM+I+G DNA substitution model, while for the ITS+LSU dataset, the GTR+I+G DNA substitution model was proposed. Bootstrap values were calculated for 1,000 replicates. For Bayesian analysis, we used for both datasets a Bayesian approach of phylogenetic inference using a Markov chain Monte Carlo (MCMC) technique as implemented in the computer program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). In each run, four incrementally heated simultaneous Markov chains were run over 2,000,000 generations using

the general time reversible model of DNA substitution with gamma distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model as recommended by Huelsenbeck and Rannala (2004). Trees were sampled every 100th generation resulting in an overall sampling of 20,001 trees. From these, the first 2,001 trees were discarded (burnin = 2,001). The trees sampled after the process had reached stationarity (18,000 trees) were used to compute a 70% majority rule consensus tree to obtain estimates for the *a posteriori* probabilities of groups of species. This Bayesian approach of phylogenetic analysis was repeated four times to test the independence of the results from topological priors (Huelsenbeck et al. 2002). Trees resulting from the LSU analyses were rooted with *Tilletiopsis pallescens* Gokhale (AB178241, AB178262, AB178263), trees resulting from

Fig. 2 Bayesian inference of phylogenetic relationships within the sampled *Exobasidium* species: Markov chain Monte Carlo analysis of a concatenated ITS+LSU alignment using the GTR+I+G model of DNA substitution with gamma distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model. A 70% majority-rule consensus tree computed from 18,000 trees that were sampled after the process had reached stationarity is shown. The topology was rooted with *Exobasidium kishianum* (AB180353/AB177577) and *E. vaccinii* (AB180362/AB177560). Numbers on branches before slashes are estimates for *a posteriori* probabilities, numbers on branches after slashes are percentage bootstrap values of 1,000 replicates. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. E. = *Exobasidium*, Eu. = *Eubotryoides*

the ITS+LSU analyses were rooted with *Exobasidium kishianum* Nagao & Ezuka (AB180353/AB177577) and *E. vaccinii* (AB180362/AB177560).

Clarification of species name in GenBank sequences attributed to *Exobasidium sawadae*

GenBank includes five sequences (AB180353, AB178241, AB177577, AB177555, AB180354) stored under the name "*Exobasidium sawadae* Nagao et Ezuka". This is an apparently provisional name, which was never published by the authors because it would cause the creation of later homonym. The name *Exobasidium sawadae* G. Yamada 1919 is already available for a completely different species. Recently, the fungus included in GenBank as "*Exobasidium sawadae* Nagao et Ezuka" was described by Nagao et al.

(2006) under the name *Exobasidium kishianum*. Thus, we used this latter name for these sequences, however, with one exception: our molecular analyses clearly showed that "*Exobasidium sawadae*" AB178241 in fact represents *Tilletiopsis pallescens* (see results and discussion).

Results

Molecular phylogenetic analyses

For both the LSU and the concatenated ITS+LSU dataset, all runs of Bayesian phylogenetic analyses that were performed yielded consistent topologies which were congruent to the results of the neighbour-joining analysis in respect to well-supported branchings (*a posteriori* probability greater than 60%, bootstrap support greater than 73%). For both datasets, we present the consensus tree of one run of Bayesian phylogenetic analyses to illustrate the results (Figs. 1, 2). Estimates for *a posteriori* probabilities are indicated on branches before slashes, bootstrap values from the neighbour-joining analysis are indicated after slashes.

The analyses of the LSU dataset placed the *Exobasidium* specimen infecting *Vaccinium reticulatum* from Hawaii as a sister taxon of *E. inconspicuum* Nagao & Ezuka within a well-supported group of four more *Exobasidium* species (*E. arescens*, *E. bisporum* Sawada ex Ezuka, *E. pachysporum* Nannf., *E. rostrupii*). Within that group, only the monophyly of *E. rostrupii* and *E. arescens* received considerable support values. The relationship between that group of species and most of the other *Exobasidium* species sampled remained unresolved using LSU sequences. Moreover, the relation between the sampled *Exobasidium* species and *Muribasidiospora indica* Kamat & Rajendren, *Arcticomyces warmingii* (Rostr.) Savile (both belonging to the Exobasidiaceae), *Kordyana tradescantiae* (Pat.) Racib. (Brachybasidiaceae), and *Graphiola phoenicis* (Moug. ex Fr.) Poit. (Graphiolaceae) was not resolved. However, the cryptobasidiaceous species *Clinoconidium bullatum* Syd. and *Laurobasidium lauri* (Geyl.) Jülich were revealed as sister taxon to all other Exobasidiales sampled. The sequence "*Exobasidium kishianum* AB178241" clustered together with the *Tilletiopsis pallescens* sequences AB178262 and AB178263 (Fig. 1). A blast search (Altschul et al. 1997) resulted in 100% identity of the sequence "*Exobasidium kishianum* AB178241" with the sequences of *Tilletiopsis pallescens* AB178263, AB178262, AJ235329, AJ235293, AJ235292, AJ235291, and of *T. albescens* Gokhale AJ235290.

In contrast to the analyses of the LSU dataset, those of the ITS+LSU dataset resolved the relationships within the cluster of six species mentioned above with good to moderate support. The *Exobasidium* specimen infecting

Vaccinium reticulatum from Hawaii clustered as moderately supported sister taxon of *E. bisporum* in a well supported group together with *E. pachysporum* (Fig. 2).
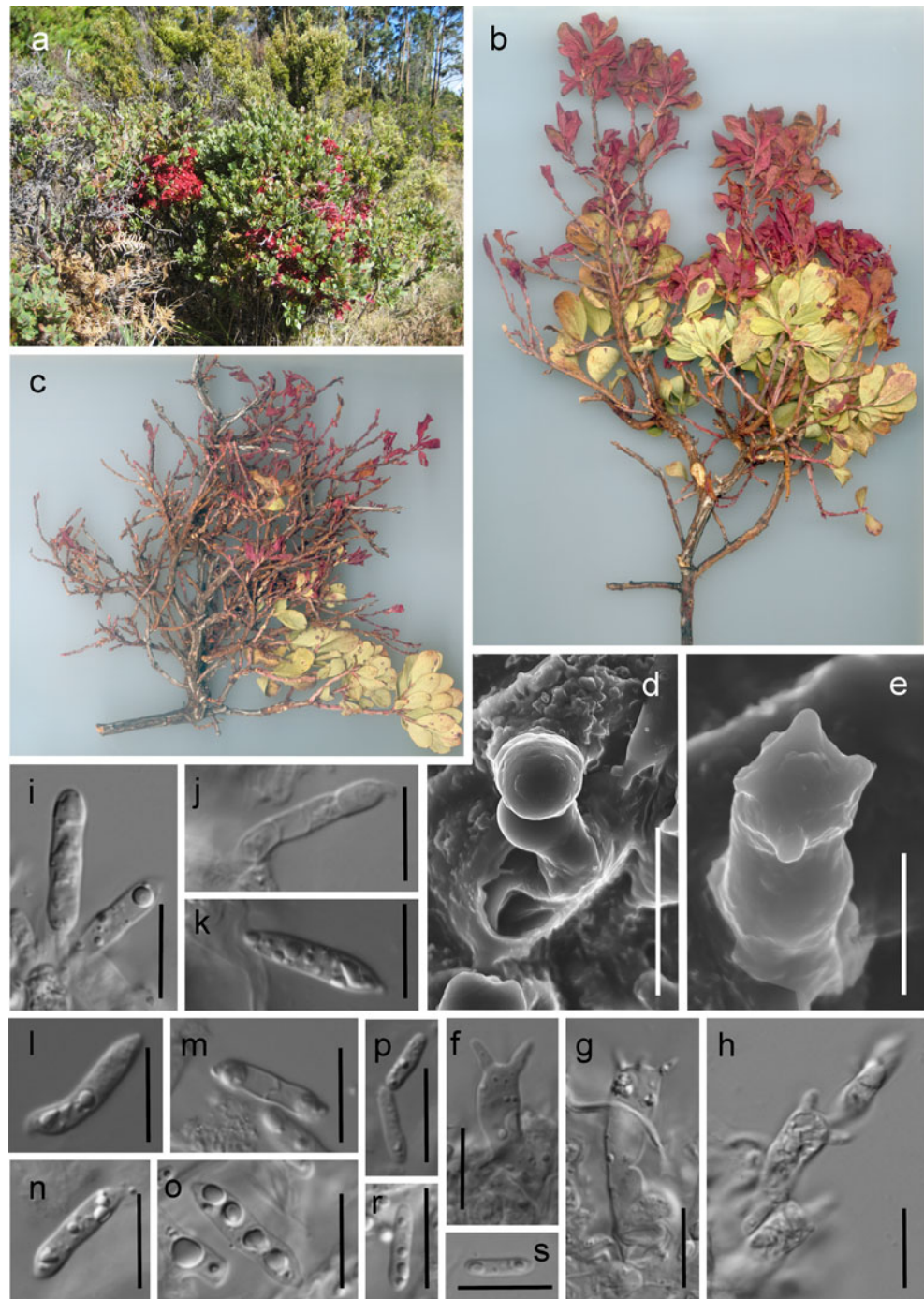
Taxonomy

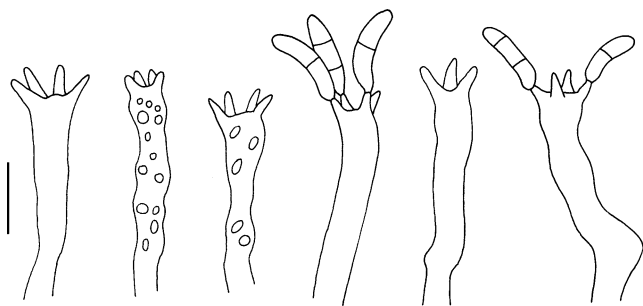*Exobasidium darwinii* M. Piątek & M. Lutz, sp. nov. (Figs. 3 and 4)

MycoBank no. MB 518226

Etymology Named after Charles Darwin (1809–1882), whose observations, among others on the oceanic Galapagos Islands, resulted in formulating the evolutionary theory.

Parasitus in *Vaccinio reticulato*. Infectio systemica. Fungus in planta hospitali per proliferationem continuam parvorum novorum ramulorum conspicuas scopas strigae facit; rami infecti saepe modice deformati, contorti cum intumescentiis associatis. Folia infecta aequaliter pallide rubra ex utraque parte, non hypertrophica et non dilatata, prae foliis validis,

Fig. 3 *Exobasidium darwinii* sp. nov. on *Vaccinium reticulatum* (all from holotype: KRAM F-47124): **a** infected plants in natural habitat, **b** infected branches of the host plant with uniformly bright red, not hypertrophied and not enlarged leaves, **c** witches' broom, **d** basidium emerging through stomatum, as seen by SEM, **e** basidium with four sterigmata, as seen by SEM, **f**–**h** basidia, as seen by LM, **i**–**o** variability of basidiospores, note the septa visible on **j** and **m**, as seen by LM, **p**–**s** bacilliform conidia, as seen by LM. *Bars* 10 μm (**d**, **f**–**s**), 5 μm (**e**)

Fig. 4 Basidia and basidiospores of *Exobasidium darwinii* (from holotype: KRAM F-47124). Note the orientation of the basidiospores on the basidia. *Bar* 10 μm

hymenium hypophyllum satis tenue, ex basidiis, basidiosporis et conidiis compositum. Basidia singulatim vel in fasciculis per stomata vel directe ex epidermi deleta emergentia, hyalina, cylindrica, 20–30 μm longa, 3.9–5.6 μm lata, apice rotundata et 3–4 sterigmatibus terminata. Sterigmata 2.2–5.5 μm longa et 1.4–1.9 μm in basi lata, ad apicem attenuata. Basidiosporae hyalinae, cylindricae, subcylindricae, musaiformes, saepe in uno apice curvatae, plerumque versus hilum attenuatae 11.5–18.5×2.5–3.8 μm, 0–3-septatae, plerumque cum paucis guttulis oleosis, paries glaber. Conidia hyalina, bacilliformia, 5–10×1.5–2.5 μm, non septata, paries glaber. Sequentiae acidi nucleici ITS/LSU typi in collectione sequentiarum acidi nucleici NCBI (GenBank) ut FJ896133/FJ866134 depositae sunt.

Type On *Vaccinium reticulatum* Sm., U.S.A., Hawaii, Maui, north facing slopes of Haleakala, near Hosmers Grove, elev. ca. 6,800 ft. (= ca. 2,072 m a.s.l.), 17 Dec. 2007, leg. P. Welton (holotype: KRAM F-47124, isotype: TUB 019166).

Parasitic on *Vaccinium reticulatum*. Infection systemic. Fungus produces conspicuous witches' brooms on the host plant through continuing proliferation of small young branches; infected twigs often somewhat deformed, contorted and accompanied with swellings. Infected leaves uniformly bright red on both sides, not hypertrophied and not enlarged in comparison with healthy leaves; hymenium hypophyllous, quite delicate, composed of basidia, basidiospores and conidia. Basidia emerging singly or in fascicles through stomata or directly from disintegrated epidermis, hyaline, cylindrical, 20–30 μm long, 3.9–5.6 μm wide, apically rounded and ended with 3–4 sterigmata. Sterigmata 2.2–5.5 μm long and 1.4–1.9 μm wide at the base, tapering toward the tip. Basidiospores hyaline, cylindrical, subcylindrical, musiform, often curved at one end, usually tapering toward the hilum, 11.5–18.5×2.5–3.8 μm, 0–3-septate, usually with several oil drops, wall smooth. Conidia hyaline, bacilliform, 5–10×1.5–2.5 μm, non-septate, wall smooth. The ITS/LSU hologenetype sequences are deposited in GenBank as FJ896133/FJ896134, respectively.

Distribution *Vaccinium reticulatum* is an endemic species of the Hawaiian Archipelago occurring most frequently on Maui and Hawai'i, and rarely on Kaua'i, O'ahu and Moloka'i at elevations from ca. 600–3,650 m a.s.l. (Wagner et al. 1990). The type specimen of *Exobasidium darwinii* was collected near Hosmers Grove in Haleakala National Park on the Island of Maui. Gardner (1985) on the basis of aerial observations concluded that *Exobasidium* on *Vaccinium reticulatum* is common throughout extensive remote areas of the Park. However, it is not excluded that his observations may at least partly refer to the reddish coloration of juvenile leaves. It is desirable that future studies look for the exact distribution of *Exobasidium darwinii* on the Hawaiian Islands in order to establish an effective conservation management of this unique species.

Discussion

The morphological observations and phylogenetic analyses in the present study show that *Exobasidium* on *Vaccinium reticulatum* in the Hawaiian Archipelago belongs to an undescribed species, distinct from *Exobasidium vaccinii* and any other species. Thus, the name *Exobasidium darwinii* is proposed for this novel taxon.

Selection of *Exobasidium* sequences for molecular analyses

GenBank offers quite a lot of sequences of *Exobasidium* species generated by various authors, including a considerable number of sequences submitted by H. Nagao in 2004. Many *Exobasidium* sequences in GenBank were apparently taken from cultures linked with permanently preserved corresponding voucher specimens, others were taken directly from voucher specimens and still others from cultures not linked with herbarium specimens. When constructing the phylogenetic trees, we (1) primarily used sequences taken from *Exobasidium* species on their type hosts (with one exception—in the case of *E. bisporum*, the sequence from the type host was not available), (2) obtained from specimens *in planta* (preferably) or cultures linked with corresponding voucher specimens, and (3) avoided (with some exceptions, when other sequences were not available) using sequences taken from cultures not linked with any voucher specimen. The reason for the two latter choices lies in the fact that the macroscopic appearance of symptoms on the host plant is essential for delimiting the species in *Exobasidium*, while absence of voucher specimens prevents future verification of species identification. It is especially important because the species concept in *Exobasidium* was and still is not fully understood in many studies. We encourage to employ a similar approach in future molecular studies on the genus

Exobasidium. This is in line with similar observation of Lutz et al. (2011) on urocystidalean species.

Not unimportantly, cultures taken from *Exobasidium* specimens can be contaminated by co-occurring *Tilletiopsis* Derx species, and, as Begerow et al. (2000) pointed out, it is difficult to obtain a pure culture of *Exobasidium*. In fact, Boekhout et al. (1995) found that the culture "*Exobasidium vexans* CBS 24752" (= sequence AJ235288) represents a *Tilletiopsis* species. Our analyses indicate that the sequence AB178241 of "*Exobasidium kishianum* MAFF 306199" was also taken from contaminated material and represents *Tilletiopsis pallescens*. Interestingly, according to GenBank data, the culture MAFF 306199 was obtained from voucher NIAES 10519, which is the holotype of *Exobasidium kishianum* (Nagao et al. 2006), included in GenBank under the provisional name "*Exobasidium sawadae* Nagao et Ezuka" (which is not *E. sawadae* G. Yamada 1919). Thus, the sequence "*Exobasidium kishianum* AB178241" should not be used in future phylogenetic studies on the genus *Exobasidium*, or considered as type sequence of *Exobasidium kishianum*.

The symptoms and infection caused by *Exobasidium darwinii*

On host plants, *Exobasidium* species can cause three different kinds of symptoms, depending on the manner of infection: circumscribed, surculicolous and systemic (Nannfeldt 1981). The infection may be monocarpic (annual, ephemeral) when the pathogen completely dies after sporulation, or polycarpic (perennial) when the pathogen survives in infected plants in the form of intercellular mycelium (Nannfeldt 1981). The circumscribed infection is a monocarpic, strictly localized infection forming leaf spots, leaf concavity galls or gall apples entirely surrounded by healthy tissues of the host plant. The surculicolous infection is also monocarpic, but in that case whole annual shoots are penetrated by the pathogen as in the case of the true systemic infection. In contrast to these two monocarpic symptom types, the systemic infection is polycarpic, and whole plants, single shoots or shoot complexes are invaded, depending on the *Exobasidium* species (Nannfeldt 1981). The main difference between systemic and surculicolous infections lies in the fact that in the systemic infection the mycelium of *Exobasidium* species survives in the host plant tissues during the winter season, while in the surculicolous infection it does not. This distinction, however, is often difficult to delineate, and even Nannfeldt (1981), who introduced these terms, pointed out that there may be transitional forms in some *Exobasidium* species. In the light of these data, the symptoms caused by *Exobasidium darwinii* could be assigned to the polycarpic, systemic infection because the pathogen surely penetrates whole shoot complexes of *Vaccinium reticulatum* leading to formation of witches' brooms within which mycelium apparently survives through the years. The infection is, however, probably limited to certain areas of the host plant since witches' brooms are formed only locally on certain branches while other branches are healthy.

Comparison of *Exobasidium darwinii* with other *Exobasidium* species on *Vaccinium* and other *Exobasidium* species causing witches' brooms

Besides *Rhododendron* L., the members of the genus *Vaccinium* harbour the largest number of *Exobasidium* species. Together with *Exobasidium darwinii*, there are 22 species and one variety described on different *Vaccinium* species (Gómez and Kisimova-Horovitz 1998a, b; Hotson 1927; Nagao et al. 2006; Nannfeldt 1981; Nickerson 1984; Piepenbring et al. 2010; Raciborski 1909; Ramakrishnan and Ramakrishnan 1949; Savile 1959). Of them, 8 species and 1 variety are clearly circumscribed taxa, while the remaining 14 species are surculicolous or systemic. *Exobasidium darwinii* belongs to the latter group, having systemic and polycarpic infections. None of the hitherto described species is morphologically similar to *E. darwinii* because none of them produces distinct witches' brooms. *Exobasidium parvifolii* Hotson on *Vaccinium parvifolium* Sm. and *V. ovalifolium* Sm. in North America forms perennial galls on the stems from which numerous cylindrical, clavarioid excrescences emerge. Nannfeldt (1981) interpreted these structures as highly disorganized shoots, which, however, cannot be confused with witches' brooms of *Exobasidium darwinii*.

The unusual morphological aspect of *Exobasidium darwinii* is the production of witches' brooms on the infected host plant (Fig. 3c). Witches' brooms are produced by phytopathogenic fungi from different, unrelated systematic groups, but were rarely observed in *Exobasidium* species. To the best of our knowledge, the witches' brooms were observed only in four species: *Exobasidium uvae-ursi* (Maire) Juel on *Arctostaphylos uva-ursi* (L.) Spreng. in Europe and North America, *E. disterigmaticola* L.D. Gómez & Kisim.-Hor. on *Disterigma humboldtii* (Kl.) Nied. in Costa Rica, *E. nobeyamense* Nagao & Ezuka on *Rhododendron wadanum* Makino in Japan, and *E. pentasporium* Shirai on *Rhododendron obtusum* (Lindl.) Planch. var. *kaempferi* (Planch.) E.H. Wilson (type host) and *R. macrosepalum* Maxim. in Japan. Additionally, Nannfeldt (1981) mentioned another, presumably undescribed, *Exobasidium* species on North American *Rhododendron macrophyllum* D. Don ex G. Don, but this species is not sufficiently known.

All these species are different from *Exobasidium darwinii*, not only because of other host plant genera but

also by metric characters of basidiospores or conidia. The basidiospores are 15–22×5–6 μm in *E. uvae-ursi* (Nannfeldt 1981), 12–21×2–5.5 μm in *E. nobeyamense* (Nagao et al. 2001) and ca. 14.4×4 μm in *E. pentasporium* (Nannfeldt 1981). Basidiospores were not observed in *E. disterigmaticola*. However, its conidia (12–15.5×2.8–3.2 μm; Gómez and Kisimova-Horovitz 1998b) are much larger compared to those of *E. darwinii*. In many groups of *Exobasidium*, basidiospore size shows only a small variance between species. Therefore, these differences distinguish these species well. Additionally, in the phylogenetic analyses, *E. nobeyamense* and *E. pentasporium* are placed in the clade comprising species infecting members of *Rhododendron*, well separated from the clade including *E. darwinii*. The sequences of *E. uvae-ursi* and *E. disterigmaticola* are still not available, and thus their phylogenetic position cannot be resolved. However, the morphological differences do not support their conspecificity with *E. darwinii*.

Hypothesis on the origin of *Exobasidium darwinii*

Three Hawaiian *Vaccinium* species: *V. calycinum*, *V. dentatum* Sm., *V. reticulatum* and the Polynesian *V. cereum* (L.f.) G. Forst. were included in *Vaccinium* sect. *Macropelma* (Klotzsch) Hook.f. (Vander Kloet 1993, 1996). Phylogenetic molecular analyses by Powell and Kron (2002) revealed that the Hawaiian *Vaccinium* species indeed form a monophyletic group that is nested within members of *Vaccinium* sect. *Myrtillus* Dumort. Powell and Kron (2002) suggested a boreal-arctic origin of the species instead of a southern Pacific origin and proposed that Hawaiian blueberries should be transferred to sect. *Myrtillus*. This view was recently accepted by Vander Kloet and Dickinson (2009).

Of the three Hawaiian *Vaccinium* species, no *Exobasidium* has so far been observed on *V. dentatum*, while the specimen on *V. calycinum* was not available for analysis. In the present study, *Exobasidium darwinii* on *V. reticulatum* is a member of a well-supported clade (LSU analysis, Fig. 1; ITS+LSU analysis, Fig. 2) containing *Exobasidium* species on different *Vaccinium* species as well as on *Eubotryoides grayana* (Maxim.) H. Hara var. *glabra* (Komatsu) H. Hara and var. *oblongifolia* (Miq.) H. Hara. The specimens used in the analyses come from Japan, Poland and Slovenia. What is interesting to note is that, except the evidently systemic *Exobasidium darwinii*, all the remaining taxa from this clade are circumscribed, monocarpic species, all producing merely leaf spots and each year renewing infection from apparently hibernated basidiospores or conidia. Thus, it is likely that the ancestral species of *E. darwinii* could have been a circumscribed species that changed its life-strategy during evolution. The geographical origin of this ancestral species is, however, difficult to indicate as the sequences of many species of *Exobasidium* on North American, South American

and South-east Asian Ericaceae are still not available. It is evident that more intensive sampling and the analysis of *Exobasidium* species from these geographical regions are necessary to determine where and on which host plants the closest relatives of *Exobasidium darwinii* actually occur.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Begerow D, Bauer R, Boekhout T (2000) Phylogenetic placements of ustilaginomycetous anamorphs as deduced from nuclear LSU rDNA sequences. Mycol Res 104:53–60

Begerow D, Bauer R, Oberwinkler F (1998) Phylogenetic studies on nuclear LSU rDNA sequences of smut fungi and related taxa. Can J Bot 75:2045–2056

Begerow D, Bauer R, Oberwinkler F (2001) *Muribasidiospora*: Microstromatales or Exobasidiales? Mycol Res 105:798–810

Begerow D, Bauer R, Oberwinkler F (2002) The Exobasidiales: an evolutionary hypothesis. Mycol Prog 1:187–199

Blanz P, Döring H (1995) Taxonomic relationships in the genus *Exobasidium* (Basidiomycetes) based on ribosomal DNA analysis. Stud Mycol 38:119–128

Blanz P, Oberwinkler F (1983) A contribution to the species definition in the genus *Exobasidium* (Basidiomycetes). Syst Appl Microbiol 4:199–206

Boekhout T, Fell JW, O'Donnell K (1995) Molecular systematics of some yeast-like anamorphs belonging to the Ustilaginales and Tilletiales. Stud Mycol 38:175–183

Burt EA (1915) The Thelephoraceae of North America 4. *Exobasidium*. Ann M Bot Gard 2:627–658

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gardner DE (1985) Red leaf disease of native Hawaiian *Vaccinium* species caused by *Exobasidium vaccinii*. Plant Dis 89:805–806

Gatesy J, DeSalle R, Wheeler W (1993) Alignment-ambiguous nucleotide sites and the exclusion of systematic data. Mol Phylogenet Evol 2:152–157

Giribet G, Wheeler WC (1999) On gaps. Mol Phylogenet Evol 13:132–143

Gómez LD, Kisimova-Horovitz L (1998a) (1997) Basidiomicetos de Costa Rica: Exobasidiales, Cryptobasidiales. Notas históricas, taxonómicas y fitogeográficas. Rev Biol Trop 45:1293–1310

Gómez LD, Kisimova-Horovitz L (1998b) Basidiomicetos de Costa Rica: Nuevas especies de *Exobasidium* (Exobasidiaceae) y registros de Cryptobasidiales. Rev Biol Trop 46:1081–1093

Hotson JW (1927) A new species of *Exobasidium*. Phytopathology 17:207–217

Huelsenbeck JP, Rannala B (2004) Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under

simple and complex substitution models. Syst Biol 53:904–913

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinforma Appl Note 17:754–755

Huelsenbeck JP, Larget B, Miller RE, Ronquist F (2002) Potential applications and pitfalls of Bayesian inference of phylogeny. Syst Biol 51:673–688

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program (outlines version 6). Brief Bioinform 9:286–298

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066

Lutz M, Bauer R, Begerow D, Oberwinkler F, Triebel D (2004) *Tuberculina*: rust relatives attack rusts. Mycologia 96:614–626

Lutz M, Vánky K, Bauer R (2011) *Melanoxa*, a new genus in the Urocystidales (Ustilaginomycotina). Mycol Prog. doi:10.1007/s11557-010-0737-7

Nagao H, Ezuka A, Harada Y, Sato T, Kakishima M (2006) Two new species of *Exobasidium* causing *Exobasidium* diseases on *Vaccinium* spp. in Japan. Mycoscience 47:277–283

Nagao H, Ezuka A, Ohkubo H, Kakishima M (2001) A new species of *Exobasidium* causing witches' broom on *Rhododendron wadanum*. Mycoscience 42:549–554

Nannfeldt JA (1981) *Exobasidium*, a taxonomic reassessment applied to the European species. Symb Bot Ups 23(2):1–72

Nickerson NL (1984) A previously unreported disease of cranberries caused by *Exobasidium perenne* sp. nov. Can J Plant Pathol 6:218–220

O'Donnell KL (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). Curr Genet 22:213–220

O'Donnell KL (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233

Piepenbring M, Espinoza J, Saldaña L, Cáceres O (2010) New records, host plants, morphological and molecular data of Exobasidiales (Basidiomycota) from Panama. Nova Hedwig 91:231–242

Posada D, Crandall KA (1998) MODELTEST, testing the model of DNA substitution. Bioinformatics 14:817–818

Powell EA, Kron KA (2002) Hawaiian blueberries and their relatives – A phylogenetic analysis of *Vaccinium* sections *Macropelma, Myrtillus,* and *Hemimyrtillus* (Ericaceae). Syst Bot 27:768–779

Raciborski M (1909) Parasitische und epiphytische Pilze Java's. Bull Int Acad Sci Cracovie Cl Sci Math 3:346–394

Ramakrishnan TS, Ramakrishnan K (1949) *Exobasidium* from South India. Proc Indian Acad Sci B 29:5–12

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Sakai AK, Wagner WL, Mehrhoff LA (2002) Patterns of endangerment in the Hawaiian flora. Syst Biol 51:276–302

Savile DBO (1959) Notes on *Exobasidium*. Can J Bot 37:641–656

Sundström KR (1964) Studies of the physiology, morphology and serology of *Exobasidium*. Symb Bot Ups 18(3):1–89

Vander Kloet SP (1993) Biosystematic studies of *Vaccinium* section *Macropelma* (Ericaceae) in Hawaii. Pacific Sci 47:76–85

Vander Kloet SP (1996) Taxonomy of *Vaccinium* sect. *Macropelma* (Ericaceae). Syst Bot 21:355–364

Vander Kloet SP, Dickinson TA (2009) A subgeneric classification of the genus *Vaccinium* and the metamorphosis of *V.* section *Bracteata* Nakai: more terrestrial and less epiphytic in habit, more continental and less insular in distribution. J Plant Res 122:253–268

Wagner WL, Herbst DR, Sohmer SH (1990) Manual of the flowering plants of Hawai'i. Bishop Museum Special Publication 83. University of Hawaii Press, Bishop Museum Press, Honolulu