Russulas of Southern Vancouver Island Coastal Forests

Volume 1

by

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B.Sc. University of Lancaster, 1991

M.S. Oregon State University, 1994

A Dissertation Submitted in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

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Abstract

The *Russula* flora of Vancouver Island is diverse, colourful, abundant, ecologically important, but poorly documented, with the literature spread in many diverse journals and books from across North America and Europe in various languages. Keys and field guides to local species emphasise macroscopic and spore characters but distinctive structures in the epicutis are not described. As Russulas are prone to environmentally affected colour variation and a number of species have a similar appearance, correct identification may require microscopic examination and a suite of chemicals, a barrier to many people. The existence of synonyms and conflicting concepts for several species adds to the frustration in identification.

Presented here are detailed illustrated descriptions of locally collected species, with discussions on nomenclatural and taxonomic issues where these cause confusion, some of these confirm past records, and some are new records or new species. Three aids to identification are examined: 1. A simplified chromatography method is described that identifies Russulas to subgenus and in some cases section and subsection level, enabling differentiation between some lookalike species without recourse to microscopy.

2. A method often used to match ectomycorrhizae with nearby basidiomata by comparing their restriction fragment length polymorphisms (RFLPs) of amplified ITS rDNA, can also be compared with virtual RFLP's from sequence data downloaded from NCBI and EMBI to aid identification. The restriction enzymes HinfI, AluI and Sau3A, resolved identities to subgeneric and section level, rarely to species. 3. Using published sequence data and Bayesian analysis, a phylogeny was sought with better resolution in the upper clades than had been found with other analysis methods. Various characters from published descriptions and from Vancouver Island collections were then examined for correlation with branching order or clade in this phylogeny, with basidia width, spore colour, pileocystidia shape and spore shape having highest correlation.

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Acknowledgments

I thank my husband Bob Trotta for his patience and support while I worked on this dissertion. I also thank my off-campus supervisor Dr. Bryce Kendrick for financial support and much useful advice. My other committee members have provided advice, assistance and laboratory facilities as needed. I am grateful to Dr. William Hintz for his help with the DNA analyses, and to Dr. Tony Trofymow for the use of office, laboratory and library facilities for a while at the Pacific Forestry Centre. I thank Dr. Shannon Berch, David Dunn and Helga Mehl for the use of laboratory space and equipment at the Ministry of Forests Research Branch.

I am indebted to the members of the South Vancouver Island Mycological Society and the members of the Pacific Northwest Key Council for collecting Russulas, and epecially to both Ian Gibson for his development of the Matchmaker program, which has been very useful, and to Ben Woo, who wrote the original PNWKC *Russula* key, collected the literature for hundreds of *Russula* species, and shared this with me.

Chapter 1

Introduction

The genus *Russula* is a member of the *Russulaceae* within the order *Russulales* (Kirk *et al.* 2001). All *Russulales* have amyloid ornamentation on the spores evident as a blue-black reaction to iodine, usually applied as Melzer's reagent. They also have gloeocystidia in the hymenium (spore-bearing tissue) and the epicutis, vascular hyphae with contents that change colour with sulphuric benzaldehydes such as sulphovanillin (SV), and the hyphae lacking clamp connections. The *Russulaceae* consists of the agaric genera *Russula* and *Lactarius* and the closely related sequestrate genera of *Cystangium, Elasmomyces, Gymnomyces, Macowanites, Martellia* (close to *Russula*), and *Archangeliella* and *Zelleromyces (*close to *Lactarius*). These sequestrate taxa are not truly separate genera; rather they are an adaptation to a different spore dissemination strategy than that of agarics (Miller *et al.* 2001). The *Russulaceae* have heteromerous trama; as well as typical cylindrical hyphae they also contain sphaerocytes in varying degrees, which as the name suggests are approximately spherical cells that give the fungi a brittle, crumbly texture. *Russula* differs from *Lactarius* in its lack of fluid latex, and some *Lactarius* species have strongly coloured flesh whereas in *Russula* the colour is restricted to the pileal and stipe cutis. Both genera have species that change colour when damaged. In temperate regions these two genera are morphologically and, in some studies, phylogenetically distinct (Simono *et al.* 2004), but with tropical species this distinction between genera is blurred (Buyck 1995). In Eberhardt (2002), Hibbett *et al.* (2005), Miller *et al.* (2001) and Moncalvo *et al.* (2002), Lactarius is a sister clade to *Russula*, with the *Lactaroides* (for example *Russula delica, R. brevipes* and *R. chloroides*) basal to both clades.

Russulas are conspicuous, fleshy, often brightly coloured species, and may be among the most abundant epigeous fungi in Pacific Northwest (PNW) forests in the fall, playing a significant role in nutrient cycling. Smith *et al.* (2002) found that of the epigeous fungi, the biomass of Russulas was greater than that of other ectomycorrhizal genera, comprising 23% in an old-growth forest, 10% in a 40-50 year old stand and 7% in a young forest. In the PNW Russulas are more abundant and diverse in old and mature forests (Durall *et al.* 1999, Roberts *et al.* 2004, Smith *et al.* 2002), although Norvell and Exeter (2004) found no significant difference in *Russula* diversity between young (26 year old), mature and late stage (55 and 150 year old) stands, much depended on the characteristics of the site. Some Russulas may also be useful indicator species of site characteristics (O'Dell *et al.* 1999, Roberts *et al.* 2004).

Russulas are all ectomycorrhizal with trees and shrubs, as far as is known, and exhibit a range of host specificity (Trappe 1962, Molina *et al.* 1992). Epigeous Russulas have been recorded as ectomycorrhizal with members of the *Salicaceae, Betulaceae, Pinaceae, Fagaceae, Rosaceae* and *Polygonaceae* (Molina *et al.* 1992). Observations made while collecting data for this dissertation and other studies have shown that while some *Russula* species are cosmopolitan and capable of forming associations with one or more hosts in a range of habitats, others were more constrained in either host or habitat or both (Roberts *et al.* 2004). Ectomycorrhizal fungi have been shown to be important in uptake of nutrients and their translocation to the host plant, increasing the viability of seedling trees over those without fungal mutualists (Trappe and Strand 1969). Simard *et al.* (1997) showed that nearby trees of different families linked by a common ectomycorrhizal system could obtain carbohydrates from one another via this link when one of the pair was under shade, an important means of survival of young trees in a mature forest until they reach the canopy.

Some *Russula* species are commonly found in wood of varying stages of decay. Tedersoo *et al.* (2003) found three *Russula* species colonizing roots in coarse woody debris, and in this study three *Russula* species were always collected from decaying wood. While it is not known if any *Russula* species can decompose wood, Leake *et al.* (2001) found that ectomycorrhizal fungi translocated to the host plant labelled nutrients from wood colonised by wood-decomposing saprotrophic fungi.

Russulas provide a seasonal food source for slugs, squirrels and deer (Cázares and Trappe 1994, Maser *et al.* 1978, Maser *et al.* 1985, Buller 1958, Keller and Snell 2002). In the Pacific Northwest, *Ariolimax columbianus*, the banana slug, preferentially consumed species of *Russula* when they were available; in a short study, 55% of all Russulas seen had slug damage averaging over 27% loss of tissue (Roberts 1997, unpublished data). Humans also collect several species of *Russula* for food, but in the

Pacific Northwest only *Russula brevipes* parasitized with *Hypomyces lactifluorum,* known as the lobster mushroom, is collected commercially.

All told, the ecological roles of Russulas are complex and may involve net movement of nutrients from soil and wood to host trees, between trees, and from host tree to various animals, probably in quite significant amounts, thus helping retain nutrients within the forest biomass.

Apart from a general appreciation of their usefulness and aesthetic value, relatively few Russulas are well known locally, and many remain unidentified. Researchers conducting diversity studies or examining reactions of mycoflora after any forest treatment need to know which and how many species are on their plots. Identification difficulties arise with Russulas because of diagnostic characters which are subjective or variable, such as taste and odour, pigments which wash out in rain, and natural colour variation within a species, as well as the paucity of adequate literature pertinent to this area. What is needed are good descriptions of local species, and different approaches to reaching an identification for people with a range of experience, equipment and needs.

In the following chapters, a simplified chromatography method and a modification of a commonly used DNA comparison method is described and their respective taxonomic values discussed. A phylogenetic tree derived using Bayesian analysis of previously published sequence data was used as a basis to examine morphological characters for evolutionary trends and clade specificity within the species used in the phylogeny, and within the Vancouver Island Russulas, for which more data could be collected. Habitat preferences were examined for species specificity and for clade-related patterns for use in the keys. Two types of key, a polychotomous and a synoptic key to the Vancouver Island species described, incorporated the most useful of these various methods and findings. A synoptic key allows more species to be added without major revision of the key, but the polychotomous key is more helpful for those unfamiliar with Russulas, as it teaches what to look for and why. Russulas used in the experimental processes were collected from Vancouver Island over an 8 year period, as mentioned below, and described in Appendix 3.

The environment of the Southern Vancouver Island coastal forests

Vancouver Island originated as a volcanic chain in the Pacific ocean, and was scraped off the Insular plate, a fragment of the Farallon plate, along with seafloor sediments when this was subducted under the North American plate 115 to 57 million years ago (Townsend and Figge, 2004). This episode also gave rise to the coastal mountains on the mainland. Vancouver Island's origins can be seen in outcrops of basalt, limestone, sandstone and other sedimetary rocks and intrusions. Most of the present-day vegetation has colonised Vancouver Island over the last 12,000 years or so since the retreat of the ice after the last glaciation, with Douglas-fir, Sitka spruce, western hemlock and western red cedar being present in varying abundances since that time, and garry oaks arriving 8,000 years ago (Hebda 1996). During the last glaciation, the Brooks peninsula on the north west coast was not covered in ice and many species found a refugium there, becoming a source for repopulation once the ice retreated (Hebda and Haggarty 1997, Rosenberg *et al.* 2003). Humans and many species of animals migrated along the coast and from mainland to islands from pre-glacial times to the present day, bringing fungal spores or mycelia deliberately or inadvertently on plant roots, as food, in the gut or in soil. Vancouver Island *Russula* populations may therefore originate from ancient populations with a different evolutionary path to their mainland ancestors, or they may have arrived recently from almost anywhere on the Earth with human immigrants bringing in favourite plants, or even in air currents if the spores remained viable long enough. We would therefore expect to find some *Russulas* that are indistinguishable from their counterparts in mainland North America, Europe and Asia, some with varying degrees of local adaptation, and some that have become indigenous, distinct species.

Most of Vancouver Island is within the coastal western hemlock (CWH) biogeoclimatic zone, except for high elevation sites and a narrow band of coastal Douglas-fir moist maritime zone (CDFmm), along the southern and southeastern coastline below 150m elevation.

The CDFmm zone is in the rain shadow of the Olympic mountains in Washington, and also covers the Gulf Islands up to and including part of Texada Island, and a strip along the mainland coast from the Puget Sound up to around Powell River.

On Vancouver island it extends from Comox south to the southern tip of the island around East Sooke Park.

The Victoria International airport weather station on the Saanich peninsula has reported mean annual temperatures range from 9.2°C to 10.5°C, with mean monthly temperatures of 3.4°C (January) 16.2°C (July and August). Although the coldest measurement made was -21.1°C, freezing temperatures do not usually last many days, and soils beneath the forest canopy rarely freeze (Brown and Hebda, 2002, Nuszdorfer *et al.* 1991).

Mean annual precipitation in the CDFmm zone varies from 647mm to 1263mm with around 5% as snow from November to April, the most rainfall is in December with a mean of 151.6mm, and the lowest rainfall in July with a mean of 17.6mm (Brown and Hebda, 2002, Nuszdorfer *et al.* 1991, Pojar and Meidinger, 1991).

Several types of forest communities occur in the CDFmm zone on Vancouver Island. *Pseudotsuga menziesii* (Douglas-fir) is commonly associated with an understory of *Gaultheria shallon* (salal) and *Mahonia nervosa* (dull Oregon-grape). Depending on the site, *Thuja plicata* (western red cedar), *Abies grandis* (grand fir), *Arbutus menziesii* (arbutus), *Alnus rubra* (red alder) and *Acer macrophyllum* (big-leaf maple) *Cornus nuttallii* (western flowering dogwood) and in some sites *Tsuga heterophylla* (western hemlock) are common tree associates. Rocky outcrops, shallow soils and open, well drained sites also support *Quercus garryana* (Garry oak), which occasionally forms almost pure stands (Nuszdorfer *et al.* 1991,). There are also a few rare *Picea sitchensis* (Sitka spruce) and occasional *Pinus contorta* (shore pine) in the area, the latter occurring on dry sites and in rare bogs. All the aforementioned trees and shrubs are hosts for ectomycorrhizal or ericoid-mycorrhizal fungi except for western red cedar and big-leaf maple. Similarly mycorrhizal common understory shrubs include *Vaccinium parvifolium* (red huckleberry), *Rosa gymnocarpa* (baldhip rose), *Rubus ursinus* (trailing blackberry) and *Symphoricarpos mollis* (trailing snowberry). The trees and plants mentioned above are just the more prevalent species; complete lists of plant associations for each site order found in the CDFmm region, together with details of soil characteristics, can be found in Nuszdorfer *et al.* (1991).

The Southern Coastal Western Hemlock very wet hypermaritime biogeoclimatic subzone (CWHvh1) extends along the west coast of Vancouver Island and inland to about 10km (Nuzdorfer *et al.*1991, Pojar *et al.*1991). Mean annual precipitation at Bamfield, approximately 120km up the west coast from East Sooke Park, is 2876mm, ranging from from 61.5mm (July) to 412.7mm (November), and mean monthly temperatures ranging from 4.4°C (January) to 14.5°C (August) with an annual mean of 9.1°C, (Brown and Hebda, 2002).

The dominant tree in this zone on Vancouver Island is of course western hemlock, and, depending on the site, associated tree species may include western red cedar, Sitka spruce, shore pine, red alder, big-leaf maple and occasional *Abies amabilis* (amabilis fir). Extensive logging has reduced the once abundant amabilis fir to an occasional tree, mostly in remaining old-growth stands. *Chamaecyparis nootkatensis,*(yellow cedar), is much less common than on mainland sites in this zone; it is occasionally found in bogs, but this habitat more commonly has shore pine and western red cedar with western hemlocks only at the dryer margins. Along the coastline, Sitka spruce becomes dominant, occasionally in almost pure stands that form krummholz along the forest-beach margin (Harcombe 1974, Pojar *et al.* 1991, Roberts *et al.* 2004). Sitka spruce and amabilis fir also support ectomycorrhizal fungi. Ericaceous plants are particularly well represented in this area, and shrubs include *Ledum groenlandicum* (Labrador tea), salal, red and evergreen huckleberry (*Vaccinium ovalifolium*), *Menziesia ferruginea, Rubus spectabilis* (salmonberry) and other small *Rubus* species, *Rosa nutkana* and, on well-drained forest edges such as sand dunes, extensive mats of *Arctostaphylos uva-ursi* (kinnikinnik). (Harcombe 1974, Pojar *et al.* 1991, Roberts *et al.* 2004).

Towards the southern part of the Vancouver Island coast, Douglas-fir also becomes an increasing component of the CWH forests as the precipitation approaches that of the CDF zone.

The areas from which the Russulas were collected for this study are principally coastal, from Clayoquot Sound and Nanaimo southwards, concentrating more on undisturbed rather than regenerating forest. A few collections were made at Cape Scott, the Cowichan Lake area, Cathedral grove and Saturna Island. Collections were given an individual code composed of the initials of the collector, year, month, date and order for the day. Collectors contributing material included Oluna Ceska, John Dennis, Ian Gibson, Pam Janszen, Bryce Kendrick, Paul Kroeger, Renata Outerbridge, Bob Trotta, and anonymous contributors to the South Vancouver Island Mycological Society shows and forays.

Most collections were photographed or illustrated, a spore print taken, chemical tests made on stipe and cap tissue, and dimensions, odour, colour, texture, bruising reactions and taste noted while the material was fresh. Specimens were sliced in half longitudinally and the caps removed from the stipes of larger basidiomata for drying, which was accomplished in a home food dehydrator. Dried material was later examined microscopically and further measurements, drawings and photographs of hymenial and cuticular characters made. Further material was used in the chromatography and RFLP analyses as described in Chapters 2 and 3. Collections were sealed in ziplock bags and stored in polythene sleeves with their data sheet pending accession to the herbarium at the University of British Columbia.

Taxonomy of the genus *Russula*

Persoon first proposed the genus *Russula* in 1796 in his *Observationes Mycologicae*, while studying fungi in Northern France, basing his new taxon on the fleshy fruit bodies, depressed cap and equal gills. He reduced it to the rank of tribe in the genus *Agaricus* in 1801. Fries similarly regarded *Russula* as a tribe of *Agaricus* in the *Systema Mycologicum* (1821), but later raised it to generic rank in the *Systema Orbis Vegetabilis*, in 1825. Gray (1821) meanwhile had also recognized *Russula* Pers. as a genus, in his *Natural Arrangement of British Plants.*

Russula is a large genus with well defined groups of species that share characters, so the advantage of splitting it into more manageable subgenera and lower taxa was soon recognized.

Fries (1836-1838) divided the genus into five groups; *Compactae, Heterophyllae, Furcatae, Rigidae* and *Fragiles* based on macroscopic characters such as the presence or absence of lamellulae, gill forking, marginal striations and fragility. The first two of these groups are still recognised today, the *Compactae* being sufficiently distinct from other *Russula* that this subgenus has undergone minimal modification over the years. Quélet (1888) felt it to be a link with the sister genus *Lactarius,* calling his version of this group *Portentosae.* In 1907, Bataille split the *Compactae* into two subtaxa: *Lactaroideae,* (later *Plorantinae*) which do not blacken, and *Nigricantes* which do. With minor modification and occasional nomenclatural adjustments, this sytem was followed by Maire (1910), Melzer and Zvára (1927); (who renamed the subgenus *Lactairoides* and the non-blackening subtaxa *Delicinae*,) Singer (1932-1935a, 1935b), Schaeffer (1935), Konrad and Josserand, (1934), Heim (1937-1938), Romagnesi (1967, 1985), Bon (1986, 1988) and Sarnari (1998). Singer added the subsection *Elephantinae* to house *R. elephantina* (= *R. mustelina*), but did not retain it in his 1986 classification. This species apparently posed some difficulty in placement as it has been moved several times. Heim (1938) added section *Archaeinae,* which is still recognised and Bon (1986) amended the *Nigricantes,* reverting to the name *Compactae* Fries.

Russulas in the remaining subgenera or sections, grouped under the *Eurussulae* (Melzer and Zvára 1927) or the *Genuinae* (Konrad and Josserand 1934), have had relatively stable lower taxa once determined (e.g. sections, subsections, and series), but the interrelationships of these groups were not so easily understood. Differing opinions on the phylogenetic importance of certain characters led to variation between classification systems.

Quélet (1888) formed two major subgeneric groups based on spore colour: *Leucosporae,* containing the *Portentosae, Ingratae*, *Sapidae* and *Piperinae*; and *Xanthosporae,* containing *Versicolores, Insidiosae* and *Tenellae*. *Ingratae* is still recognised, allbeit with a narrower concept, (*Portentosae* approximates Fries' *Compactae* and *Sapidae* the *Heterophyllae*). Massee (1893) recognized the taxonomic usefulness of taste of the pileal flesh as a base for infrageneric groupings. These important characters of taste and spore colour were subsequently adopted by most mycologists and are still two of the most useful macroscopic taxonomic characters today.

Arnould and Goris (1907) found sulphovanillin useful for examining spore walls, and Maire (1910) made an important contribution in which the microscopic morphology and histochemical reactions were described for their taxonomic value. In particular he discovered pileocystidia and other cuticular hyphal structures. Maire (1910) reduced the scope of the *Ingratae* to more or less its current circumscription, and *Heterophyllae* to the white-spored species currently in this group. He introduced subgenus *Polychromae* containing the *Decolorantes, Integrae* and *Urentes,* and subgenus *Alutaceae*, with a wider range than in more recent classifications. The latter has been removed from this subgenus but the former two remain as lower taxa. Kauffman (1918) retained the *Compactae* and *Fragiles* of Fries but divided the rest of the genus into *Rigidae* and *Subrigidae*, based on the macroscopic appearance of the cuticle and lamellae.

Melzer (1921-1924) described the preparation and use of an iodine solution, now known as Melzer's Reagent, which stains the ornamentation on the spores of the *Russulales,* making it easy to see under the microscope. Melzer and Zvára based their 1927 classification on taste, spore colour, gill shape and texture, reactions to bruising, $FeSO_4$ and sulphovanillin, and macroscopic and microscopic characters of the pileal cutis and spores. They divided the subgenus *Eurussulae* (all taxa other than the *Compactae*) which was further divided into *Ingratae* and *Gratae.* Melzer and Zvára's concept of the *Ingratae* included the *Emeticinae, Rubrinae* and *Sanguininae* as well as the *Foetentinae* and *Felleinae*. Within the *Gratae* they separated out the *Indolentes* (= *R. cyanoxantha* group) and *Viridantes* (= *R. xerampelina* group), from the *Rutilantes*, based on differing $FeSO_4$ reactions. They did not follow Maire's separation of the *Heterophyllae* at subgeneric level, instead this group appears as *Lividinae* within the white-spored *Immutatae* (non-blackening species) of *Rutilantes*, along with the *Lilaceinae,* that lacked pileocystidia, and *Lepidinae.* This latter had previously been included in the *Heterophyllae* because of its firm texture and velvety cap, which gave it a superficial resemblance to *R. virescens*. Melzer later discovered the use of SV for staining the incrustations on primordial hyphae (Melzer 1934).

Crawshay (1930) published a book including spore print and gill colours, and detailed illustrations of the spores of 92 European Russulas, showing beautifully the taxonomic usefulness of their ornamentation as seen in Melzer's reagent. In the preface to Crawshay's book, Bataille credits the following mycologists (other than those mentioned above) with contributions towards *Russula* taxonomy: Gillet and Cooke each began to give spore measurements in their descriptions of Russulas in the period 1877 to 1895; Bresadola and Patouillard each gave measurements of basidia and hymenial cystidia in *Russula* descriptions made between 1881 and 1892; Ricken in 1915 and Rea in 1922 followed suit. Unfortunately these measurements have not been consistently given in descriptions since.

Singer (1932, 1935a, 1935b) had studied American and tropical *Russulas* prior to making his classification, and had sections rather than subgenera as the highest taxon level. In addition to refining (and in some cases renaming) some of the taxa of Maire and of Melzer and Zvara, he separated out subsections *Chamaeleontina, Subcompactinae* (≈ *Griseinae* Schaeffer) and *Puellarinae* within his section *Constantes;* subsection *Melliolentinae* within section *Decolorantes*; and subsection *Olivaceinae* within section *Rigidae.* The three sections do not directly correlate with sections or subgenera of other authors, and Romagnesi (1967) commented on the rather heterogeneous nature of Singer's sections, which relied more on macroscopic characters than microscopic ones. In his 1986 classification, Singer had narrowed the concepts of sections *Compactae*, *Decolorantes* and *Rigidae,* but the latter remained a rather mixed group, united only by a velutinous cutis; those with a viscid, gelatinous cutis were placed in section *Russula.* (The term *Rigidae* has since been used for *R. cyanoxantha* and related species by Hongo (1960), and for the *Lepidinae* by Bon (1988), and its use should be discontinued in favour of more definitive terms.)

By contrast, Schaeffer (1935) essentially refined Melzer and Zvara's classification, breaking the *Integrinae* into two: the *Paludosinae* with filamentous pileocystidia, and the *Russulinae* Shroeter, without. Other contemporary classifications were made by Konrad and Josserand (1934), Lange (1940) and by Heim (1938) who introduced section *Fistulosinae* in Group *Ingratae,* and Group *Pellicularíae*, both originally of "exotic" species (from Madagascar). Bills and Miller (1984) regard *R. ballouii* Peck, from the Southern Appalachians to be in *Fistulosinae.*

The value of macrochemical reactions in identification and systematics was investigated by Bataille (1948), who published the results of putting KOH, NH_4OH, guaiac, phenol, SO_4OH, SV and aniline on gills, trama or stipe of 106 *Russula* species and varieties. Grund (1965) similarly investigated the reactions of Washington state Russulas.

This first half-century of *Russula* classification saw a gradual refinement of the lower, and some higher taxa into comparatively homogeneous groups, and the use of "rest groups", taxa housing those species that did not fit elsewhere. Since then, there has been a greater effort to understand the phylogeny, culminating in recent years in the study of DNA sequence relationships.

Perhaps the most comprehensive monograph on European and North African Russulas is that of Romagnesi (1967, 1985), who documented 467 species and varieties, the majority with a detailed description and illustrations of microscopic features. His 1967 classification was based on macroscopic, chemical, ecological and microscopic characters of hymenium and pileal cutis, and has since undergone relatively minor modifications by other authors. His understanding of phylogenetic relationships is borne out by the similarities between his groupings and those determined by DNA analyses such as those of Miller *et al.* (2001) and Eberhardt (2002). Romagnesi originally split the genus between subgenera *Compactae* Fries and *Russula,* which was subdivided into eight supersections; these he raised to subgenera in 1987. Romagnesi united the mild tasting species with incrusted primordial hyphae into his subgenus *Incrustatula,* but left the *Lepidinae* and *Rubrinae*, also with epicutal incrustations, in subgenus *Russula*, along with the pale-spored, peppery species. Following the studies of *Russula* pigments by Gluchoff (1969), Romagnesi (1985) transferred three species out of the *Ingratae*: *R. ochroleuca* was put with the *Incrustatae*, and *R. consobrina* and *R. fellea* were placed into the *Piperinae*. He considered these latter two species to be a bridge between the *Ingratae* and the *Piperinae*. Bon split section *Plorantes* of subgenus *Compacta* into *Delicinae* and *Pallidosporinae*, a division subsequently supported by DNA analyses (Miller and Buyck 2002) but unfortunately not followed by Sarnari (1998), who wrote the most recent monograph on the European Russulas.

In volume 1 of his monograph, Sarnari covers his classification of genus *Russula*, and illustrated descriptions of 138 species in the subgenera *Compactae, Heterophyllidia, Amoenula, Ingratula* and *Russula* section *Russula*. This is the system followed in this dissertation. Sarnari felt that *Russula amoena* and related species different enough from the parent group, the *Heterophyllidia,* to warrant their own subgenus, the *Amoenula.* This group is characterised by a velutinous cap, coloured stipe, lack of SV reactive structures and other microscopic features. He also moved subsection *Olivaceinae* Singer into subgenus *Incrustatula*, and out of Romagnesi's *Polychromidia,* as he recognised the presence of (non-incrusted) primordial hyphae in the epicutis. Species in the *Olivaceinae* are unusual in having a bright purple reaction with phenol solution and an epicutis lacking cystidia.

Miller and Buyck (2002) and Eberhardt (2002), each generated a phylogenetic tree of European Russulas based on the internal transcribed spacer of the ribosomal DNA, which has sufficient variation at the infrageneric level. These phylogenies supported many of Romagnesi's and Sarnari's groupings, although not necessarily at the taxonomic levels accorded to them in these traditional nomenclatures. Some clades did not relate to any previous grouping and require further investigation; these are discussed in chapter 3 of this dissertation. A limited number of morphological characters were mapped onto phylogenetic trees in these studies, but more useful characters remain to be considered.

The issue of whether a particular group of species is defined as a subgenus, section, subsection and so on may soon be irrelevant as a clade-based nomenclature, termed the phylocode, has been proposed as a more stable alternative to hierarchical divisions (Cantino and de Queiroz 2006).

A summary and comparison of the classifications of Romagnesi (1987), Bon (1988) and Sarnari (1998) follows at the end of this chapter; the sections and subsections within each subgenus have been rearranged from the order in which they were published to better align them with one another. A description of each taxon is given in the original literature, and in some cases in the keys and species descriptions in Appendix 3 of this dissertation.

North American knowledge of Russulas

In America, Murrill described several new species of Russulas from Florida between 1938 to 1945, but unfortunately did not stain the spores with iodine and consequently did not describe in detail the ornamentation. Peck published the New York Species of *Russula* in 1907 and Burlingham (1915) compiled the known North American species, publishing them in her paper *Russula* which appeared in North American Flora; she also published several more papers on the genus, some of which related to Russulas of the Pacific Northwest. Beardslee (1918) published a monograph of the Russulas of North Carolina, and Kauffman (1918) included Russulas of the north midwest in his book *The Agaricaceae of Michigan*. Singer (1957) published descriptions and redescriptions of many species. Unfortunately many of these early descriptions lack details of some of the characters considered necessary in today's taxonomy. Hesler (1960) addressed some deficiencies by publishing descriptions and illustrations of the epicutis microscopic structures and spores of 159 North American Russulas, originally published by Singer, Murrill, Kauffman, Burlingham, Peck, Beardslee and others. In 1961, Hesler published a further 34 descriptions of Julius Schaeffer's collections from Europe. Several more of these early descriptions have been redescribed in various publications and dates by Shaffer, Singer, Bills and Fatto.

Recent useful contributions to the identification of North American Russulas include keys and descriptions by Kibby and Fatto (1990), and Fatto (1998, 1999, 2000, 2002), these deal mainly with eastern and northern species. Kibby and Fatto (2005) also have an on-line synoptic key to Russulas that includes both European and North American species.

Shaffer published descriptions of 14 North American species and forms of Section *Compactae* subsection *Compactae* in 1962, 10 species of subsection *Lactaroideae* in 1964, 18 of various sections in 1970, 10 of section *Ingratae* subsection *Foetentinae* in 1972, and 8 of subsection *Emeticinae* in 1975. All Shaffers' descriptions were based on North American collections of new and known species, some of which occur in the Pacific Northwest (the taxonomic groupings here mentioned are as he published them). Other eastern species descriptions have been published by Bills and Miller (1984). On the west coast, Harriet Peters (1962) documented Russulas of the San Francisco area in her Masters thesis and Grund (1965) those of Washington State. In Grund's doctoral thesis a number of species were given provisional names as they were assumed to be previously undescribed, just five of these were subsequently validly published, four by Grund (1979) and one by Thiers, who also published one of Peters' discoveries (Thiers 1997a). Thiers (1997b) published a monograph with succinct descriptions of 102 Russulas of Northern California, and Woo (1989) published the PNW Key Council keys to PNW Russulas that had been documented as locally present in various publications.

The amount of detail and quality of illustration in descriptions ranges from a relatively brief summary with a sketch of the spores as in Thiers (1997) to a detailed treatise with ample drawings of hymenial and cuticular structures, supplemented with SEM photographs of spores, as in Bills and Miller (1984), Buyck and Ovrebo (2002) and Kong *et al.* (2002).

Woo compiled the literature for approximately 325 species of *Russula* reported or described from North America by numerous authors and mycological societies, and which he made available in 1997 to members of the Pacific Northwest Key Council interested in Russulas. This compilation has been of great assistance in the identification of several Vancouver Island collections described in the appendix of this dissertation, since the searching through abundant, sometimes hard to obtain literature was much reduced. One of the main problems facing a researcher is that of simply knowing of the existence of a description. This has historically led to a species being described more than once, under different names, and these are only slowly being weeded out as herbarium type collections are re-examined. An example is *Russula atropurpurea* (Krom.) Britz., which Sarnari (1998) estimates to have 9 synonyms and to have been described as different varieties and forms of these synonyms at least 23 times. Local species can and do sometimes vary in a given character from the specifications given in the original descriptions, and such variations should be explicit in descriptions. In some cases this variation from the type is large enough and sufficiently consistent to warrant a new variant name, or to be considered a new species. Currently, for collections that do not match existing North American descriptions, European and Asian publications may provide useful information if the language is not a barrier. In addition to those texts mentioned above, collections of useful descriptions and/or keys include those of Arora (1986), Blum (1963), Bon (1987, 1988), Buczacki (1992), Courtecuisse and Duhem (1995), Einhellinger (1987), Moser (1983), Phillips (1991), and Rayner (1977).

The current level of knowledge of Russulas in western Canada

Redhead (1997) estimated that only 1% of B.C. macrofungi had been systematically studied, and only 20 *Russula* species had been documented for B.C. Of the 150 or more species in the genus originally estimated to be in the Pacific Northwest

(PNW) by Grund (1965), 78 (agarics) have been recorded (Woo, 1989, Gibson *et al.* 2006), with 37 reported from Vancouver Island and the Gulf Islands, mostly from observations made during forays by members of the South Vancouver Island Mycological Society, Vancouver Mycological Society and the PNW Key Council (Gibson 1998). Some of these records were unconfirmed, and they are also incomplete. Roberts *et al.* (2004) recorded 34 species of *Russula* in the forests around Clayoquot Sound between 1997 and 2001. Some of these species and those found elsewhere on Vancouver Island were difficult to identify, since one or more of their characters did not match available published descriptions.

Questions addressed by this dissertation

1. Are there are more species of *Russula* to be found on Vancouver Island than are currently recorded from local foray data and from published accounts of Pacific Northwest collections, and what are the characteristics and habitats of local forms of recorded and newly observed species?

2. While traditional identifications rest on morphological characters, can the RFLPs of an amplified piece of DNA, specifically the ITS region of the rDNA gene be usefully compared with virtual restrictions of published sequence data to confirm an identification, and can paper chromatography of cuticle pigments be employed in the identification of taxonomic groups?

3. How does the suite of morphological characters used in traditional classification within the genus *Russula* relate to the clade structure within a phylogenetic tree based on DNA sequences, and can a more efficient identification key be based on such characters sorted by the taxonomic level at which each is useful?

Summary of three recent classification systems.

S ome taxa that approximate one another but with dissimilar names are linked with shading. S = Section, Ss = Subsections.

Romagnesi 1987	**Bon 1988**	**Sarnari 1998**
Subgenus *Compacta* (Fr.) Bon.	**Subgenus *Compacta* (Fr.) Bon.**	**Subgenus *Compacta* (Fr.) Bon**
Type *R. nigricans.*		
S.: *Nigricantinae* Bat.	S.: *Compactae* Fries	S.: *Compactae* Fries
Plorantinae Bat.	*Plorantes* (Bat.) Sing.	*Lactarioides* (Bat.) Konr. & Joss.
Archaeinae Heim: Romagn.	Ss..: *Delicinae* Bat.	*Archaeinae* Heim ex Buyck & Sarnari
	Pallidosporinae Bon	
Subgenus *Ingratula* Romagn.	**Subgenus *Russula***	**Subgenus *Ingratula* Romagn.**
Type: *R. foetens*	S.: *Ingratae* Quélet	S.: *Ingratae* (Quél.) Maire
S.: *Foetentinae* Mlz. & Zv.	Ss..: *Foetentinae* Mlz. & Zv.	Ss..: *Foetentinae* (Mlz & Zv) Sing.
Groups: *R. foetens*	*Pectinatae* Bon	*Farinipedes* Sing.
R. pectinata		S.: *Felleinae* (Mlz. & Zv) Sarnari
S,: *Felleinae* Mlz. & Zv.		*Subvelatae* Sing.
		Fistulosae Heim
		Subgenus Heterophyllidia Romagn.
Subgenus *Heterophyllidia* Romagn.		S.: *Griseoflaccidae* Sarnari ad int.
Type:*R. grisea*	S.: *Heterophyllae* Fries	*Virescentinae* (Sing.) Sarnari
S.: *Virescentinae* Sing.	Ss..: *Virescentinae* Sing.	*Heterophyllae* Fries
Indolentinae Mlz. & Zv.	*Indolentinae* Mlz. & Zv.	Ss..: *Cyanoxanthinae* Sing.
Heterophyllinae Maire	*Heterophyllinae* (Fries) Schaef.	*Heterophyllae* (Fr.) Schaeff.
Griseinae Schaef.		*Griseinae* Schaeff.
Ilicinae Romagn.	*Griseinae* Schaef. ex Bon	*Ilicinae* (Romagn.) Buyck
Amoeninae Sing.	*Amoeninae* Sing.	**Subgenus *Amoenula* Sarnari.**
		Type : *R. amoena*

Romagnesi 1987 (continued)	**Bon 1988** (continued)	**Sarnari 1998** (continued)
Subgenus *Russula* (Pers.) Fries amend.	**Subgenus *Russula*** (continued)	**Subgenus *Russula* Romagn. amend.**
Romagn. Type:*R. emetica*	S.: *Rigidae* Fries	**Type: *R.emetica*.**
	Ss.: *Lepidinae* Mlz. & Zv.	S.: *Russula* (Romagn.) Sarnari
1. Piperinae-rubroflavae:	*Rubrinae* Mlz. & Zv. amend.	Ss.: *Consobrinae* Sarnari,
S.: *Lepidinae* Mlz. & Zv.	Romagn.	*Rubrinae* (Mlz. & Zv) Sing.
Rubrinae Sing. ss. str.	S.: *Russula* (=*Fragiles* Fries)	*Russula* (Romagn.) Sarnari
Citrinae (Romagn.) Bon	Ss.: *Felleinae* Mlz. & Zv.	*Sardoninae* (Sing.) Sarnari
Emeticinae Mlz. & Zv. ss. str.	*Citrinae* (Romagn.) Bon	*Urentes* Maire
Persicinae Romagn.	*Emeticinae* Mlz. & Zv.	*Violaceinae* (Romagn.) Sarnari
2. Piperinae-variecolores:	*Atropurpurinae* Romagn.	
S.: *Atropurpurinae* Romagn.		S.: *Viscidinae* (Sarnari) Sarnari
Violacinae Romagn.	S.: *Violaceae* Romagn.	*Messapicae* Sarnari
Exalbicantinae Sing.		*Paraincrustatae* Sarnari
Sardoninae Sing.	S.: *Firmae* Fries	Ss.: *Integrae* (Maire) Sarnari
	Ss.: *Exalbicantinae* Sing.	*Lepidinae* (Mlz. & Zv) Sing.
	Sanguininae Mlz. & Zv.	
Subgenus *Insidiosula* Romagn.		
Type: *R. veternosa*	S.: *Insidiosae* Romagn.	
S.: *Urentinae* Maire ss. str.	Ss.: *Cupreinae* Bon	
Maculatinae Konr. & Joss.	*Urentinae* Maire	
Subgenus *Coccinula* Romagn.	S.: *Russulinae* (Schroet.) Sing.	
S.: *Decolorantinae* Maire ss. str.	Ss.: *Integrinae* Maire	
Paludosinae Schaef.	*Paludosinae* Schaef.	
Laetinae Romagn.	*Laetinae* Romagn.	

Romagnesi 1987 (continued)	**Bon 1988** (continued)	**Sarnari 1998** (continued)
Subgenus *Polychromidia* Romagn.	S.: *Polychromae* Maire	S.: *Polychromae* (Maire) Sarnari
Type: *R. integra.*	Ss.: *Decolorantinae* Maire	Ss.: *Auratinae* Bon,
S.: *Melliolentinae* Sing,	*Melliolentinae* Sing.	*Melliolentinae* (Sing.) Sarnari.
Integroidinae Romagn.	*Laeticolorinae* Bon	*Integriforminae* (Bon) Sarnari.
Olivaceinae Sing. ss. str.	*Integriforminae* Bon	*Paraintegrinae* Sarnari,
Integrinae Maire ss. str.		*Xerampelinae* Sing.
Viridantinae Mlz. & Zv.	S.: *Viridantes* Mlz. & Zv.	
	S.: *Alutaceae* Maire	
	Ss.: *Olivaceinae* Sing.	
	Auratinae Bon	
Subgenus *Tenellula* Romagn.		
Type: *R. puellaris.*	S.: *Tenellae* Quélet. amend. Romagn.	S.: *Tenellae* Quél.
S.: *Sphagnophilinae* Sing.	Ss.: *Sphagnophilinae* Sing.	Ss.: *Betulinae* (Romagn.) Sarnari
Rhodellinae Romagn.	*Rhodellinae* Romagn.	*Rhodellinae* (Romagn.) Bon
Laricinae Romagn.	*Laricinae* (Romagn.) Bon	*Laricinae* (Romagn.) Bon
Puellarinae Sing.	*Puellarinae* Sing.	*Puellarinae* Sing.
	Odoratinae Bon	
Subgenus *Incrustatula* Romagn.		**Subgenus *Incrustatula* Romagn. emend.**
Type: *R. lilacea*	S.: *Lilaceae* (Mlz. & Zv.) Konr. & Joss.	S.: *Lilaceinae* (Mlz. & Zv) Konr. & Joss.
1. Incrustatae-leucosporae	Ss.: *Roseinae* Sing.,	Ss.: *Roseinae* Sing. ex Sarnari
S.: *Roseinae* Sing.,	*Lilaceinae* Mlz. & Zv.	*Lilaceinae* (Mlz. & Zv) Schäff.,
Lilaceinae Mlz. & Zv. ss. str.	*Amethystinae* (Romagn.) Bon	
2.Incrustatae-xanthosporae	*Chamaeleontinae* Sing.	S.: *Amethystinae* (Romagn.) Sarnari
S.: *Amethystinae* Romagn.	*Integroidinae* (Romagn.) Bon	Ss.: *Amethystinae* (Romagn.) Bon,
Chamaeleontinae (Sing.) amend. Romagn.	*Ochroleucinae* (Romagn.) Bon	*Chamaeleontinae* Sing.
Ochroleucinae Romagn.		*Integroidinae* Romagn. in Bon,
		Olivaceinae Sing.

Chapter 2

Chromatographic analyses of cuticle pigments

Introduction

The colours of *Russulas* are amongst the most confusing of characters in the genus, since the incident light and weather affect the development of pigments and their relative longevity in the cutis. Many species are very similarly coloured, but at the same time the colour can vary dramatically between basidiomata of the same species.

The analysis of cuticle pigments by paper chromatography was examined for its potential in differentiating between taxa, and for finding natural groupings among species which could be compared with other data used for taxonomic divisions within the genus, such as those based on classical morphology and those arising from phylogenies based on DNA analyses. The initial experiments with cuticle pigments suggested that a simple procedure using widely available materials worked reasonably well, and as such could be of use to ecologists, forestry workers and amateur mycologists involved in diversity studies to aid identification when only a temporary field laboratory is available.

Comparison of naturally occurring pigments with watercolour pigments.

In the preparation of the watercolour illustrations for this thesis it became apparent that for most of the variably coloured Russulas three and occasionally four pigments provided the most accurate rendition of the colours of the cap cuticles; a primary red, yellow, a greenish blue and an ochre yellow. The behaviour of these watercolour pigments in mixtures and dilutions was similar to the behaviour of the *Russula* pigments in situ. Carmine red (St. Petersburg artists' watercolour), is close to the magenta ink found in ink jet printers, but gives a brighter red in concentrated form such as that of *R. sanguinea* and *R. americana* var. *modicaspora* nom. prov., as well as a more purple pink in dilution as in *R. fragilis.* The blue pigments found in Russulas are of a greenish blue, duplicated in paint by phthalocyanine blue (DaVinci artists' watercolour, although most quality brands will be similar, since this pigment is a standardized manufactured product); this blue is darker and greyer than the cyan ink used for ink-jet printers. In Russulas the blue pigment was rarely, if ever, found alone, and both the natural and the watercolour blue pigments produced the range of blues, greys, violets and purples when in combination with the red, greens when combined with yellow and browns and black when combined with yellow and red. Although seven yellow pigments have been found in Russulas, discussed below, usually only two or three of these were found in any one species. The yellows were either a bright clear lemon-yellow or a browner shade of yellow. For most illustrations quinacridone gold (Daniel Smith artists' watercolour) duplicated both the deeper and lighter yellows and those in brown, green and mixed colour Russulas. Arylide yellow (DaVinci), similar to yellow ink-jet printer ink, was used when a clear citrus yellow was required, such as for *R. gilva*.

Russulas in the subgenera *Compactae* and *Ingratae* were better illustrated with a different set of pigments: ivory black and the earth colours raw and burnt umber, and raw and burnt sienna (either Winsor and Newton or Maimeri artists' watercolours).

In mixing the above colours and diluting them an appreciation was gained for the variety of hues possible with two and three pigment mixes, so that published descriptions of species listing colours ranging from green to grey to pink to brown became easier to visualize and understand. With experience, duplicating the colours of a fresh collection in watercolour pigment yielded information on whether one, two or three pigment groups (i.e., whether just reds, or reds and yellows, etc.) were likely present, and their approximate relative amounts. While such information could be confirmed only by chromatographic analysis of the actual cuticle extract, in practice this quick paint analysis sometimes facilitated the identification process. Analysis of colours by image manipulation programs on a computer of photographs of mushrooms was less reliable because the variation in light and shade on such three-dimensional objects affects local colour, although it is possible to reduce this source of variation by flattening a section of the mushroom cap onto the bed of a scanner and scanning in the image.

In the field, *Russula* pigments varied in their response to weather and light conditions. In some species, light is necessary for the development of the red pigment, as in *R. fragilis* (personal observation) and *R. xerampelina* (O. Ceska, personal communication). The red pigments also were more water soluble in the living mushroom than the blue and yellow – at least in the subgenera *Russula* and *Incrustatula* (sensu

Romagnesi 1987 and Sarnari 1998). The yellow colouration was sometimes observed within the dermatocystidia, where it appeared to be held more tightly than the red vacuolar pigments, and in age a dull yellowish hue appeared in many species as tissues degenerated and oxidized; a change not due to pigmentation. A general tendency was for an enrichment and darkening of pigmentation as the immature basidioma matured and emerged above the litter, followed by weathering, in which rain washed out the colours, diluting them toward a grey to yellowish-grey hue, or yellow to white in species lacking blue pigment. In dry weather colours sometimes changed toward brownish hues as decomposition set in. In very dry weather, the colours were often preserved as the mushroom dried before decomposition affected it. With this understanding of the behaviour of *Russula* pigments and of paint pigments, it was easier (but not infallible) to avoid making either mixed species collections or to split collections unnecessarily in the field, and also to recognize a particular species in the field in part by the weathering/aging pattern of its pigments.

Pigments from the cuticles of Russulas have been separated chromatographically and analyzed spectroscopically by Gluchoff, (1969, 1975) and Iten *et al.* (1984). In these studies, the chemical identity of the pigments was sought and isolation of each fraction was brought about by chromatographic separation on paper, thin layer silica gel, and columns of silica gel, using a variety of solvents including water, ethanol, acetic acid, pyridine and phenol. Identification of the reds was through spectrographic analysis and comparison with known compounds. Gluchoff (1969) was interested both in the chemical identity and taxonomic potential of the pigments and isolated three red pigments which she termed Russularhodines (RrI, RrII and Rra); three blue pigments termed Russulacyanines (RcI, Rc II and Rc d'*Heterophyllae*); and seven yellow pigments termed Russulaxanthines (Rx I to VI and Rx d' *Heterophyllae*). No green pigments were detected. Gluchoff isolated cuticle pigments from 37 species of *Russula* spread among eight supersections of subgenus *Russula* sensu Romagnesi (1967). These analyses showed, with some notable exceptions, that the *Ingratae* and members of the *Heterophyllidia* Romagnesi lacked the suite of pigments found in the other groups. The exceptions, namely *R. ochroleuca* and *R. consobrina* in *Ingratae* and *R. amoena* and *R.*

violeipes in *Heterophyllae*, have recently been reclassified by Sarnari (1998) following Gluchoff's findings: *R. ochroleuca* into the subgenus *Incrustatula*, *R. consobrina* into subgenus *Russula* section *Russula* and the last two into a new subgenus, *Amoenula* (Sarnari 1998, Miller and Buyck 2002). Gluchoff (1969) also concluded that the supersection *Piperinae* sensu Romagnesi formed a natural group having two red and two yellow pigments in common, and within this group, all members of the section *Sardoninae* also had a blue pigment in common. This pattern was not exclusive of species in other groups.

Russularhodine I is a strongly polar hexose or pentose sugar-based nitrogenous heterosidic molecule found to be very soluble in water and 5% acetic acid; Rr II is soluble in 50% acetic acid or 50% pyridine, less soluble in water. A third red, Rr a, migrates along with the other two reds on paper in 5% acetic acid and superimposes on them. These pigments all fluoresced orange in UV light (360nM) (Gluchoff, 1969).

Russulacyanine I and II can be separated in a bidimensional paper chromatograph by running first with 40% acetic acid and secondly in the perpendicular direction with a butanol/pyridine/water mixture. Neither of these pigments fluoresce in UV light. The russulacyanine specific to the *Heterophyllae* sensu Romagnesi fluoresces orangish in UV light Gluchoff (1969).

Of the russulaxanthines, Gluchoff (1969) found Rx I and V to be water soluble; whileRx V and Rr I were found to be interconvertible; Iten *et al.* (1984) confirmed that three of the yellow compounds (lumazines) were derived by the hydrolysis of a red pigment. Rx VI was isolated from *R. vesca* and a further yellow from *R. cyanoxantha,* both in the *Heterophyllae*. Gluchoff did not discuss the other yellow pigments except to give the colour in daylight and UV light. Gluchoff also found a colourless pigment that fluoresced violet in UV light which she termed 'I.V.'. A table of the visible and UV fluorescence properties is reproduced from Gluchoff (1969) in the appendix. Also reproduced is her table of the pigments found in each of 37 *Russula* species using Whatman no1 paper with 40% acetic acid and silica gel with 4:1 phenol:water. Iten *et al.* (1984) analyzed colourless and yellow pigments, which they termed Russupteridines, from *Russula* collections, and found several ribitol compounds, some of which are intermediates between two others. One of the yellows was riboflavin, a common pigment in *Russula* cuticles. Iten *et al.* (1984) commented on the difficulty of obtaining separations, stating that 1kg of Sephadex gel (in a column) was required to separate 10mg of previously purified fraction. Their methods also used single species of *Russula* in 200 to 300kg amounts. The chromatography methods worked out below are designed to make use of the limited amount of material that is normally available in a collection, which can sometimes consist of only one basidioma, and a very simple procedure that nevertheless gives useful taxonomic information.

Materials and Methods

Extraction of Pigments

The pigments in *Russula* are found within the cutis, which in many species will separate readily from the underlying tissues at least at the cap margin in fresh specimens. The pigments in most species are vacuolar. In the Ingratae the pigment is in the hyphal walls, and in *R. ochroleuca* (and perhaps other encrusted groups) a yellow pigment encrusts the outside of the hyphae (Gluchoff 1969, Romagnesi 1967). Slivers of cuticle totalling approximately 1cm^2 and cleaned of underlying tramal tissues from either fresh or dried collections were placed in a small boiling tube or 1.5ml Eppendorf tube and covered with distilled water. The amount of tissue used depended upon the amount available, retaining sufficient undamaged material for deposition in an herbarium. In practice this meant using between 1 and 4cm^2 of cuticle tissue and about 0.5 to1.5ml of water. For single small samples a quick method was to microwave the contents of the boiling tube at maximum power for 10 seconds if 0.5ml water was used. For several or larger samples the mixture was heated in a heating block at 60-65°C if in an Eppendorf or on a hotplate set on low to medium (use a thermometer in a beaker of water to determine setting) if in a glass flat-bottomed tube, until the pigments were seen to be released into the water at about 60°C. This happened quite abruptly, and after 2 to 5 minutes in hot water very little colour remained in the tissue, so this was removed. The pigment solution was then reduced to 50-100μl to sufficiently concentrate the colour, by leaving it on the hotplate or placing it in an oven at 50-60°C and checking frequently. Completely dried extracts formed a crystalline deposit which will keep in a closed container away from light for several weeks without deterioration. Gluchoff (1975) analysed the pigments of ten collections of *R. norvegica*, some of which were fresh and others of various ages from a herbarium and found the pigment profiles sufficiently similar to assume chemical stability in dry storage. Dried extracts could be rehydrated with about 100µl water depending on colour density and consistency of the deposit. With some species, particularly members of the *Ingratae,* the concentrate was somewhat gelatinous and needed to be diluted more than that of other groups. This gelatinous substance sometimes rendered part of the pigment spot immobile on the chromatograph baseline.

Chromatography

Whatman No. 1 paper was cut into strips approximately 8mm wide by 30cm long and marked with a horizontal pencil line 5cm from one end to denote the starting point, and labelled at the other end. The use of strips rather than a sheet limited the horizontal spread of the pigments, a necessary precaution when minute amounts of pigment require maximum concentration if they are to be seen. An occasional disadvantage was that a colour band was pushed to the side of the strip rather than progressing upwards, necessitating a repeat. The extract was spotted onto the line. It was necessary to allow the spot to dry and repeatedly apply it to concentrate the pigments further on many samples, particularly pale species.

The strips were suspended with the lower 2.5cm in 2% acetic acid solvent in a closed glass jar at room temperature, and the solvent front allowed to progress 21-24cm up from the baseline, which took approximately 3 hours. The chromatographs were removed from the solvent, the solvent line marked, and the strips allowed to dry. Pigment bands visible in daylight were noted, then those fluorescing in blue light emitted from a blue light-emitting diode (LED) flashlight at a wavelength centered on 470nm, and finally those fluorescing in ultra-violet light (UV), at a wavelength of 365nm, (as in a portable counterfeit currency detector light.) The blue LED and the UV observations were made in a darkroom. It was necessary to shine the blue LED onto the chromatograph for 20-30 seconds to impart sufficient energy to allow fluorescence, then move it slightly aside to check for a reaction. Red and orange LED lights were also tried but these have a longer wavelength and consequently lower energy, so they were not expected to incite fluorescence, and did not do so.

Results

There was little difference in the number and colours of bands formed when the running solvent was distilled water or 5% acetic acid, although the acetic acid generally gave slightly cleaner separation of yellow bands. When 5% ammonia was used, bands tended to smear more, except for the magenta bands for which separation into two fractions was slightly improved. The solvent ultimately chosen for further work was 2% acetic acid, which gavesufficiently clean band separation in the yellows and still allowed the reds to move off the blue pigments.

UV light caused several pigment bands to fluoresce, including some not visible in daylight. No fluorescence was seen with red, green or orange LEDs, although blue pigments appeared dark with red or orange, and non-fluorescing brownish or orange pigments appeared dark under blue light. No bands were seen under blue LED light that were not visible under either daylight or UV light, although the yellow pigment appearing about 4/10 of the way up the chromatograph, which was sometimes too pale to see in daylight, could still be detected with the blue LED. The pigment bands and the effects on them of UV and blue light are summarized below in the pattern diagrams.

Pigment bands fell into five basic patterns. Pattern 1 (fig. 1) and its variations were associated mostly with subgenera *Russula* and *Incrustatula* and is shown below. In general this pattern showed in daylight a blue-green to blue-grey at the baseline, two red pigments, not well separated, a bright yellow, a pale orange-brown spot and a diffuse pale brownish-orange up to the solvent front.

The red pigments did not usually separate, but appeared as one elongated band which was orange-magenta at the lower end and a pure magenta at the upper end.

The bright yellow band at 4/10 of the way up varied in intensity with different species (as did the other pigments), and in some species had a poorly differentiated band directly below it which fluoresced a green-blue in UV light. It was not clear if this was simply the yellow pigment that had smeared or another fraction, since the yellow shone greenish-yellow in UV light when fairly intense in the visible spectrum, becoming bluer at weaker intensities. This yellow fluoresced orange with blue light from an LED, being visible in this and UV light when too weak to be seen in daylight. Other yellow pigments included a pale orange-brown that migrated half way to the solvent front, usually in a well defined spot, which showed as a dark spot or band in UV light just above the yellow and below the blue-green fluorescing band when the latter was present. A third yellow which appeared in visible light as a pale brownish-orange migrated in a diffuse band up to the solvent front, where it was most concentrated. In UV light a band of blue-green was visible just above half-way in some species, and a band of bright violet at the 2/3 to 3/4 mark; above this band to the solvent front the pigment had a low light orange fluorescence, becoming bluer when very weak, and in some cases with fluorescence only in the lower half. These dull yellows did not fluoresce in blue LED light (470nm).

Variations in **Pattern 1** (fig. 1) involved the balance of various pigments, such that some were much reduced compared to others. In the *Xerampelinae* the red pigments fluoresced in UV light but not under blue light from an LED. Gluchoff (1975) found that in many groups of Russulas, including the *Xerampelinae,* there was a large proportion of Rr. I, with or without one or both of the other two reds. The *Xerampelinae* she tested lacked a second red or had only minor amounts of one, so the lack of fluorescence in blue light can be taken as indicative of the presence of Rr. I alone. Other Russulas tested by Gluchoff showed one or both of Rr. II and Rr. a in addition to Rr. I, and unfortunately the first two could not be separated by these simple methods. Collections of *Russula aeruginea* (or *R. stenotricha*) of subgenus *Heterophyllidia,* showed a pattern intermediate between pattern 1 and pattern 3, where other members of this subgenus were placed.

Pattern 2 (fig. 2), e.g., *R. occidentalis,* was similar to pattern 1 but with fewer bands. Pattern 2 had a blue baseline pigment which appeared somewhat brownish to purple-grey, was not fluorescent in UV light, but orange-pink in blue light. Above it and incompletely separated was an orange-pink spot which fluoresced that colour in UV light. As these two pigments partially overlaid one another, the fluorescence or lack thereof may have been due to the optical mixing. About 1/4 of the way up was a yellow-brown spot which fluoresced light blue in UV and orange-pink in blue light. At half-way was a pale yellowish band fluorescing yellow-green to green-blue, similar to that of pattern 1. From there to the solvent front was a diffuse pale orange-brown area which contained a strongly fluorescent violet band directly above the yellow, and above that a dimly bluish to pale orange fluorescence in UV light, in one case with a non-fluorescent area above this.

Pattern 3 (fig. 3), e.g., *R. medullata* and *R. brunneola,* was much simplified, with a greyish or brownish spot on the baseline which fluoresced orangeish in UV but not in blue light, and which seemed to be equivalent to Gluchoff's (1969) Russulacyanine specific to the *Heterophyllae* sensu Romagnesi. Above that at about 1/3 to 1/2 way was a yellow-brown spot which fluoresced bright blue in UV but with a non-fluorescent spot in the lower half. Neither of these fluoresced in blue light.

Continuing up to the solvent front was a diffuse very pale dull yellow which was undifferentiated into bands in UV light but fluoresced blue to violet-blue.

Pattern 4 (fig. 4), e.g., *R. crassotunicata,* was simpler still with a brownish blue-grey spot at the baseline which fluoresced orange in both UV and blue light, and about 1/3 of the way up to the solvent front was a brownish yellow which fluoresced light orange in UV light.

Pattern 5 (fig. 5), e.g., *R. adusta* and *R. cerolens,* had a brownish blue-grey which fluoresced orange in UV light at the baseline, 1/4 of the way up was a colourless band which fluoresced orange in UV light; above that lay a pale yellow band to 1/2 way up; which fluoresced yellowish white in UV light, and above that to the solvent front was a brownish yellow area which fluoresced orange in UV light. There was no fluorescence with the blue LED.

Table 1 *Russula* species with pattern 1 pigmentation.

Pattern 1	Blue-grey	Magenta2 UV+ BlueLED(+)	Magenta3 UV+ BlueLED(+)	UV Cyan	Bright yellow UV+, blueLED+	Pale orange-brown UVneg	UV cyan	UV violet	Pale orange UV+	Brownish, UVneg
R. aeruginea LB	Tr	Tr	Tr	Tr	+	?			+	
R. aeruginea LB	+	Tr	Tr	+	+	+		+	+	
R. aeruginea Kok	+	+(led-)		+	Tr	+			++	Tr
R. viscida	++	++(led-)	+	+	++	Tr	+	+	+	
R. raoultii	Tr	Tr				+?		Tr	+	
R. fragilis	+	+	+		+	+	+	+	+	
R. fragilis	++	++	++		+	+	+	+	tr	
R. laccata	+	+	+		+	+	+	+	+	
R. silvicola	Tr	++	++	+	+	+	+	+	+	
R. silvicola	Tr	++	++	tr	++		+	+	+	
R. sanguinaria	+	++	++		Tr	Tr		Tr	+	+
R. sanguinaria	+	++	++		+	+		+	+	
R. lutea	Tr	+	+	Tr	++	++	Tr			
*R. lepidiformis**	Tr	++	+	+	+ led-	Tr	+	+		Tr
R. murrillii	++	+(led-)	+(led-)		++		Tr	+	+	
R. veternosa	+	+	+	Tr	+	+		+	+	
R. veternosa	+	+	+	Tr	+	+	+	+	+	
R. veternosa	+	++	+	Tr	++	+	+	+	+	
R. veternosa	+	+	+	+	++	+		+	+	
R. olivaceae	++	+	+	+	+	+	+	+	+	
R. xerampelina	++	++ (led-)	++ (led-)	Tr	+	+	+	+	0	Tr
R. xerampelina	++	++ (led-)	++ (led-)	Tr	+	+	+	+	0	Tr
R. semirubra	+	++ (led-)	++ (led-)	0	+	+	0	+	0	
R. cf. *pruinosa*	++	+ (led-)	+ (led-)	+	+	+		+		+

Pattern 1	Blue-grey	Magenta2 UV+ BlueLED(+)	Magenta3 UV+ BlueLED(+)	UV Cyan	Bright yellow UV+, blueLED+	Pale orange-brown UVneg	UV cyan	UV violet	Pale orange UV+	Brownish, UVneg
R. eleodes	++	+ (led-)	+ (led-)		++	+		+		+
R. isabelliniceps	+	+ (led-)	+ (led-)	+	+	+		+		+
R. isabelliniceps	++	+(led-)	+(led-)	+	+	+		+	Tr	Tr
R. abietina	++	+	+ (UV&led-)		++	+		+	tr	
R. cessans	++	+	++		+	+		+	Tr	
R. aureofulva	+	+	Tr	+	+	+	Tr	+	++	
R. nauseosa	++	+ (led-)	+ (led-)		+	Tr		++		
R. aeruginoides	+	+	Tr	Tr	Tr	++			Tr	

Abbreviations and symbols: LB =Longbeach area, near Tofino, Pacific Rim National Park; Kok=Koksilah river valley, north side; * the yellow not visible in daylight, or LED, only UV.

Table 2 *Russula* species with pattern 2 pigmentation.

Pattern 2	Brownish -blue-grey, LED-orange	Magenta	Yellow-brown UVblue	Pale yellowish UV+	UV greenish	UV violet	Pale orange UV +	Brownish, UVneg
R. occidentalis	+	+	+	+	+		+	+
R. occidentalis	+	+	+	+	+	+	+	
R. occidentalis	+	+	+	+	Tr	+	+	
R. occidentalis	+	+	+	+	Tr	+	+	

Table 3 *Russula* species with pattern 3 pigmentation.

Pattern 3	Pink-brown UV orange	Yellow-brown UV negative	Yellow-brown UV blue	Pale dull yellow UV-orange - white
R. brunneola	+	++	++	++
R. brunneola	+	?	+	+
R. medullata	+	+	+	+
R. parazurea	+	+	+ (UV yellowish)	+
R. sublevispora	+	Tr	++	+

Table 4 *Russula* species with pattern 4 pigmentation.

Pattern 4	Brownish -Blue-grey, UV Or, Blue LED-orange	Brownish yellow, UV Or,(upper 2/3)
R. crassotunicata	+	++
R. crenulata	+	++

Table 5 *Russula* species with pattern 5 pigmentation.

Pattern 5	Grey, UV & blue LED-ve	Colourless, UV orange, blue LED-ve	Faint yellow, UV yellowish white	Brownish yellow, UV Or	Grey-brown, UVnil
R. adusta	Tr	+	+	++	
R. adusta	Tr	+	+	++	
R. dissimulans	+			++	
R. dissimulans	+			++	
R. cf. *pectinata*	+			++	
R. cerolens	Tr		+	+	+
R. fragrantissima	+		+	Tr	

Explanation of symbols in Tables 1 to 5; + = present, ++ = present and relatively abundant, Tr = trace amounts, ? = difficult to tell if there was a trace amount because of masking from other pigment bands.

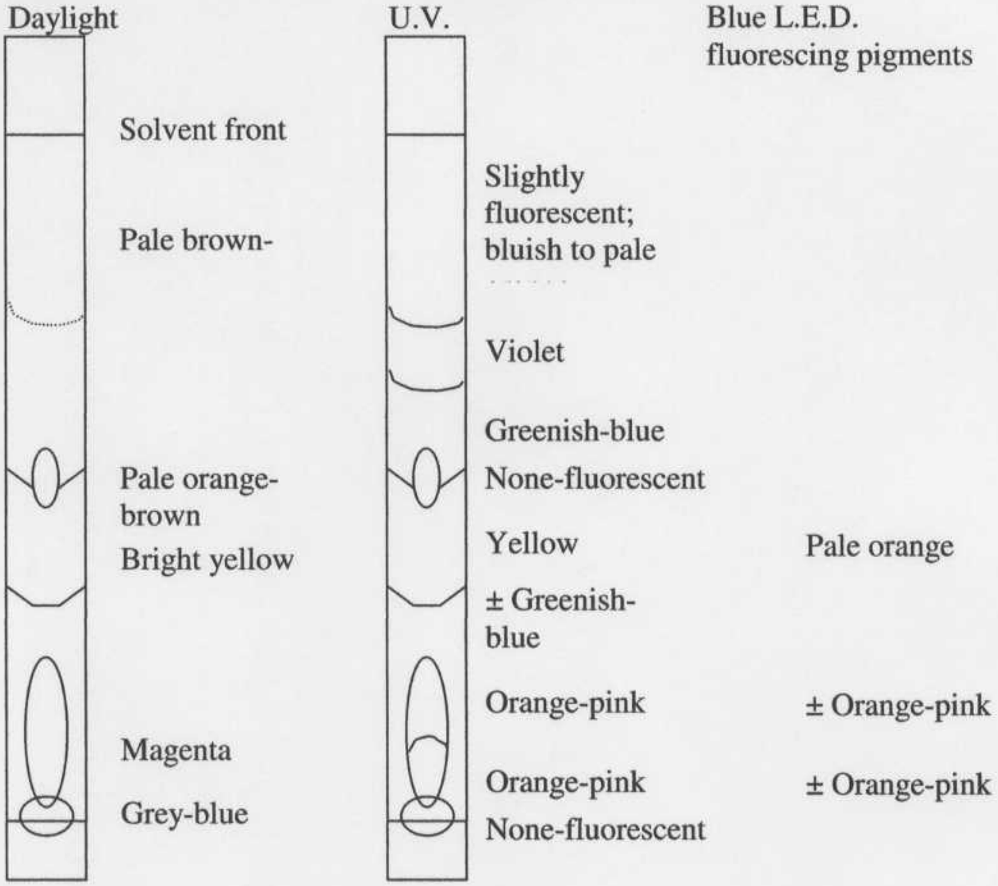


Figure 1 Pattern 1 e.g. *R. veternosa*.

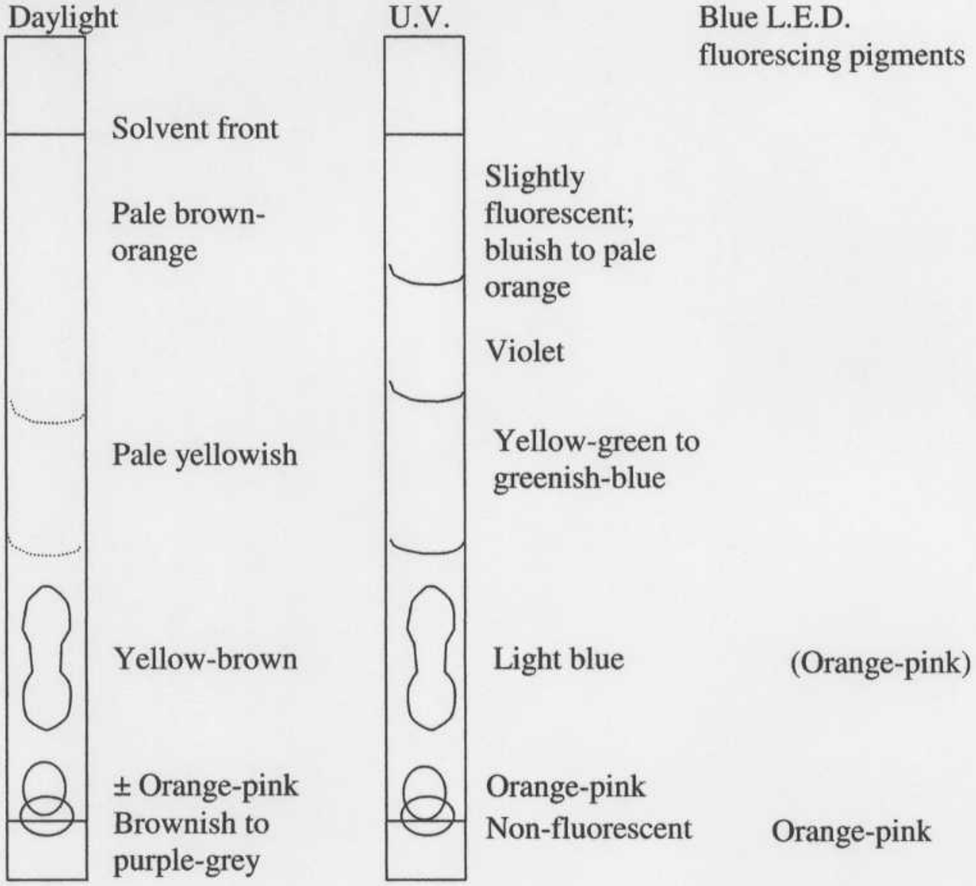


Figure 2 Pattern 2, e.g. *R. occidentalis.*

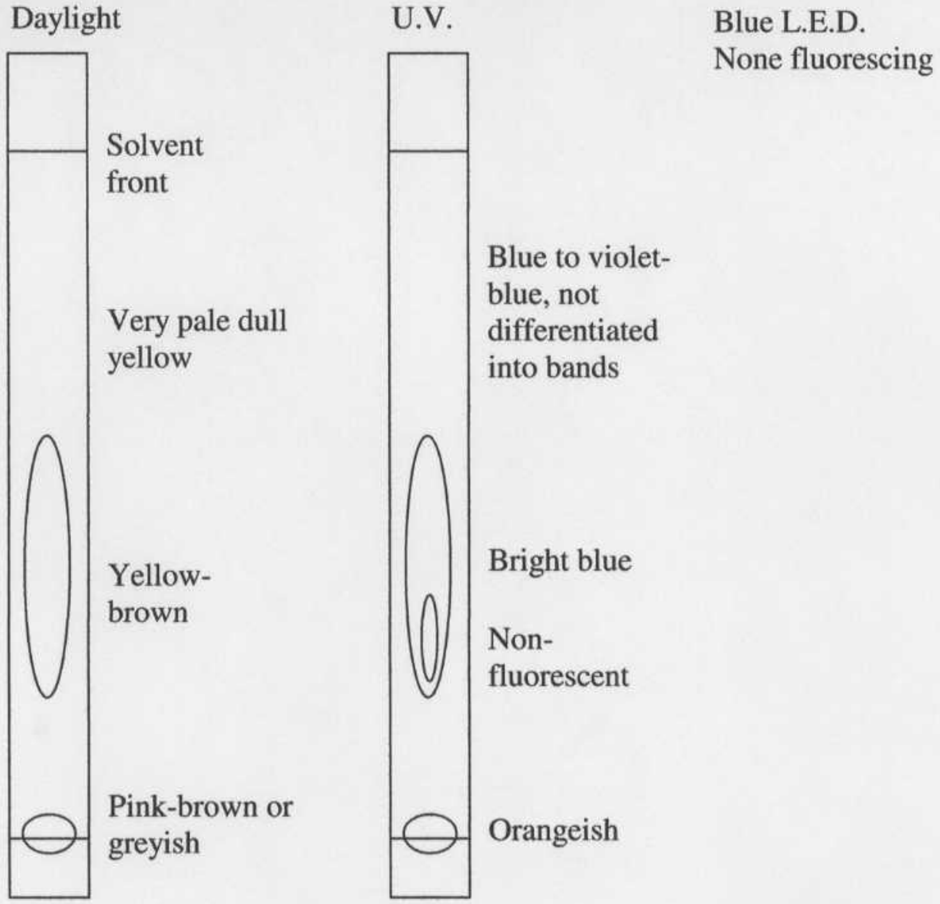


Figure 3 Pattern 3 e.g. *R. medullata.*

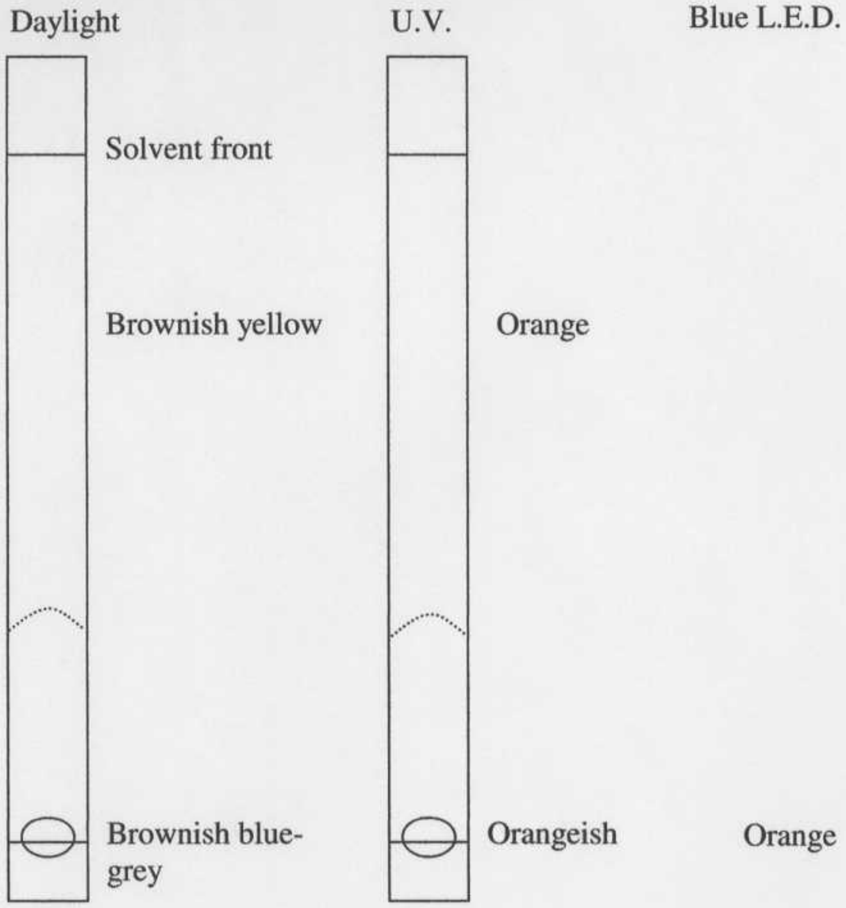


Figure 4 Pattern 4 e.g. *R. crassotunicata.*

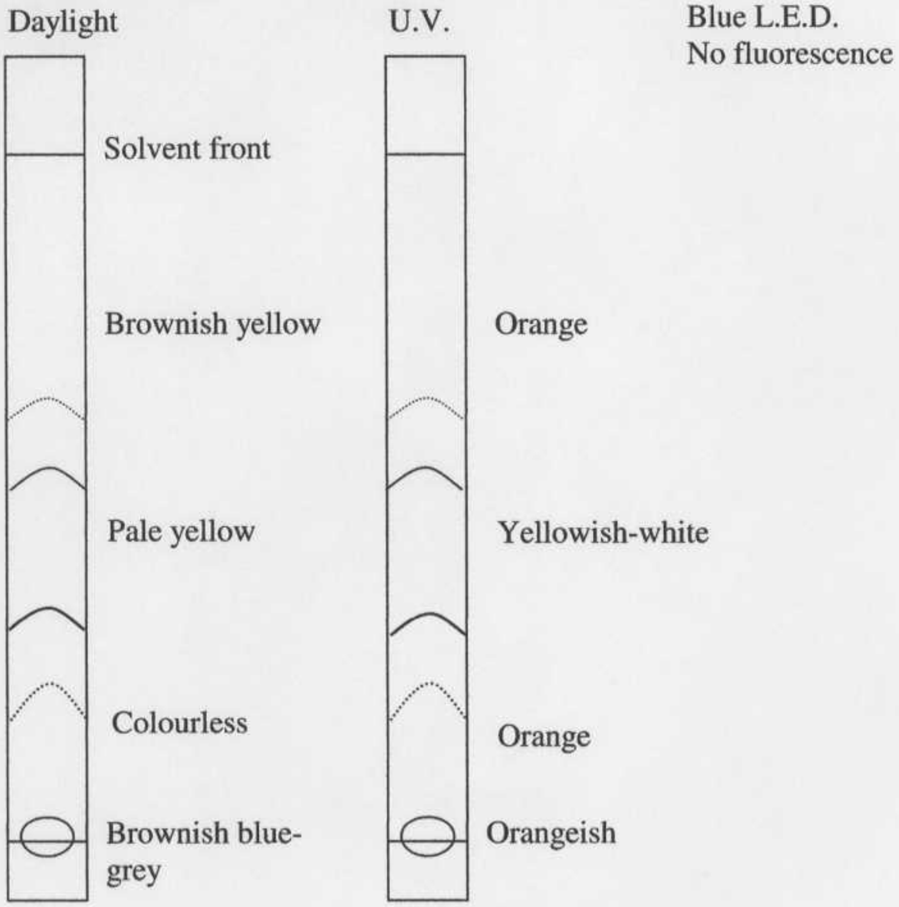


Figure 5 Pattern 5 e.g. *R. adusta*

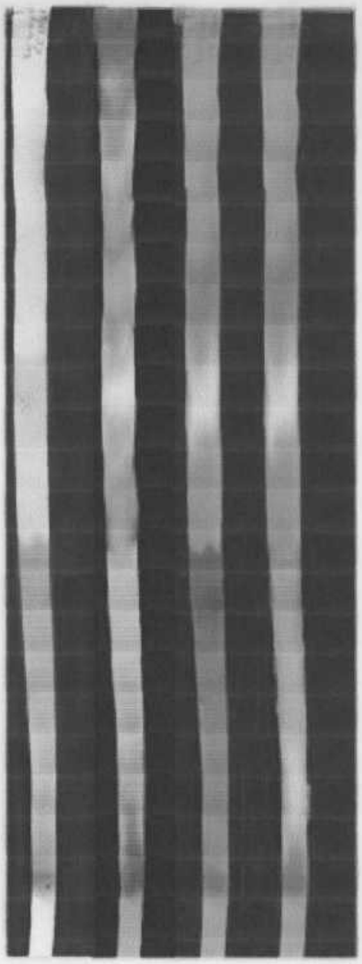


Figure 6 Paper chromatograph under three light conditions, on left, *Russula xerampelina* pigments under natural light, second from left, in UV light, third from left, under a blue LED light, and right, the effect of a blue LED light on a chromatograph with the red pigment fluorescing, for example *Russula silvicola.* The chromatographs shown were done with a high concentration of pigment, extracted from about half of a large basidioma, with the colour digitally saturated with photographic manipulation software (Arcsoft Photostudio 3.0), in order to show the colour bands, which are normally much paler. The chromatographs shown in UV and blue LED light were difficult to photograph because of the low light levels and the colours that the eye detects were not apparent on the photograph. In order to show what the eye sees, colours were digitally superimposed where appropriate on the chromatograph.

Conclusions and Discussion

In searching for effective extraction and chromatography solvents, acetone, chloroform, ethanol, ethanol-water mixtures, 5% ammonia solution and 5% acetic acid solution were tried, but gave results inferior to water. Both the blue and the red pigments are altered by strong acids and alkalis while in the cutis, and even 5% ammonia solution can in some species effect a colour change in the tissues, so their use in extraction had potential problems.

The extraction method above contrasts with that of Gluchoff (1969) who used ethanol:water in a 1:1 ratio followed by multiple aqueous pyridine extractions at room temperature. The method adopted in the present study was simple and used easily obtainable non-toxic materials, and since the isolation and chemical analysis of individual pigments was not the intent, it proved adequate for defining overall patterns associated with taxonomic groupings. It is possible that not all of the pigments were extracted with hot water, and that some additional cell proteins were included since the heat may break cells. However, similarities with the pigment patterns obtained by Gluchoff (1969, 1975) suggest that this hot water extraction was quite effective.

The use of narrow waveband light such as can be emitted by LED lights shows promise as a highly portable spectrum examination system. The blue light at 470nM had less available energy than a UV light to excite the electrons to a higher orbit in fluorescent materials, so fluorescence tended to be lower, a different colour, or affected fewer pigments. Different molecules fluoresce under different and precise spectra and if the molecule and the wavelength required to excite it are known, in theory a light of that specific wavelength should be able to detect its presence or absence in a chromatograph.

Direct comparisons with the pigments detected by Gluchoff (1969, 1975) and Iten *et al.* (1984) could not be done without subjecting the cuticles to the same procedures they used, which required considerably more material than the few mm^2 of cutis used here. However, some similarities exist: specifically, the red which did not fluoresce under blue LED light appeared to be analogous to Rr I, and those that did fluoresce in both UV and blue light were similar to Rr II and Rr a, which do not easily separate anyway and were identified spectroscopically by Gluchoff (1975). Most of the species tested in 1975 by Gluchoff had only one of two blue pigments; Rc I for the majority, and

Rc II for *R. nauseosa, R. heterochroa* and *R. maculata,* plus the blue specific to the *Heterophyllae* sensu Romagnesi, which fluoresced in UV light, and which was evident in the Vancouver Island species in this group. In view of the fluorescence with a blue LED of the blue pigment in four of the collections of *R. occidentalis* further investigation with narrow waveband blue light could well find specific patterns of fluorescence in these pigments. Of the seven yellow pigments Gluchoff (1969) isolated, some of which Iten *et al*. (1984) suggested may be intermediate products or decomposition products of the other yellow pigments, one that seemed to be in most species of both Vancouver Island and Gluchoff's (1969, 1975) collections, and the first yellow to be eluted in water was the pale orangish colour that ran up to the solvent line, probably analogous to Rx I, and which matched her description of a dull yellow fluorescence in UV.

The bright clear yellow that ran about 1/3 to 1/2 of the distance to the solvent front was most likely analogous to Rx V, which was found in varying quantities in all but the *Ingratae* and *Heterophyllae* in Gluchoff (1969, 1975), and in the same groups here, and also in *R. aeruginea*. The identity of the other yellows was not clear.

Interestingly, *R. aeruginea* in section *Heterophyllae* had a pigmentation pattern intermediate between that of subgenus *Russula* and that of the other members of subsection *Griseinae*. This, together with the reduction in the cutis of multiple septae in the epicutis hyphae and few inflated epicutal hyphal cells, and the relatively large spore ornamentation for this subgenus, suggests that local *R. aeruginea* could be be a link between the two subgenera.

Russula adusta, a member of subgenus *Compactae* (Fries) Bon, Section *Compactae* Fries, showed traces of the yellow pigment in the same position on the chromatograph as the yellow in those of subgenus *Russula.* There were also traces of pigment that were not visible in daylight but showed slightly orange in UV light in the same position as the UV+ magenta in red and purple Russulas. These pigments were not seen on the chromatograph of *R. nigricans*, a species in the same section as *R. adusta* and with a similar blackening of damaged flesh. The pigment patterns point to a closer relationship between *R. adusta* and subgenus *Russula* than is found in other sections of subgenus *Compactae*. The *Foetentinae* shared the same basic pattern as these members of the *Compactae*. Miller and Buyck (2002) in their phylogenetic analysis of the genus

Russula found that the clade with *R. adusta* and *R. nigricans* did not fall within the other *Compactae* examined but branched off after the *Heterophyllae* and *Foetentinae* clade, and was basal to the subgenus *Russula* sensu Romagnesi (1967), a position supported by this pigment analysis, (at least for *R. adusta*).

Members of the *Xerampelinae* (*Viridantinae* sensu Romagnesi 1967) including *R. xerampelina* var *xerampelina, R.* cf. *pruinosa, R. eleodes, R. isabelliniceps* and *R. semirubra* all shared a similar pattern and with the exception of a *R.* cf. *abietina* were the only ones so far tested that had a strong red pigment that did not fluoresce in blue LED light at 470nM. Their close relationship was confirmed by the similarities in the RFLP patterns of the ITS rDNA region, both within Vancouver Island collections and collections deposited in GenBank from Europe and North America.

The reduced pattern of the four *R. occidentalis* may be an artifact of the spotting technique on the chromatography paper, the extracts for most of these collections were very pale and needed repeated application with air drying in between applications to obtain sufficient depth of colour to be visible when separated out. This may also have had the effect of concentrating the viscid substance generated by the cuticular hyphae, which appears to interfere with the ability of pigments to elute up the paper. *Russula occidentalis* is in the subsection *Integroidinae* (Romagn.) Bon and was unfortunately the only member of this group to be collected in this study, so comparisons cannot be made to ascertain which of the patterns is correct. Gluchoff (1969) examined the pigments of *R. claroflava,* a yellow species also in the same subsection and clade as *R. occidentalis*, finding no red and blue pigments, but four yellow pigments and the colourless pigment that fluoresces violet in UV light.

Russula crassotunicatae and *R. crenulata* had a pattern not yet seen in another species, supporting the separation of these species into subsection *Crassotunicatae* Singer within the *Ingratae* (Quélet) Maire.

With the exception of *R. aeruginea,* which had a pigment pattern closer to pattern 1, species in subgenus *Heterophyllidia* Romagnesi had pattern 3 which lacked strong reds and clear yellows, even though greens and pinks appeared in the whole cutis. Evidently these pigments were not extracted by hot water, or they did not retain their original colours in the processing. Either way, the pattern formed seems to be indicative of members of this subgenus.

The pigment pattern analyses using the simple method outlined above on the whole supported the newer taxonomic divisions of Sarnari (1998) but also raised new questions regarding anomalous species. As an identification tool, it quickly resolved whether a species was in the *Compactae-Foetentinae* group, the *Heterophyllidia,* or the other subgenera of *Russula* and *Incrustatula* sensu Sarnari 1998, although these latter two are not differentiated by this tool. Even though these groups are all fairly well defined in terms of microscopic morphological characters, it is not uncommon to encounter a collection in which one or more characters are ambiguous, particularly if the collection is very young, weathered, or at one extreme of the character diversity, and especially if it is one which the researcher has not encountered before. The *Xerampelinae* have in common red pigments lacking fluorescence under the blue LED light, a characteristic also found in some of the *Incrustatula,* some of which form a basal clade and some a sister clade to the former (Chapter 2). Any tool that can eliminate a range of possibilities increases the chance of an identification being made, and whilst this method of pigment analysis has its limitations, it also presents the potential for further developments and adaptations to make it more specific.

Chapter 3

DNA analyses Part 1: Evaluation of the RFLP of the ITS region of Russulas as a means of differentiating among them, and confirming their identity by comparison with published sequence data.

Introduction

In making an identification of a species of *Russula* the usual method of matching morphological characters of the species in hand with published descriptions does not always result in a satisfactory conclusion. Part of the problem is that descriptions vary in the amount and clarity of the information given, and in the accessibility and language of the journals in which they are published. Viewing holotypes is not always practical, especially when a number of species are being questioned, therefore, other methods of either confirming an identification or placing an unknown *Russula* species into a taxonomic unit within the genus would be helpful.

In the Pacific Northwest, in addition to purely local species, there are many species of Russula that are also found in the temperate zones of Europe and Asia. Increasingly, DNA sequences of parts of the genome of these species are being made publicly available in databases such as GenBank at NCBI and the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI).

DNA sequences are an additional source of useful characters in systematics with different parts of the genome varying to different degrees. In general, more conserved regions are useful for elucidating phylogenetic relationships at higher taxonomic levels, while more variable regions are useful at lower taxonomic levels. Published sequences are, therefore, another set of characters available for comparison with an unidentified species.

Sequencing genes is becoming faster and more cost effective, but is still difficult for many researchers, particularly those who do not have ready access to sequencing facilities. Comparing restriction fragment length polymorphisms (RFLP) of an amplified gene or spacer DNA is one alternative with less precision than sequencing, but with the power to differentiate between taxa at the subgeneric level (Kernaghan *et al.* 1997, Suga *et al.* 2000). The results can also be compared with published RFLP data and with sequence data available through GenBank. Such comparisons to confirm or discover the identity of a basidioma would also be useful in ectomycorrhizal studies, since the below-ground fungal population often does not conveniently produce fruiting bodies with which to compare DNA (Gardes and Bruns 1996, Durall *et al.* 1999).

The use of rDNA genes and spacer regions for RFLP comparisons

An appropriate region of the genome to use for RFLP comparison needs to have sufficient variation to distinguish between species but little intraspecific variation. Phylogenetic studies have focused on the mitochondrial ribosomal DNA genes (rDNA) (Peter *et al.* 2001) or on nuclear rDNA as these sequences have both conserved and variable regions which can be selectively amplified (Gardes & Bruns 1993, Vilgalys and Hester 1990, White *et al.* 1990).

The ribosomal large sub-unit (LSU or 28S), a very conserved region of DNA, has been preferred for taxonomic analyses above the genus level (Drehmel *et al.* 1999, Eberhardt 2002, Henkel *et al.* 2000, Miller *et al.* 2001, Montcalvo *et al.* 2000, 2002). The small subunit (SSU or 18S) has similarly been used to establish phylogenetic relationships among the fungi (Bruns *et al.* 1992).

The internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) gene is amongst the most widely sequenced DNA regions in fungi (Vilgalys 2001), as it is often used to determine intrageneric phylogenies (Kretzer *et al.* 1996, Aanen *et al.* 2000, Høiland and Holst-Jensen 2000, Miller *et al.* 2001, Miller and Buyck 2002, Eberhardt 2002) and between isolates of a species or species complex (Gomes *et al.* 2000, Methven *et al.* 2000, Zervakis *et al.*2004). Gottlieb and Lichtwardt (2001), however, found it too variable to infer phylogenies in the *Harpellales*.

The internal transcribed spacer region (figs.7 and 8) is a variable region about 600-1000 base pairs long; in *Russula* it is usually between 800 and 920nt, containing two non-coding spacers between the highly conserved 18S and 28S subunits, and flanking the conserved 5.8S subunit of the ribosomal RNA gene in the nuclear genome (Gardes and Bruns 1993, 1996). The whole 18S-ITS1-5.8S-ITS2-28S complex occurs in multiple repeating units each separated by two intergenic spacers, IGS1 and 2, which flank the 5S subunit (fig. 7).

In *Schizophyllum commune,* the variation between repeating ITS units was found to be very low but variation was seen between IGS repeats (James *et al.* 2001). The non-coding region may be much more variable than the subunits of the gene, which has been a problem in its use for inferring phylogenies in some studies (Gottlieb and Lichtwardt 2001). In other cases, the variation between species has been found to be low, less than 1% in some groups of *Hebeloma* (Aanen *et al.* 2000). In fungi, variation among both the ITS and IGS regions can occur within a species (James *et al.* 2001, Pantou *et al.* 2003), and even between repeats within an individual (James *et al.* 2001, Gomes *et al.* 2002), but this is usually less than that between species. The large sub-unit (LSU or 28S), has been used for higher levels of taxonomic analyses than the ITS region (Henkel *et al.* 2000, Miller *et al.* 2001, Montcalvo *et al.* 2000, 2002, Eberhardt 2002). But the ITS region was used for infrageneric phylogenetic analyses of *Russula* as it has an appropriate level of variability for this purpose (Miller *et al.* 2002, Eberhardt 2002).

Primers

Gardes and Bruns (1993) developed the basidiomycete-specific primer pair ITS1-F/ITS4-B (table 6), which reduces the possibility of amplifying contaminant moulds, often a problem with field-collected specimens of fungi. *Russula* basidiomata are frequently infested with insect larvae, sometimes even in the primordial stage, and should not be assumed to be sterile inside the trama. Growing pure cultures of *Russula* from spores or even from basidiomata is still unreliable and difficult, and growth extremely slow (Ali and Jackson 1989, Hintikka and Niemi 1999), so fresh or dried basidiomata were used in this study. Primer pair ITS1-F/ITS4-B yields an amplification product about 14nt longer at the 5' end than the forward primers ITS5 and SR6R, and about 137nt longer at the 3' end than ITS4 (fig. 8), this latter being commonly used in published sequences.

Dunham *et al.* (2003) used the primer pair ITS1-F and ITS4 (rather than ITS4-B) to amplify DNA from *Cantharellus* species after testing both reverse primers. Of 152 ITS1 sequences of *Russula* species held in the GenBank and EMBI databases, 38 commenced within the ITS1 primer site and 87 within the ITS5 site, the remainder commenced downstream of these sites. Of 150 *Russula* sequences containing the complete ITS2 region, 13 include or go beyond the ITS4-B recognition site, 90 terminate within the ITS4 recognition site and one terminated at the LR1 site. A more recent set of sequences placed in Genbank and listed in appendix 2, begin within ITS5, 20nt downstream from the 5' end of the ITS1-F site, and terminate beyond the ITS4-B site. Four *Russula* sequences covered the entire ITS1-F to ITS4-B region, three of these were from B.C., Canada (Durall *et al.* unpublished). The sequence string between the forward primers ITS1-F, ITS5 and ITS1 is within the conserved 18S subunit, and that between ITS4 and ITS4-B is within the conserved 28S subunit: both these regions show little variation among *Russula* species. Some commonly used ITS primers are shown in table 1, below, and their recognition sites are demonstrated in an example sequence of *Russula xerampelina* in fig. 8.

Table 6 Primers developed for the ITS region and shown in Figure 8

Primer	**Target sequence 5' to 3'**	**Developed by**
ITS1	TCCGTAGGTGAACCTGCGG	White *et al.* 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* 1990
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns, 1993
SR6R	AAGWAAAAGTCGTAACAAGG	Vilgalys lab*

Reverse primer	**Target sequence 3'to 5'-(same strand)**	
LR1	AGGAAAAGAAACCAACC	Vilgalys and Hester, 1990
ITS4	GCATATCAATAAGCGGAGGA	White *et al.* 1990
ITS4-B	CTGGACCGTGTACAAGTCTCCTG	Gardes & Bruns, 1993

*Unpublished data available on <http://www.botany.duke.edu/fungi/mycolab>

Restriction enzymes used in ITS-RFLP studies

Farmer and Sylvia (1998) tested seven restriction enzymes for their ability to differentiate among 69 species in 17 genera of ectomycorrhizal fungi, using the ITS1-F/ITS4 fragment. They found that almost all isolates had a restriction site for AluI, CfoI, HinfI and TaqI, but needed more than one enzyme to distinguish them, since members within a genus often shared restriction fragments. The ITS1-F/ITS4-B fragment, cut with the enzymes AluI and Hinf1, has been successfully used to identify the fungal partners of ectomycorrhizae by matching the RFLP patterns with those of various species whose basidiomata were collected nearby (Gardes and Bruns 1996), and for separating two species of *Lactarius* (Kraigher *et al.* 1995). Goodman *et al.* (1996) routinely include the HinfI, AluI and Rsa I RFLP patterns of the ITS1/NL6Bmun fragment, which includes slightly more of the large subunit gene than ITS4-B, for their descriptions of ectomycorrhizae. The restriction enzymes HinfI, AluI and NdeII or its isoschizomer MboI, have been used on the amplified ITS region in the identification of ectomycorrhizae (Smith and McKay personal communication, Sakakibara *et al.* 2002). NdeII, which recognises the GATC motif, has nine isoschizomers including DpnII and Sau3AI. Kernaghan *et al.* (1997) used HinfI, AluI and RsaI and HhaI in their study of Russulaceous ectomycorrhizae and concluded that RFLP of the ITS region using these enzymes gave an "appropriate level of resolution for the taxa studied". They also noted that RsaI did not always cut the sequence. This was the experience of Suga *et al.* (2000) using RsaI in addition to three other enzymes to fragment *Fusarium solani* rDNA ITS region. The restriction sites for HinfI, AluI and Sau3AI in a sequence of *Russula xerampelina*, together with the primer recognition sites listed above it, are given in fig. 8. All of these enzymes recognise a 4-base pair sequence motif hence cut DNA more frequently than enzymes that recognise 6 or more base-pair targets.

Extraction protocols

Extraction protocols vary depending on the source organism and the purpose for which the extracted DNA will be used. Different organisms vary in the amounts and type of contaminants, such as proteins and polysaccharides, which are extracted with the DNA: sometimes these contaminants interfere with amplification and have to be removed. In other cases simple dilution of the template solution solves problems. Extraction of DNA involves the physical or chemical breaking open of cell walls to release the cell contents. This is accomplished by freeze-drying, nitrogen freezing and crushing, freeze-thaw cycles or grinding tissue with or without sand. The breaking of cell membranes to release the nuclear material is usually accomplished with a detergent such as sodium dodecyl sulphate (SDS) or cetyl trimethyl ammonium bromide (CTAB), the latter also inhibiting the action of DNAase. Finally the released DNA needs to be separated from the extraction chemicals and cell debris.

Many extraction methods use phenol and chloroform to remove proteins from the extraction mixture (Moller *et al.* 1992, Gardes and Bruns 1993, Bergemann and Miller 2002), and most protocols use several Eppendorf tubes for each sample as the DNA and cell debris are separated. Efforts have been made to reduce the use of hazardous chemicals and the number of transfers to new tubes, a particular consideration for automated systems. Aljanabi and Martinez (1997) used salt instead of chloroform to precipitate out proteins and carbohydrates from a variety of organisms including fungi. They obtained DNA of a quality and quantity suitable for multiple PCR reactions. Norvell (2000) extracted sufficient DNA from fresh spores of *Phaeocollybia* species by freeze-thaw cycling in TE buffer, which was then used directly as the template solution to amplify the ITS region without further modification. This method worked only on very fresh spores (Norvell, pers. communication). Similar techniques are applied to plant tissue using commercially prepared lysis buffer and a short heat-cool series in a thermocycler before being used directly as a PCR template (Burr *et al.* 2001). Shimono *et al.* (2004) used an SDS-based extraction buffer containing proteinase K for their phylogenetic study of members of the *Russulaceae*. After incubation with homogenised tissues and separation of the cell debris by centrifugation, the extraction buffer was directly used as template DNA. The many protocols and variations reported in the literature suggest that a certain degree of experimentation is required to extract DNA from a given organism adequate for the intended purpose, and that some steps may prove to be optional.

The objectives were: 1) To develop a method for isolating genomic DNA from fresh and air-dried *Russula* basidiomata, and for the amplification and digestion of the internal transcribed spacer (ITS) region, and 2) To evaluate the usefulness of the ITS region and its restriction fragment length polymorphisms (RFLPs) as a means of differentiating among species of *Russula*, as a tool for grouping similar species, and as a means of confirming identifications through comparison with published sequence data.

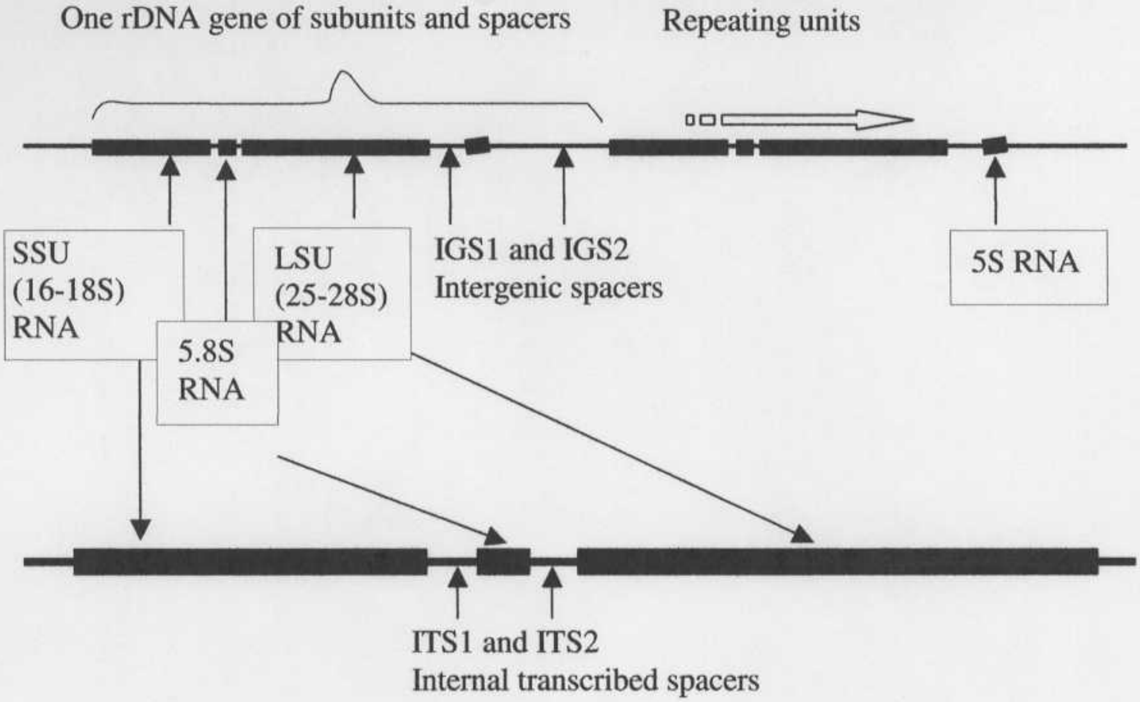


Figure 7 The rDNA coding subunits, internal transcribed spacers and intergenic spacers. Diagrammatical representation of repeating units of the ribosome genes, genes are separated by two intergenic spacers: IGS1 and IGS2, one either side of the 5S coding unit. The remaining three coding units are separated from one another by transcribed spacers: ITS1 lies between the small (18S) and the 5.8S subunits, and ITS2 lies between the 5.8S and the large (28S) subunits.

ITS1-F

CTTGGTCAATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG**GATC**ATTATCGTACAACG

ITS5, SR6R ITS1

GAGGTGTGAGGGCTGTCGCTGACCTTTAGAGGTCGTGCACGCCCGAGCACTTTCATACAATCCATCTCACCCCTTTGTGC

ATCACCGCGTGGGTCCCCTTTGCGGGAGGGCCCGCGTTTTCACATAAAACTCGATACAGTGTAGAATGTTCTTTTGC**GAT**

CACACGCAATCAATACAACTTTCAACAACG**GATC**TCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGT

AATGTGAATTGCAGAATTCAGT**GAATC**ATC**GAATC**TTTGAACGCACCTTGCGCCCCTTGGCATTCCGAGGGGCACACCCG

TTTGAGTGTCGTGAAATCCTCAAAAACCCTTTTCTTGTCAGGAAAGGGATTTTTGGACTTGGAGGTTCAATGCTCGCCTTT

CACTTTTGAAAGTG**AGCT**CCTCTCAAATGAATTAGTGGGGTCCGCTTTGCT**GATC**CTTAACGTGATAAGATGCTTCTACG

TTTTGGACCCGGCTCTGTCCCTTGGATGCCTGCTTCTAACTGTCCCACGGACAATGATGGTGCTTCGGTCACTGCCATCTA

CATTGGCGGGAGGCTGGACCCACAAAATGAAAACCTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGC

ITS4

ATATCAATAAGCGGAGGAAAAGAAACT*AACAAG**GATTC**CCCTAGTAACTGCGAGTGAAGCGGGAAA**AGCT**CAAATTT

LR1

AAAATCTGATGGTCTTTGGCCGTCCGAGTTGTAATTTAGAGAAGCGTCTTCCGCGCTGGACCGTGC*ACAAGTCTCCTG

ITS4-B

Figure 8 An example of the ITS 1 and 2 sequence with the forward primers ITS1-F, ITS5, SR6R and ITS1 and reverse primers ITS4, LR1 and ITS4-B under or overlined, differences between the primer nucleotides and the sequence nucleotides are marked with an asterisk. In bold shaded text are the restriction sites of HinfI (GANTC), AluI (AGCT) and Sau3aI (GATC). The sequence is from *Russula xerampelina* var. *xerampelina,* GenBank accession number DQ367916.

*Base differs from that of the primer.

Materials and Methods

Russula species were collected from coastal forests of southern Vancouver Island over the course of five fruiting seasons. A fruiting season generally runs from mid to late summer through fall and winter, with some Russulas withstanding light frosts but not fruiting at temperatures below freezing. Species were identified using the following references: Romagnesi (1967, 1985), Sarnari (1998), Gibson (2000), Thiers (1997), Woo (1989), Phillips (1991), Hesler (1960, 1961), Grund (1965), and Shaffer (1964, 1970, 1972). As collections were destined eventually for an herbarium they were dried in a warm-air food drier; and for many Russulas this was done before a sample was taken for DNA analysis. Other collections made in the weeks before the DNA analyses were carried out had a sample removed and stored frozen in a 600µl aliquot of CTAB buffer or were extracted and the precipitated DNA stored dry and frozen at -20°C.

Selection of restriction enzymes

An initial selection of ITS sequences from 17 species of *Russula* and one each of a *Martellia, Lactarius* and *Rozites* were trimmed to a common start and end point. The location of all occurrences of the recognition site of each of 6 enzymes was found and the fragment sizes for each enzyme gave a virtual RFLP pattern for each sequence, which was examined for uniqueness among those of other sequences. The first enzyme, HinfI, which recognizes the motif GANTC, was found in all *Russulales* sequences at a double site within the 5.8 S conserved region, each cut site being 8 base pairs apart, effectively separating ITS1 from ITS2. Some sequences also contained additional sites in the ITS2 region. However, this enzyme alone was insufficient to differentiate among all sequences.

The second enzyme AluI, which recognizes the motif AGCT, was found to have up to three recognition sites in ITS2, one of which consistently appeared around 87 base pairs before the downstream end of the ITS4-B primer site. Some sequences also contained a recognition site for AluI in ITS1. Whilst this enzyme in addition to HinfI provided much more resolution among sequences, there remained a few that were not differentiated from one another. Three more enzymes, RsaI (GTAC) , MaeI (CTAG), and HaeII (GGCC) were found to have recognition sites in some but not all sequences.

The enzyme Sau3AI, which recognizes the GATC motif, cut all sequences from 1 to 6 times, and provided good further resolution between test sequences. One site around 60nt downstream of the ITS1-F primer site was found in most sequences. The three enzymes finally selected for the study were HinfI, AluI and Sau3AI, which gave three levels of resolution.

DNA extraction procedure

Fresh and dried *Russula* tissues were extracted, amplified and digested using an adaptation of the basic protocols of Kårén (1999), after also testing the methods of Aljanabi and Martinez (1997) which use sodium dodecyl sulphate (SDS) as the lysis agent and a saturated solution (6M) of NaCl to precipitate the polysaccharides and proteins.

In a trial using two collections of *Russula*; *R. brevipes* and *R. xerampelina*, comparisons between the SDS and CTAB extraction buffers, the salt and the chloroform method of precipitating cell debris, and the ethanol with the isopropanol precipitation of DNA were assessed. Test extractions were diluted to 1:10, 1:100 and 1:1000, amplified and electrophoresed on 1.5% agarose gel. The results indicated that the CTAB buffer was more reliable than the SDS, that the salt precipitation worked about as well as the chloroform regardless of buffer, and that ethanol at 2.5 volumes was more successful than isopropanol at precipitating amplifiable DNA without salt also precipitating out (5/12 test extractions for isopropanol, 20/24 for ethanol). The salt precipitated cell debris did not form a solid pellet when centrifuged and was easily disturbed while removing the supernatant, requiring further cleaning of the supernatant with chloroform in several cases. Due to this and occasional problems with salt contamination of the DNA the salt precipitation method was not used for the main analyses.

A volume of about 3-5mm^3 of fresh tissue or its equivalent of dried tissue was taken from the youngest and cleanest specimen of a *Russula* collection and placed in a 2ml Eppendorf tube. As the moisture content varied in fresh material, weighing was not helpful. The gills of a basidioma have a greater concentration of genetic material per unit volume of tissue than the cap or stipe trama, since the latter contains many sphaerocytes. Gill tissue was therefore expected to release more DNA per mm^3 than stem or trama tissues and so was preferred. With mature, infested or dried specimens, judgment must be made in terms of the tissues and volume selected for extraction. The pattern of decay caused by contaminants such as moulds and insect larvae varies between individual basidiomata, but almost every *Russula* collected contained arthropod larvae. Specimens that had been dried for more than two years, as in herbaria, needed either more tissue or longer processing or both. None of the collections used for DNA analysis were freeze dried since this destroys their microscopic structures and renders them useless for herbarium specimens.

To the tissue in the Eppendorf tube, only 2 to 3 drops of 2% CTAB extraction buffer were added and the tissue ground by hand using a clean blue disposable pestle or a pestle made of 1/4-inch Teflon rod shaped with a pencil sharpener to fit the Eppendorf tube. A pounding, twirling and squashing motion homogenized the tissues most effectively. Fresh tissue could be ground immediately, but dried tissue was better left for up to an hour at room temperature (or overnight in a refrigerator at 4°C) to soften in no more than 1/2 ml of CTAB extraction buffer before grinding. Once a smooth paste was formed, more CTAB buffer was added to the tube contents to bring them to approximately 600μl and any remaining visible lumps carefully macerated with the pestle. Acid-washed sand was used as a grinding agent only as a last resort on somewhat rubbery samples, since it prevented adequate crushing of the tissues. Proteinase-K added to the CTAB solution (at 400μg/ml final concentration) was used when extracting from dried collections, particularly those over two years old, as the DNA becomes bound to the nuclear proteins more strongly over storage time and proteinase helps release it. In all cases amplifiable DNA was extracted without the use of β-mercapto-ethanol.

Each sample was heated to 60-65°C in a heating block or oven and maintained at that temperature for a minimum of one hour for macerates of fresh tissues, and up to overnight for macerates of dried tissues. Each tube was shaken manually at intervals. Removal of cell debris from the buffer solution was accomplished by filtering, since this gave a cleaner supernatant than centrifugation. Filters were made using cotton wool tightly packed into 1ml blue automatic pipette tips to a depth of about 10mm: the flat end of a clean disposable pestle worked well for this. The cotton wool was first soaked for an hour in two changes of 3% hydrogen peroxide, rinsed in three changes of sterile distilled water and oven dried. The filters were autoclaved prior to use. The filtrate was gathered into a new 2ml Eppendorf tube and the cotton filter rinsed through with sufficient CTAB buffer to bring the volume up to 500µl. DNA was then precipitated out with 2.5 volumes (1.25ml) of ice-cold ethanol or 1.5 volumes of ice-cold isopropanol. Ethanol precipitation was the preferred method since it brought down less salt than isopropanol (Aljanabi, personal communication). If DNA was seen forming as a jelly-like or cottony substance the tubes were immediately centrifuged, otherwise they were kept at -20°C for an hour to overnight until DNA was visible. Kårén (1999) recommended a 30 minute spin but usually 5-10 minutes at 13,000 rpm brought down a visible pellet. The supernatant was then carefully drained off so as to retain the pellet, which was then washed with 3 changes of 70% ethanol, each of about 0.3-0.5ml, depending on the size of the pellet, and taking care not to dislodge it. Any pellets that became loose were spun at 13,000 rpm for 30 seconds to 1 minute to redeposit them. Tubes were placed upside-down onto paper towels to drain then left on their sides (to prevent contaminants from falling in) for an hour or two to air-dry. At this point the dried DNA could be stored at -20°C for several weeks.

In cases where the extraction solution was dark, cloudy, or the sample had failed to amplify on a previous attempt, the DNA was cleaned of proteins and lipids after filtering with a wash of 600µl of 24:1 chloroform: isoamyl alcohol, added under a fume hood and mixed well by hand. Cell debris collected and remained in the lower, discarded layer after centrifuging for 5 minutes at 13,000 rpm. The clear Eppendorf tubes sometimes burst in the centrifuge when used with chloroform at or above 10,000 rpm, but could be safely spun for 4 minutes at 7,000 rpm. The upper phase was removed to a clean tube and precipitated with ethanol as above. When this method was used on second attempts to extract amplifiable DNA after an earlier failure, it yielded an improvement in only two cases. With many *Russula* samples it did not make much difference whether a chloroform wash was used or not, because they amplified without this extra step.

Amplification protocol

For amplification, the DNA pellet was resuspended in 100µl of sterile double distilled water, which was found to give more consistent amplification success than TE buffer. When only a very small pellet of DNA was recovered, 50µl sdd H_2O was used and for large or dark-coloured pellets, 200µl were used for resuspension. Tubes were flicked with a finger to mix and left about an hour at room temperature or overnight at 4°C to dissolve the DNA. A dilution of 1:100 or occasionally 1:50 of the above solution as the DNA template in an amount of 10µl per 20µl PCR mix was usually successful.

For amplification a master mix of PCR reagents was prepared and portioned into labelled PCR tubes in 10µl aliquots, followed by the template. All reagents and samples were kept on ice while mixing and until placed in the thermocycler. In each thermocycler run a blank containing the PCR mix and water instead of template DNA was included.

PCR mixes:-

For a 20µl reaction at 1.5mM $MgCl_2$, the following reagents were added in order listed:

3.2µl ultra-pure water
2µl 10x Mg free buffer
2µl dNTP stock 2mM
1µl Primer ITS1-F 50µM
1µl Primer ITS4-B 50µM
0.6µl 50mM $MgCl_2$ soln.
0.2µl Taq DNA polymerase
10µl DNA template

For buffers supplied with Mg already in, the above recipe was followed except that 3.8µl of water was added instead of water and $MgCl_2$ soln.
For a 3mM $MgCl_2$ PCR reaction mix the recipe was as above but used:
2.6µl water
1.2µl 50mM $MgCl_2$

Henegariu *et al.* (1997) examined the effects of different concentrations of $MgCl_2$ on amplification and found an optimum at 3mM which resulted in more amplification product than when the more usual 1.5mM concentration was used. A 3mM $MgCl_2$

concentration was tried on three collections, one of *R. mustelina* which had previously amplified only weakly, one of *R. crassotunicata* which had previously failed, and one of *R. aeruginea* which had amplified normally. The results, shown in figure 9, of a comparison of 1.5mM in the first of each pair of lanes, and 3mM in the second, show an increase in the amount of amplified DNA for *R. mustelina*, lanes 1-4, and no improvement or difference in the *R. crassotunicata* lanes 5 and 6, or *R. aeruginea,* lanes 7 and 8. This information was found after completing the bulk of the DNA work, but a final run of 16 previously failed samples resulted in 7 of them amplifying.

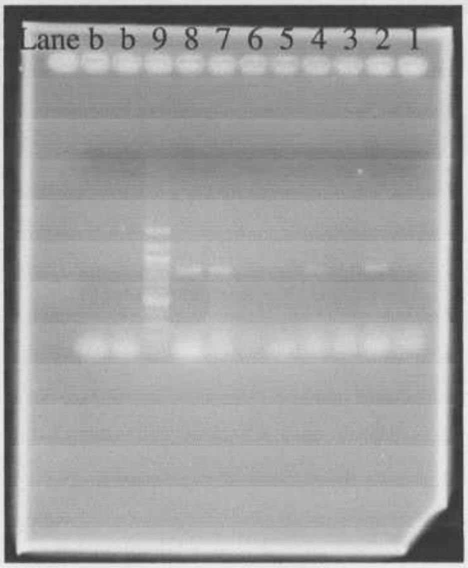


Figure 9 Comparison of two concentrations of $MgCl_2$ used in the PCR reaction. Lanes are in pairs starting from top right with the first of each pair using $MgCl_2$ at 1.5mM and the second at 3mM. In lanes 1 and 2 a fresh extraction of *Russula mustelina* and in lanes 3 and 4, an older extraction of the same specimen which had amplified only weakly in a previous run. Lanes 5 and 6 contain a fresh extraction of *R. crassotunicata* which had previously failed, and lanes 7 and 8, *R. aeruginea* which had successfully amplified in a previous run. Lane 9 is a 100bp ladder, brighter bands are at 1000 and 500bp. The last two lanes are blanks. Lane 6 shows very little terminal "cloud", suggesting the gel well leaked. The higher $MgCl_2$ concentration resulted in an increase in the amplification product of both *R. mustelina* extractions but about the same for those of *R. aeruginea*. An additional PCR product of approximately 250bp is apparent in lanes 7 and 8, the origin of which is unknown.

Amplification conditions:-

The PCR mixture was topped with a drop of mineral oil to prevent evaporation, spun briefly (about 15 seconds) to deposit the reagents at the base of the tube and placed in the thermocycler.

For ITS1-F and ITS4-B primers, the following thermocycler conditions were developed by Gardes and Bruns (1993.) and followed in this study:

Denature at 94°C for 1 minute, then cycle through the following denaturing, amplification and annealing stages:-

95°C for 35 seconds
55°C for 55 " 13 cycles
72°C for 45 "

95°C for 35 sec
55°C for 55 sec x22
72°C for 120 sec

95°C for 35 sec
55°C for 55 sec x8
72°C for 180 sec

72°C for 10 minutes

hold at 4°C

Following this, the oil was removed by freezing the tubes, and while the aqueous phase was still frozen the oil was drawn off with a sliver of clean paper towel.

The amplification product was visualized on a 1% agarose gel using 3 µl of the PCR solution in a 12µl gel-well, the remainder of the volume consisting of 5µl sterile distilled water and 4µl of a 0.2X solution of running dye. The running dye was diluted with 30% glycerol solution from a 6 X dye mix containing 0.25% of bromophenol blue and 0.25% xylene cyanol FF in a 30% solution of glycerol in water. This lower dye concentration was still visible when electrophoresed into its component blue and violet dyes, yet was not so dark that it masked any underlying DNA bands. A 100bp DNA ladder and a blank was run on each gel.

Gels were electrophoresed in TAE running buffer at 100V and 400 amps for about 1 hour 20 minutes or until the violet dye band was about half-way along the gel. Gels were stained with ethidium bromide, destained in water then viewed and a digital photograph taken under UV light.

Digestions

Aliquots of the PCR product were added to the restriction enzymes in 20µl reaction volumes as per the recipes below.

Digestion Recipes:

HinfI (10,000 units/ml or AluI (8,000 units/ml)– per 20µl reaction:

0.2µl HinfI (or AluI)
2µl 10x buffer
13.8µl sdd water
4µl PCR product

Sau3AI (4000 units/ml):

0.5µl Sau3AI
2µl 10x buffer
0.2µl 100x BSA
13.3µl sdd water
4µl PCR product

Most reactions required 4µl PCR product in 20µl reaction, however, when the amplification produced a relatively abundant amount of DNA as observed by a very bright band under UV light on the stained gel, aliquots of 1-2µl were digested and the reaction volume adjusted to 20µl with sterile double-distilled (sdd) water. Digestion mixtures were incubated overnight at 37°C. Aliquots of 10µl of digestion product mixed with 2µl 0.2X running dye were loaded onto 1% agarose gels and electrophoresed as for the PCR product above but for approximately 1hour 45 minutes or until the violet dye (which runs at about the same rate as the 500bp band) had travelled about 1/2 the length of the gel. Aliquots of the amplified blank were digested as for the other samples and run on each gel, a 100bp ladder was also included in each gel. Gels were stained and photographed as above.

Determining the size of DNA fragments

A subset of gel photographs were manually analysed using an interpolation method which determined the size of a band by comparing the distance it had travelled with that of the next smaller and next larger bands within the ladder according to the following equation:

$$X=B^{(1-Z)}* S^{Z}$$

Where X was the size of the unknown DNA, B was the size of the larger flanking band, S the size of the smaller flanking band and Z the ratio of the distance the unknown band travelled past the larger band over the distance travelled by the smaller band past the larger. This equation assumed a straight line relationship between the log of the fragment size and the distance of band migration, but only between adjacent bands. The interpolation equation was found to be within 12bp and more usually within 3bp when estimating for example the 800bp ladder DNA from the 1000 and 900 bands, compared with an accuracy which varied up to 50bp for a best fit line method. This method was particularly useful on gels where the migration rate of DNA was uneven as indicated by the fuzzy terminal band of primer and incomplete amplification products, seen in fig. 9. This terminal band was a useful marker of the wave front of migrating bands and was retained in the digestions by using the entire PCR product. The band covered digestion products in the range of 50 to 150bp, however, there was sufficient variability in the larger digestion products to differentiate most species. McKay and Smith (personal communication) did not use any band below 100bp in their analyses of ectomycorrhizae using the same suite of restriction sites as in the present analysis.

Two software programs were used in reading the bulk of the gels: LabImage version 2.7.1. (1999-2004 Kapelan GmbH) and TotalLab version 2003.03 (Nonlinear Dynamics Limited). The LabImage analyses were interpolated using the bicubic option, which gave results very close to the manual interpolation method (usually less than 10bp difference), and was simple to use provided the gel had run evenly across its width. This program did not have the option for corrections due to "smiling", a problem that occurred to a lesser or greater extent in about two thirds of the gels and appeared to be due to the expansion and consequent slight distortion of the gel as the electrophoresis bath became slightly warmer during the run. This was dealt with in two ways. The first involved manipulation of the image in a graphics program (Arcsoft Photostudio 3) to skew and stretch or shrink selected areas of the image to obtain a straight line-up of the terminal bands, prior to measuring band sizes with LabImage. The second involved measuring the unadjusted image with the TotalLab program which had a means of manually adjusting the reading frame to the shape of the distortion. Whilst TotalLab gave results consistent with the manual interpolation and the best fit to the 100bp ladder bands using the cubic spline interpolation option on undistorted gels, the results for the distorted gels could vary as much as 50bp between reading methods. The problem with distorted gels was a lack of clarity concerning the point where one section of the gel began to migrate faster than another, so measurements were therefore an estimate of the DNA size. The worst affected samples were re-run on new gels, otherwise an average of readings was used.

The fragment sizes were entered into a spreadsheet from which the charts in figures 12 and 13 were created. Comparison with the virtual RFLPs from sequence data described below and shown in figures 10 and 11 was initially visual, since this gave an immediate sense of both the variation in RFLP patterns across species groups and the similarities or otherwise between query and known species. Following this the calculated fragment sizes were compared with those derived from sequence data.

Creation of RFLP data from published sequences

Sequence data of the ITS-rDNA of *Russula* species were selected from the GenBank and EMBL-EBI databases, imported into the BioEdit software (version 7; Hall 1999) and trimmed to the ITS1-F to ITS4-B sites when longer. The BioEdit restriction site recognition function was used to find cut sites. The number of nucleotides between cut sites was calculated to give the fragment lengths, and were entered into a spreadsheet (Excel 2000, Microsoft Corporation) (figs 10, 11). The sequences chosen included representatives of the species found on Vancouver Island where available, otherwise species within the same taxonomic groups were selected. Not all sequences extended to both sites, in which case the end fragments were incomplete and not comparable with real RFLP data. It is therefore possible that there was an underestimation of the number of cut sites for any enzyme in these shorter sequences. Where available, other sequences of the same species, covering a further part of the sequence, were aligned alongside the first to supply additional fragment data. While it is possible to splice two such sequences to get an estimate of all fragment sizes, there are two potential pitfalls to this: the length of the added piece from the donor sequence may differ from that of the recipient, and there may be site substitutions at a cut site erroneously included. The data must therefore be cautiously or conservatively interpreted.

Sequences beginning between the 3' end of the ITS1-F site and the 3' end of the ITS1 site are indicated on the charts (figs. 10 and 11) by a grey fragment size marker rather than a black one, to indicate that these are shorter by 20 to 57bp than the equivalent fragment from the V.I. RFLPs. A dot above such markers indicates the estimated fragment size were these missing nucleotides included. (Where the dot is above a black line there are two fragments of the same size at this position). This whole 57bp section is covered by various primer sites (fig. 8) and is set within a conserved region, so such estimates are likely correct within 1-2bp.

Many sequences ended at or just before the "gagga" motif within the ITS4 primer site, which is approximately 140bp short of the ITS4-B primer site and within the 28S subunit. End fragments from such short sequences were not used for comparison, as these are incomplete compared to the RFLPs of V.I. collections, and can vary, particularly in the presence or absence of a HinfI cut site around 10bp downstream of the

ITS4 primer site. This unfortunately means that the fragment patterns for these shorted sequences are incomplete, and it is harder to see species- or group-specific patterns. Shorter sequences which may only cover either ITS1 or ITS2 completely are so indicated in figures 10 and 11. Those sequences beginning from ITS1-F to ITS1 and ending at ITS4-B -full length sequences or nearly so, are labelled in bold type. Sequence data shorter than 600bp in total is not shown to avoid confusion with fragment data. A table of Genbank accession numbers, authors and place of origin of sequences used in these analyses are given in Appendix 2. Also in Appendix 2 is a table of the collection numbers of V.I. species used in the RFLP analyses, further particulars of which are given in the descriptions of each species in Appendix 3.

Results and Discussion

The species were grouped into clades arranged from basal to upper levels as shown in figures 10 to 13, according to their relationship to the Russulas used in the phylogenetic analysis.

The calculated size of DNA bands varied between reading methods by 0 to 50bp, the latter problem arising when the gels ran unevenly. Repeat amplifications also varied slightly, as in the two versions of *R. mustelina* in fig. 9 and 12, which were extracted and amplified from the same basidioma hence experiments were repeated until consistent results were obtained. There were also variations between collections of a species in the size of the restricted fragments both in the GenBank and Vancouver Island species; for example the Genbank *R. nigricans* showed two patterns in the HinfI fragments due to a site substitution at a recognition site, with the 366bp fragment of the second being equivalent to two fragments of 284 and 78bp of the first (fig.10, 78nt fragment not shown). Several Vancouver Island (V.I.) collections also showed variation in Sau3AI fragment patterns; for example, *R. fragilis*, *R. silvicola* and *R. sanguinea,* in fig. 12. In other cases there was very little variation between collections of the same species. In fig. 13, the *R. xerampelina - eleodes - isabelliniceps* complex, there is little differentiation between these closely related species. With these factors in mind, when considering the pattern of a particular species, reference also needed to be made to its phylogenetic neighbours.

The PCR product

The ITS region in general averaged around 860bp for the V.I. Russulas with a range of 778-932. The GenBank full-length sequences ranged from 828-870bp with little distinction in size range between taxonomic groups. (Note that figures 10 and 11 full-length sequences are denoted by bold type for the species name, other sequence lengths are shorter than the V.I. species' PCR product, and sequence lengths below 600bp are not shown). The notable exception to these figures was *Russula olivacea*, which had a PCR product of 1141bp in the V.I. collection, comparable to sequence lengths of 1002nt and 979nt (ending circa 140nt before the ITS4-B site) from the GenBank collections of European specimens. The insertion of 250bp of novel sequence in the ITS1 region had not been observed to-date in any other *Russula*. Its PCR product alone can quickly identify this one species.

One V.I. collection of *Russula brevipes* and the Genbank *R. pallidospora* had a slightly smaller ITS than others in the *Lactaroides*. The former specimen was collected on the mainland for use as a standard, but did not amplify well enough for the restriction fragment patterns to be visible on the gels. (A collection of *R. xerampelina* was subsequently used as a standard). The two V.I. *R. brevipes,* both var. *acrior*, had ITS regions of 885bp: this variety was much more common locally than *R. brevipes* var. *brevipes*.

HinfI

The HinfI restrictions showed four main patterns that were more or less indicative of a taxonomic group but with some overlap into neighbouring clades. The Genbank sequences showed that the main cut site divides ITS1 from ITS2, with one or two more cut sites in ITS2 being fairly common. Extra cut sites in ITS1 were more rare, and occurred in *R. pallidospora* and *R. aquosa* (latter not used in figures).

Pattern 1 showed two fragments of similar size, less than 40bp apart, in the region of 350-400, which appeared on the gels as a single, sometimes heavier band. This pattern occurred in the GenBank *Russula adusta*, *R. nigricans* 2 and *R. albonigra,* of the *Compactae* (fig. 10), also in the V.I. *R. albonigra,* all the V.I. *Ingratae* (*R. cerolens* to *R. farinipes*) and *R. sublevispora* of the *Heterophyllae* (fig. 12). The Genbank

Heterophyllidia with this pattern included *R. cyanoxantha, R. heterophylla, R. parazurea* and *R. aeruginea.* The V.I. *R. aeruginea* and most others in this group had patterns closer to those of the Genbank *R. virescens* and R. *vesca,* in which the fragment in ITS2 had an extra cut site around 275bp downstream of the double HinfI site in the 5.8S subunit.

Whilst pattern 1 seemed to be a good indicator of basal clades, the V.I. *R. stuntzii*, a *R. silvicola, R. queletii, R. integra* and *R. abietina* and the Genbank *R. raoultii* (the first one), *R. fragilis R. betularum, R. persicina, R. cavipes* and *R. sanguinea* also had bands within 40bp of each other, making them difficult to place accurately within a taxonomic framework using HinfI alone (figs. 10, 11, 12 and 13).

Pattern 2 showed two bands, one of which lay in the 350-405 range, the other between 220-270, with at least 120bp difference. The GenBank *R. chloroides, R. pectinatoides, R. farinipes, R. grisea* showed this pattern, as did the two V.I. *R. brevipes,* the V.I. *R. aeruginea, R. brunneola,* and several species of subsection *Russula.* An unusually high number of cut sites occurred in the Genbank *R. pallidospora,* resulting in the largest fragment of 231bp with five other fragments ranging from 95 to 148bp, no other species showed this pattern.

Pattern 3 was found in most of the remaining members of the subgenera *Russula* and *Incrustatula,* and consisted of two bands between 40 and 100bp apart, but most within 60-70bp, with some variability in the sizes of the band pair. The larger band was generally in the 380-425 range but as low as 364bp in a V.I. *R. queletii.* The smaller band generally ranged between 325 and 364 but was as low as 303 in a V.I. *R. isabelliniceps.*

A number of species had double band patterns somewhat intermediate between those of pattern 3 and pattern 2, notably *R. mustelina* and *R. virescens* in the GenBank set and most of the V.I. *Heterophyllae* and the *R. raoultii-fragilis-silvicola* group. The V.I. *R. raoultii* collections had one smaller fragment of around 275bp as opposed to that of the Genbank collection of North American origin, which contained an extra cut site dividing that fragment in two.

There were some species-specific patterns such as that of *R. olivaceae*, which showed bands in the regions of 230-240 and 180bp in the Genbank and V.I. collections, representing 2 fragments of similar size. A band around 140bp also appeared in both Genbank *collections*.

In figures 10 and 11, *R. ochroleuca*, *R. violacea* and *R.* cf. *maculata* have bands at around 350, 230 and 150, and, except for the smaller band, in the V.I. *R. brunneoviolaceae,* one *R. puellaris* and *R. cessans*, indicating that this fragment pattern is associated in general with the upper clades but is no more specific than that. In summary, the *Ingratula-Compacta* group, the *Lactaroides* and the subgenus *Russula* each had patterns shared by many members of the group, but these were not completely exclusive to the group and could occur in closely related groups.

AluI

The AluI restriction patterns closely echoed the HinfI patterns in terms of group specificity. By far the most common pattern was of a band around 500nt ± 25 for the GenBank sequences, more variable for the V.I. sequences, ranging from 456 to 569bp, with a band around 210bp smaller, in the range of 274-294bp for the GenBank species and between 250-333bp in the V.I. species. These two bands occurred in the majority of the subgenera *Russula* and *Incrustatae,* which included the V.I. species from *R. stuntzii* onwards in fig. 12 through all of fig. 13 and the Genbank *R. ochroleuca* on fig. 10 onwards through fig. 11. Amongst the Genbank *Ingratae* and the *Heterophyllidia* of both datasets a smaller fragment of around 220-265bp was more common. A Genbank and the V.I. *Russula olivacea* each had three fragments differing slightly in size from other species in the upper clades; at 420-451, 363-379 and 242-269bp, the two larger bands also occurred in *R. lepida* (fig. 11). A fragment in the mid 300's also occurred in one V.I. *R. americana* var. *modicaspora*, *R. occidentalis* and as an extra fragment in one *R. abietina. Russula turci* in the GenBank sequences had a double band around 300 and one at 190bp, the former appears to be equivalent to the band in the low 300's occurring in the closely related V.I. *R. murrillii* (fig. 13).

The subsection *Ingratae* was also divided into two patterns, corresponding to the *R. cerolens-granulata* group and the *R. fragrantissima-farinipes* group (fig. 12). The former group were first segregated from the latter in the classification system of Romagnesi (1987), which was followed by Bon (1988) and Sarnari (1998) albeit at slightly different taxonomic levels. They belong to the series *Pectinata* (ad int. Sarnari 1998). The V.I. *Pectinata* and the Genbank *R. amoenolens,* closely related to *R. cerolens*, had an extra AluI cut site in ITS1, giving three fragments over 100bp long. However the Genbank *R. pectinatoides,* also in the *Pectinata,* lacked this cut site and showed a pattern like that of *R. foetens.* Two V.I. *R. fragrantissima* and the *R. farinipes* were in agreement with the GenBank *R. laurocerasci*, *R. farinipes* and *R. pallescens*. The V.I. *R. fragrantissima* collections were intermediate morphologically between that and *R. laurocerasi,* in common with several North American collections examined by Shaffer (1972).

A distinctive AluI pattern was seen among the Genbank *Compactae* and *Lactaroides,* in which in addition to the fragment around 500bp, one or two small fragments around 127 and 155bp occurred instead of the circa 250bp band seen in other species. *Russula albonigra,* one *R. nigricans, R.* aff. *delica,* both *R. brevipes* and *R. chloroides* all showed these smaller fragments. Among the V.I. set one *R. dissimulans* (a close relative of *R. nigricans*) and two *R. brevipes* showed only the larger band, the smaller double band was not distinguishable on the gels but as no mid-range band was visible, this would suggest a similar pattern to the aforementioned Genbank species. A V.I. *R. dissimulans* and the Genbank *R. nigricans 4* had fragments of 705 and 640 respectively, larger than in all other species examined except the Genbank *R. parazurea,* and due to the lack of one cut site in ITS2. Neither the other V.I. *R. dissimulans* nor the *R. albonigra* amplified strongly enough to give a visible digestion product, a problem also encountered in several attempts to amplify *R. adusta.* An incomplete digestion is a possible reason for the faint extra fragment of 367bp occurring in the V.I. *R. cascadensis.*

Sau3AI and overall patterns

The Sau3AI patterns gave far more resolution between species within a group and showed some group-specific patterns, but as with the other enzymes, group-related patterns were not unique to a group. One noticeable pattern was of widely different fragment sizes as in the Genbank *R. fragilis* to *R. emetica* group (fig. 10) with fragments around 600 and 215bp. This pattern was also evident in three V.I. *R. silvicola, R. laccata* and *R. crenulata* and surprisingly, in the Genbank *R. decolorans* (fig. 11) and in the V.I. *R. cessans and R.* cf. *cessans,* unlike any of the GenBank *Tenellae* (fig. 12). The V.I. *R. albonigra* and one *R. dissimulans* had fragments of 632 and 647 respectively but no smaller bands were resolved on the gels. A similar pattern with fragments of around 550 and 235bp was seen in the GenBank *R. nigricans* and *R. aeruginea* (fig. 10), one V.I. *R. dissimulans, R. medullata* and *R. sublevispora* (fig. 12). The latter also had an extra fragment of 346bp. One of the V.I. *R. dissimulans* showed the same restriction fragment patterns for all enzymes as the GenBank collection of *R. nigricans 4*, -a closely related European species, the other did not, although these distinctive species were unlikely to have been misidentified. The one fully successful *R. dissimulans* amplification and restriction was from a freshly collected specimen, whereas the others in the *Compactae,* including several unsuccessfully amplified *R. adusta,* had been collected and dried 1-2 years prior to extraction.

Of the *Lactaroides,* the two V.I. *R. brevipes* (fig. 12) a Sau3AI fragments read as 226 for one and 245bp for the second, may represent two or three fragments of 200, 247 and 257bp, as in the Genbank sequences of this species. *Russula* aff. *delica* and *R. chloroides,* both European species, had similar patterns to those of *R. brevipes* (fig. 10).

Russula cascadensis showed a pattern dissimilar to others in this group and more like that of the European *R. pallidospora*.

Sau3AI gave further differentiation between *R. cerolens, R.* cf. *pectinata* and *R.* cf. *pectinatoides*, in the V.I. *Ingratae,* but showed *R. granulata* with the same overall pattern as that of *R. cerolens.* The three V.I. collections of *R. cerolens* differed slightly in the Sau3AI fragments, and the first two, both collected from the same spot in Royal Roads University but two years apart, also differed in AluI fragment patterns (fig.12). Shaffer (1972) considered *R. cerolens* the western equivalent of *R. amoenolens,* which displays different patterns in the two Genbank sequences. The European *R. amoenolens* has two fragments analogous to those of the Genbank *R. pectinata,* but contains an extra cut site not found in the latter in the fragment downstream of these (not shown as they are incomplete). The North American sequence (*amoenolens 2*) is unusual in having a Sau3A1 cut site due to a missing base just beyond the ITS4 site, where many other species have a HinfI cut site, the second V.I. *R. cerolens* shows similarities in the larger fragment sizes. Wickaninnish collections in this group were slightly darker but otherwise morphologically identical to the Royal Roads collections of *R. cerolens*, but had a pattern more like that of *R. pectinata,* a species which had been previously reported from Crescent City in Northern California by Singer but subsequently determined as *R. cerolens* by Shaffer (1972). The collection was originally keyed out to *R. amoenolens* from which it differed only in having more reticulations on the spores, and unfortunately the RFLPs have not unambiguously clarified the identity. Series *Pectinata* consists of species with very slight differences not agreed upon by all authors, and in which Romagnesi (1987) and Shaffer (1972) consider *R. amoenolens* to be *R. sororia* in the sense of Schaeffer (1952) and *R. pectinata* in the sense of Singer (1957). This raises the question of whether differences between sequence data of a given species is due to natural variation or taxonomic confusion between species.

The V.I. *R.* cf. *pectinatoides,* which had an extra fragment of 586bp, which, when added to the other fragments was more than the amplification product, indicating either infragenic variation or incomplete digestion (fig. 12). The V.I. collections of *R. fragrantissima* show variation in fragment size, as do the two Genbank *R. laurocerasi,* due to a cut site in the second sequence that is lacking in the first. The V. I. collections identified as *R. fragrantissima* have several characters intermediate between that species and *R. laurocerasi,* and the variability of Sau3A1 restriction fragments in each species does not clarify matters further. The GenBank *R. pallescens* and V.I. *R. farinipes* had similar RFLPs for all enzymes, the similarities and differences between these two species are discussed in the notes following the description in appendix 3.

Amongst the *Heterophyllae* two V.I. species: *R. mustelina* and *R. aeruginea,* had equivalents in the GenBank set (figs. 10 and 12); the former was a confirmed identification, but showed slight fragment size variation between the two extractions of the same basidioma. *Russula aeruginea* had fragment sizes closer to those of the GenBank *R. grisea.* Interestingly, the collections of *R. aeruginea* from the Clayoquot area appeared to be intermediate between *R. aeruginea*, placed in subsection *Ilicinae* in Sarnari (1998) and *R. stenotricha*, placed in subsection *Griseinae* along with *R. grisea*. Although the morphology did not support the identification of this collection as *R. grisea,* the RFLPs suggested it belongs in *Griseinae* and is more likely *R. stenotricha. Russula medullata*, again in subsection *Griseinae*, had a Sau3AI and AluI pattern like the GenBank *R. aeruginea* but a HinfI pattern like *R. grisea.* Of the two collections of *R. brunneola* (fig.12), one had an extra AluI cut site, and because of the variation in the

Sau3AI fragments this species could not be assigned to a subsection. Shaffer (1970) commented on the variability of this species, which he had had difficulty in identifying. *Russula* cf. *basifurcata* had a similar RFLP pattern to that of *R. brunneola* and *R. mustelina,* these three species also have the same cuticular micromorphology. The two V.I. *R. crassotunicata* differ in the Sau3AI pattern, the first collection matches those of the Genbank *R. crassotunicata* but differs slightly in one AluI and both HinfI fragments. The second V.I. collection, labelled *R.* cf. *crassotunicata,* failed to show any AluI digestion products. This collection was morphologically intermediate between *R. crassotunicata* and *R. compacta*. The latter had no full ITS1-ITS2 sequence available through GenBank at the time of writing.

In the subgenus *Russula* subsection *Russula,* the V.I. *R. stuntzii, R. laccata* (in the *R. fragilis* complex), *R. betularum* and three of the *R. silvicola* showed a similar Sau3A1 pattern to those of the GenBank *R.* aff. *fragilis, R. betularum* and *R. emetica,* but could not be further differentiated with these three enzymes. The V.I. *R. crenulata* also showed this pattern rather than that of the GenBank *R. ochroleuca*, a superficially similar species, as did the Genbank *R. decolorans* in a different taxonomic group. The three V.I. *R. raoultii* showed slight fragment length variations between them for all three enzymes, and the third collection had the smaller AluI fragment cut down to 124bp compared with the first two collections. The GenBank *R. raoultii* showed two closely sized fragments of 223 and 250bp, more or less analogous to the smaller of the two V.I. fragments of this species. The V.I. collections show a fragment of 326-346bp, which appears to be the end portion of the ITS2 section, only 195bp of this fragment in the Genbank collection were available in the sequence, so comparisons cannot be made. *Russula raoultii* can be difficult to identify with certainty since there are a number of closely related white species separated mainly by spore size, and some of the red species in this group have white forms.

The *R. fragilis* in the Pacific Northwest is very variable in taste and in microscopic but not macroscopic or habitat characters. Collections were made which bore a range of characteristics associated with *R. atropurpurea* such as pileocystidial shape, weakly peppery taste and pure white spore print, through to typical *R. fragilis* characters. The Genbank *R. fragilis* had two closely sized smaller fragments of 213 and 245bp, and a larger one of 338bp. This compared with the first three V.I. collections of this species, which had a smaller fragments ranging from 317 to 260bp, it was difficult to clearly see two separate bands on a gel when they were this close in size. The comparative larger fragments of five of the V.I. collections ranged from 305 to 354bp.

The remaining group on figs. 10 and 12 show the *Sardoninae*. Two V.I. *R.* cf. *fuscorubroides,* two *R. queletii* and the *R. cavipes* showed similar Sau3A1 fragment patterns, the remaining two *R.* cf. *fuscorubroides* and one *R. queletii* shared similar restriction fragment patterns. In the Genbank *Sardoninae*:- *R. persicina* to *R. cavipes,* there were fragments in three size ranges of 185-215, 245-266 and 307-338bp, with variation between species and no common pattern. The double band seen in *R. queletii* was not detected as separate fragments in the V.I. collections, however three of them had fragments in the 253-270bp range, close in size to those of the Genbank collection.

In figure 13, the two collections of *R. americana* var. *modicaspora* nom. prov. , the first from Mount Douglas Park in Victoria, the second from the Clayoquot rainforest, had uncorrelated patterns and may be different species. Whilst neither had RFLPs that completely matched other species in this group, the Mount Douglas collection had most similarity to the GenBank *R. violacea* RFLP pattern. The first V.I. *R. sanguinaria* (from Saturna Island) showed a similar pattern to that of the last V.I. *R. queletii,* although all fragments were slightly smaller, the second had one fragment of similar size at 277bp to one of the Genbank *R. sanguinea* (fig. 11). Unfortunately of the several Genbank sequences of *R. sanguinea* and *R. rosacea*, most were too short for direct comparison with V.I. RFLPs, and were also quite variable. *Russula rosacea* and *R. sanguinaria* (as *R. sanguinea*) were considered synonymous by Romagnesi (1967), Sarnari (1998) and Thiers (1997b), but there is clearly much variation amongst populations of this species complex.

The majority of the remaining species in figure 13 had a Sau3AI fragment averaging 335bp and a smaller one around 220bp, the latter was also common in the Genbank set as it was within ITS1, which was complete in more sequences than was ITS2, in which the larger fragment occurred. A third fragment was more variable, in some cases being intermediate between the former two fragments, and in others, smaller than the 220 fragment and between 140 and 200bp in the V.I. set, and down to 106bp in the Genbank set (below the level of accurate resolution in the gels used for V.I. collections). In the V. I. set, the smaller two bands could not always be differentiated, and when a single band appeared it may have represented two closely sized fragments.

The next two groups included species in subgenus *Incrustatae* and sections *Paraincrustatae* and *Polychromae* of subgenus *Russula*, which were grouped together on the basis of the phylogenetic analysis (Part 2 of this chapter). *Russula albida* and *R. murrillii* had no GenBank equivalent. *Russula lepida* - a red-capped species, is closely related to the white *R. albida*, and there was a similarity of the Sau3A1 overall pattern, but the fragment sizes were all 40-60bp larger in the V.I. *R. albida,* and the AluI patterns differed. The Genbank *Russula olivacea* showed three small Sau3AI fragments in the 115 - 167bp range and one in the 333 - 336bp range. The V. I. *R. olivacea* showed the larger fragment but the smaller ones were not differentiable, showing as a fragment of 195bp. *Russula integra* showed a broad HinfI band on the gels between about 360 and 380bp which was close to the size of the HinfI fragment in the GenBank material, conversely, the Sau3AI digest revealed only a single band corresponding to the GenBank material's double bands at 225 and 257bp.

The V.I. *R. veternosa* collections showed some fragment size variability, mostly in the AluI fragments, but the overall pattern was consistent between collections (fig. 13). The two fragments of the GenBank *R. veternosa* were similar to those of the V.I. collections but were not distinct from those of the GenBank *R. firmula* or *R. amethystina* in the previous group (fig. 11). This is consistent with the morphology, which shows a close relationship of *R. veternosa* to the *Incrustatae*. The V.I. *R. integra* differed from *R.* cf. *integra* in fragment sizes for all three enzymes, the latter bearing a closer resemblance to the GenBank *R. velenovskyi,* which differs mainly in cap colour. The Genbank *R. integra* showed a double Sau3AI fragment of 225 and 257bp where only one of 260bp was detected in the V.I. *R.* cf. *integra* collections, the first of these also showed a double HinfI band of 360 and 380bp, the smaller of which was comparable to a GenBank fragment.

The GenBank *R. vinosa* and *R. occidentalis* had identical Sau3AI restriction fragments for the first part of the sequence, but the smaller fragment of the V.I. *R. occidentalis* was larger at 251bp than the largest ITS1 fragment in the GenBank set. The

GenBank and V.I *R. occidentalis* had a similar sized fragment in the ITS2 region (figs. 11 and 13). *Russula occidentalis* and *R. vinosa* have blackening flesh and a unique reaction to phenol, so are unlikely to be confused with other species.

Interestingly, the position of *R. occidentalis* among species with incrusted pileal hyphae prompted a re-examination of the cutis with acid fuchsin rather than 5% KOH, whereupon incrustations were found. This was not mentioned in Singers' 1957 description, nor in Grund (1965) or Thiers (1997), but Thiers recognised it as a member of the *Coccineae* Romagn. which contains species with incrustations.

Russula sphagnophila and *R. brunneoviolacea* are related to the *Xerampelinae* according to the analysis in Part II of this chapter, yet they show different overall RFLPs from this group in both the GenBank and V.I. collections, except for two Sau3AI fragments in the V.I. *R.* cf. *brunneoviolacea* (figs. 11 and 13). The V.I. collection of *R. sphagnophila* has fragments that differ by up to 40bp from those of the GenBank collection, yet they have approximately the same relationships between fragment sizes. The two collections of V.I. *R. abietina* have somewhat varied RFLPs and were all from different locations. This species has an ambiguous original description and no GenBank representatives, the RFLP pattern shows similarities with both *R. sphagnophila* and *R. nauseosa,* it is placed among the *Sphagnophilae* because its cuticular pigments are like others in that group (see the notes under this species' description in appendix 3).

The V.I. collections of *R. xerampelina* and its close relatives *R. elaeodes* and *R. isabelliniceps* show similar patterns for all enzymes, and these match those of the GenBank collections of *R. xerampelina* (figs. 13 and 11). Another member of this group, *R. semirubra* differed only in the lack of a Sau3AI fragment around 270nt, presumably due to an extra cut site in this piece.

An immature, mild tasting *Russula* with undeveloped spores and flesh that stained blue-green in $FeSO_4$, was identified as *R. xerampelina* from its RFLP patterns, which were identical with those of a mature collection of this species.

The last group of V.I. species were of the *Tenellae*. The GenBank group also included *R.* cf. *maculata* in the *Urentes*, a species whose taxonomic position was not unambiguously resolved by the phylogenetic analysis in part II of this chapter, but which shows a Sau3AI pattern like those of *R. occidentalis* fig. 11). *Russula aureofulva,* a new species with mild taste and large spores which had some morphological characteristics of both the *Tenellae* and the *Urentes,* and showed most RFLP similarity with the GenBank *R. cuprea,* an acrid species of the *Urentes* with spore morphology and cap colours like those of *R. aureofulva.* The V.I. *R. cessans* and *R.* cf. *cessans* had a Sau3AI fragment around 600nt and a HinfI fragment of 249bp that were not cut (fig.13), in contrast to that of the GenBank *R. cessans* (fig. 11). The *Macowanites* sp. could not be unambiguously identified from its RFLP pattern, the largest Sau3AI fragment appears to be incompletely digested as the fragment sizes add up to a greater length than that of the amplification product. The remaining Sau3AI and HinfI fragments place this species close to *R. aureofulva.* With the exception of the smaller HinfI fragments at 160bp, and some variation around the Sau3AI 215bp fragment, the second and third V.I. *R. puellaris* RFLPs are close to those of the GenBank collection. The GenBank sequence was not long enough to assess whether the circa 300bp Sau3AI fragment was present, but the lack of a cut site in the remaining nucleotides and a similar lack in a 28S sequence (AF325315, not shown), suggests this fragment is present. The first V.I. *R. puellaris* (CR021016-11) failed the Sau3AI digestion, and shows more similarity in HinfI fragments to those of the V.I. and Genbank *R. nauseosa,* and these show different Sau3AI patterns. *Russula puellaris* and *R. nauseosa* can be hard to differentiate as they are closely related and share many characters, differing principally in the degree and extent to which they bruise yellow. The remaining V.I. species labelled with a collection number CR001007-04, an acrid species with incrustations, showed similarity to one of the V.I. *R. abietina*, a mild species lacking incrustations that is in the *Tenellae*; otherwise it had a very generalised pattern that could have placed it in several groups.

Conclusions

The three enzymes functioned overall in a nested division of Russulas, with HinfI making divisions at roughly the subgeneric level, AluI making further divisions in some but not all groups and Sau3AI subdivided these and in some cases provided divisions not made by AluI. Occasionally, as in the *Russula nitida-xerampelina* complex, HinfI provided a greater resolution than AluI. The three enzymes were not always sufficient to resolve all the identities, however collating information derived from all three enzymes would be expected to give the best resolution of identities. In the higher clades particularly, between-species variation was low and there may not be an enzyme that would differentiate them. In such cases, resolution was limited to group level, but this was useful for confirming relationships between known and query taxa. To increase resolution, more enzymes could be employed, but the time and materials required for each additional restriction have to be weighed against the cost of sequencing, which returns far more information. Infrageneric variation occurred in several clades, some due to restriction-site substitutions; such variation may be explained by geographic separation, and sometimes by uncertainty in identification. In the collections of *R. veternosa,* small variations reflected population differences with the first collection from Breitenbush in the Oregon Cascade mountains, and the second and third from two southern Vancouver Island Douglas-fir forests.

The use of published sequence data to confirm an identification by comparing virtual RFLP patterns with those obtained by amplification and restriction using the enzymes HinfI, AluI and Sau3AI was most appropriate when full-length sequences were available. Species with several analogous or partially overlapping sequences published were less useful. The gathering of published sequences and the process predicting enzyme recognition sites enabled an overview of the variability in RFLP in a given species or clade, and the origin of that variability. The use of sequences from different collections of a species, as mentioned above, brings a realisation that substitutions of a single base can result in changes in the fragment pattern, which makes identification using RFLP matching sometimes uncertain. Since these enzymes sample such a small portion of the overall sequence any difference can be interpreted as a large degree of divergence. Fragment sizes that changed due to a single site substitution tended to affect Sau3AI more than AluI or HinfI because several of the recognition sites for Sau3AI were in most variable regions of the target DNAs. With query collections in which the sequence information was not available, adding the smaller fragments to see if they resulted in a missing larger fragment was one way of assessing whether a single extra cut site was a likely cause of the difference. Infrageneric variation in RFLPs was also seen between the pairs of GenBank *R. nigricans* and *R. laurocerasi* in fig. 10, and among the V.I. *R. fragilis* and *R. silvicola.*

Unfortunately, the resolution of bands below 150nt by agarose gel electrophoresis was a problem as incomplete amplification products and small restriction fragments produced a diffuse band. The elimination of the terminal band by using the DNA in the band from the PCR assessment gel brings in more steps and consequently more risks of contamination and sample loss. For many species, this step would not bring further resolution, as it was seen from the GenBank sequences that some of the smaller bands below 120nt were similar among species and originated in the conserved region.

In some species the fragment size distribution was remarkably stable, as in the GenBank and V.I. *R. xerampelina* complex, indicating little genetic variation even between geographically separated populations.

There were collections that had been identified as one particular species until the RFLP pattern showed a non-match either with other V.I. collections or the GenBank representative of that species or group. In these cases the characters of the V.I. collection were reviewed and some characters re-examined, and either the original identification confirmed or other possibilities considered. One such collection was assumed to be *R.* cf. *sanguinaria* but the RFLP pattern suggested otherwise, and on re-examination, this time using acid-fuchsin on the cap cuticle, incrustations were found which placed it as *R.*cf. *rubra*. This was a validation of this approach.

Vilgalys (2003) commented on the frustrating problem of misidentified sequences in the GenBank and EMBL-EBI databanks and Bridge *et al.* (2003) found up to 20% of published ITS sequences of *Helotiales, Amanita* and *Phoma* had been misidentified. Eberhardt (2002) found a GenBank sequence identified as *R. mairei* that was more likely to have been *R. compacta,* and in the phylogenetic analysis below, two sequences of *R. amethystina* fell into different clades, one of which (AY061653) consistently appeared on the same branch as *R. firmula*. As some of the collections of Vancouver Island Russulas were difficult to identify either because of variation from the published descriptions or due to the occasional conflicting or confusing description, the same difficulties must exist for other authors, whose concept of a species may also vary. Therefore, when the RFLPs of a collection in hand failed to match those of a published species, or indicated a different species, some judgement was required to assess both the accuracy of the published data (by comparing it with that of sibling species), and the accuracy of the identification of the local collection. That said, the methods outlined above for treatment of published sequence data provided a useful framework into which query species could be placed, and in many cases verified. The method also found three misidentifications in the Vancouver Island collections that were subsequently confirmed as members of a different group. There were some disagreements between GenBank and V.I. RFLPs for a given species, but the local collections agreed with the published descriptions for these species (e.g. *R. cessans*).

Uncovering the reasons for such differences would require sequencing of local collections and comparisons with the GenBank sequences. The use of RFLP comparisons, as described here for identification of the *Russula* partner of ectomycorrhizal root tips, shows that in most cases the level of identification possible is within that of subgenus, sometimes down to subsection, and in rare cases to species level. A matching pattern does not necessarily return the correct identification, nor does a mismatch necessarily indicate a wrong identification. The degree of uncertainty should decrease as the sequence databases grow and anomalies are weeded out or re-examined.

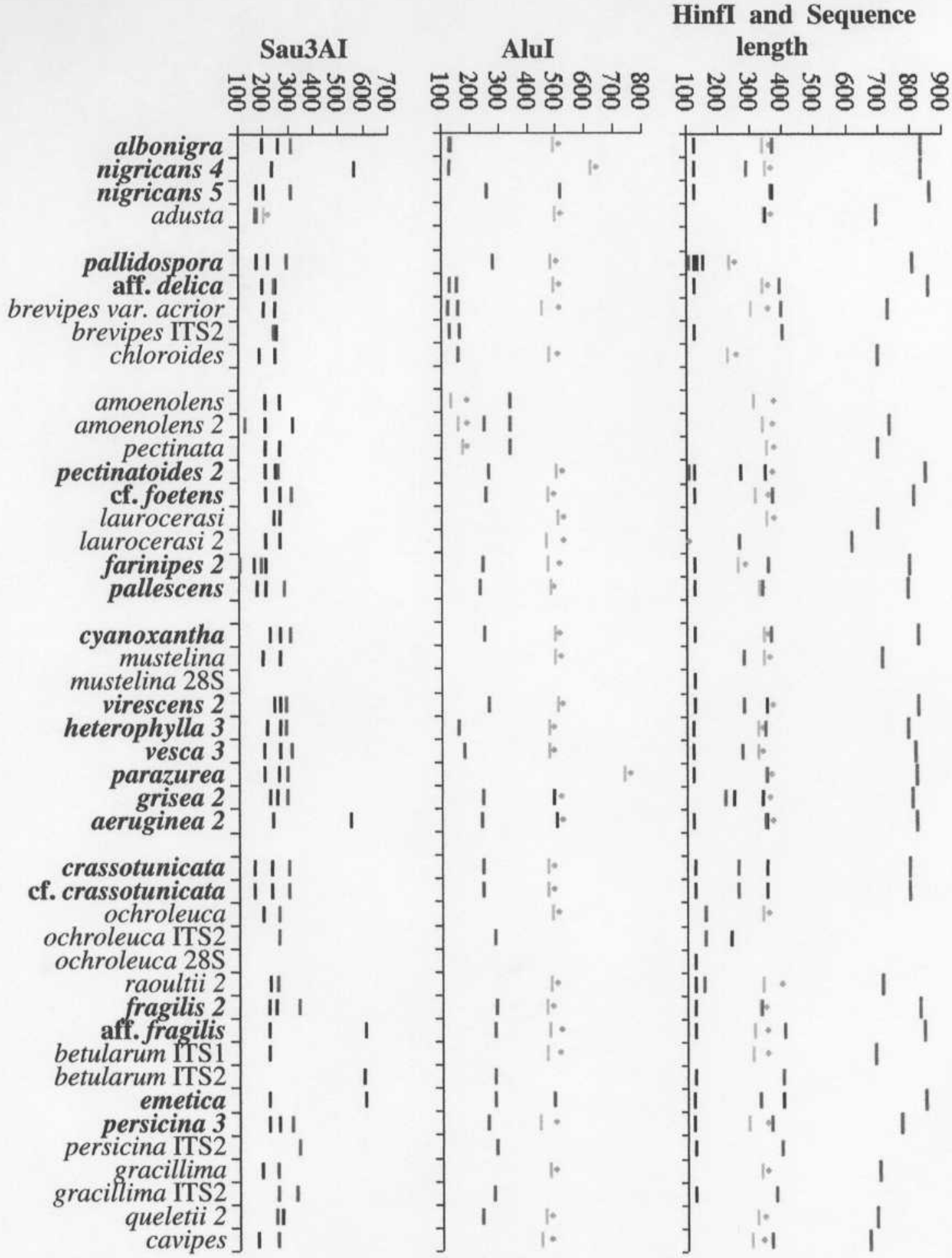


Figure 10 GenBank *Russula* ITS-rDNA virtual RFLP's of the *Compacta, Ingratula, Heterophyllidia, Russula* (subsections *Russula* and *Sardoninae*). Black bands are complete fragments, grey bands are fragments beginning between the 5' end of the ITS1-F primer site and the 3' end of the ITS1 primer site, with a dot indicating the estimated size were they to extend to the ITS1-F site. Only sequences in bold are full length, running between ITS1-F (or ITS1) to the end of ITS4-B.

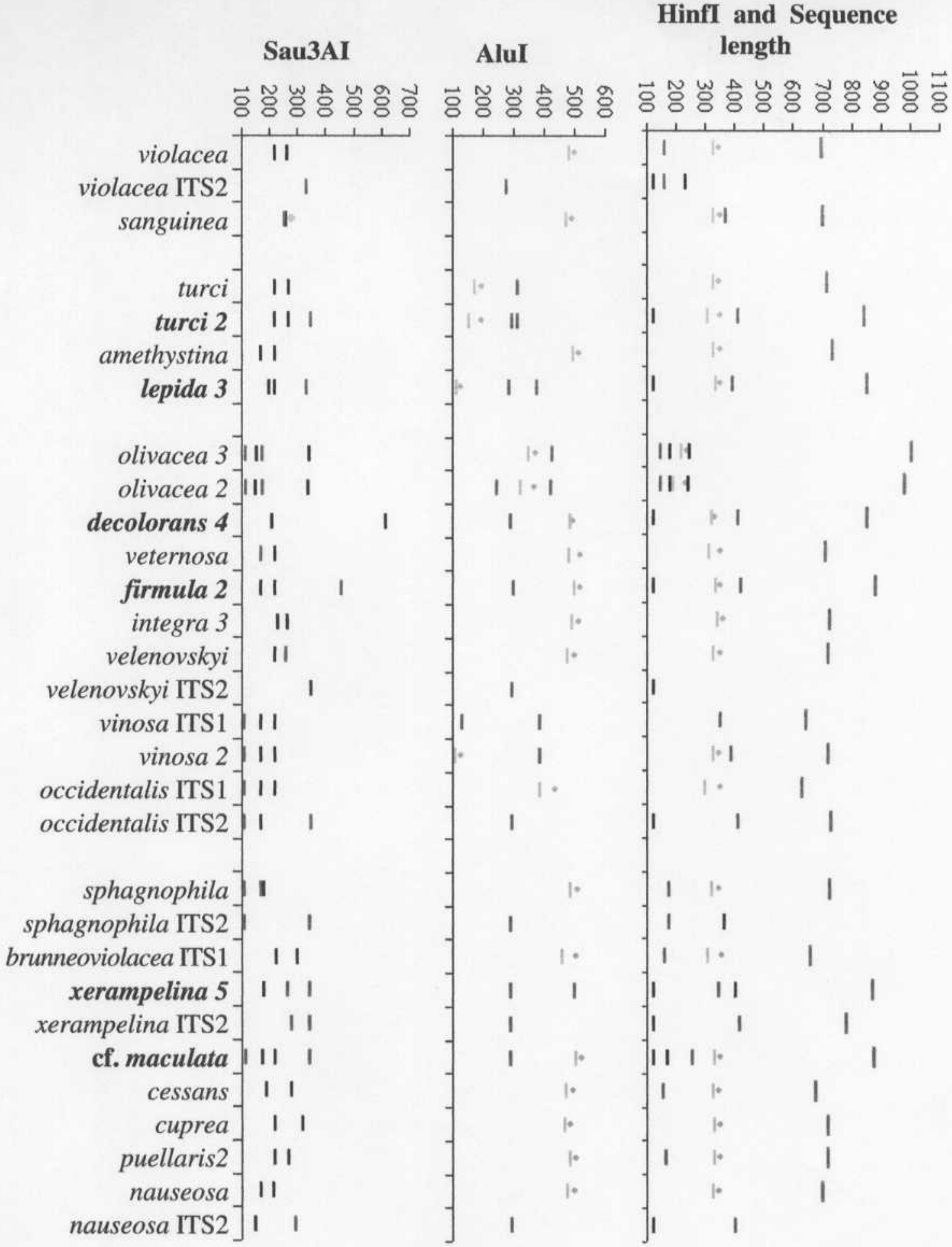
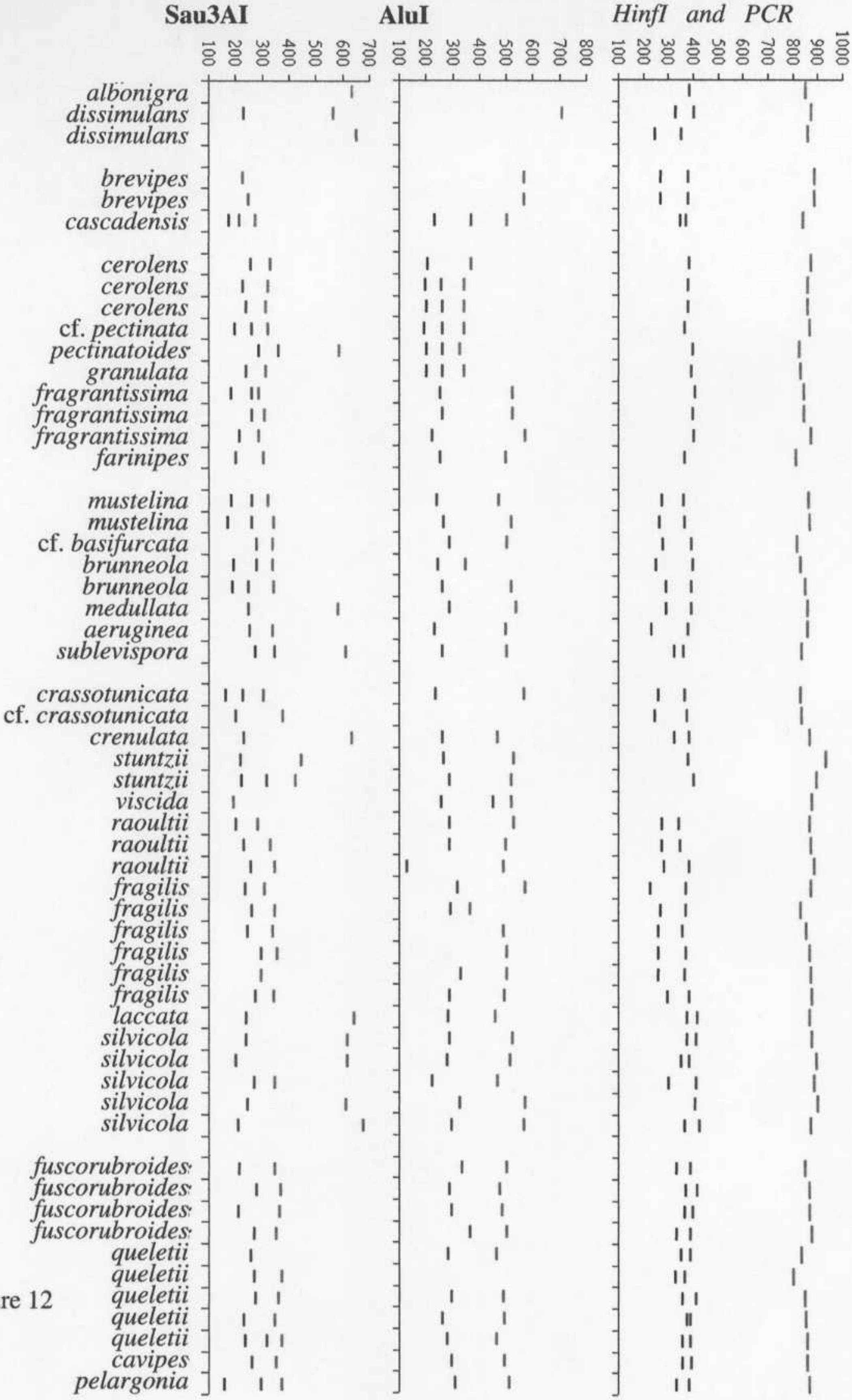


Figure 11 GenBank *Russula* ITS-rDNA virtual RFLP's of subgenus *Russula* sections *Paraincrustatae, Polychromae* and *Tenellae; Russula* subsections *Violaceinae* and *Urentes,* and subgenus *Incrustatulae.* Black and grey bands, dots and bold type as in fig. 10.

Figure 12 Overleaf, Vancouver Island Russula ITS-rDNA RFLPs: subgenera *Compacta, Ingratula, Heterophyllidia, Russula* (subsections *Russula* and *Sardoninae*).

Figure 13 page 80, Vancouver Island Russula ITS-rDNA RFLPs: Subgenus *Russula* sections *Paraincrustatae, Polychromae, Tenellae* and *Russula* subsections *Urentes, Violaceinae* and *Rubrinae*, and subgenus *Incrustatula.*


Sau3AI
AluI
HinfI and PCR
100
200
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400
500
600
700
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800
900
1000
albonigra
dissimulans
dissimulans
brevipes
brevipes
cascadensis
cerolens
cerolens
cerolens
cf. pectinata
pectinatoides
granulata
fragrantissima
fragrantissima
fragrantissima
farinipes
mustelina
mustelina
cf. basifurcata
brunneola
brunneola
medullata
aeruginea
sublevispora
crassotunicata
cf. crassotunicata
crenulata
stuntzii
stuntzii
viscida
raoultii
raoultii
raoultii
fragilis
fragilis
fragilis
fragilis
fragilis
fragilis
laccata
silvicola
silvicola
silvicola
silvicola
silvicola
fuscorubroides
fuscorubroides
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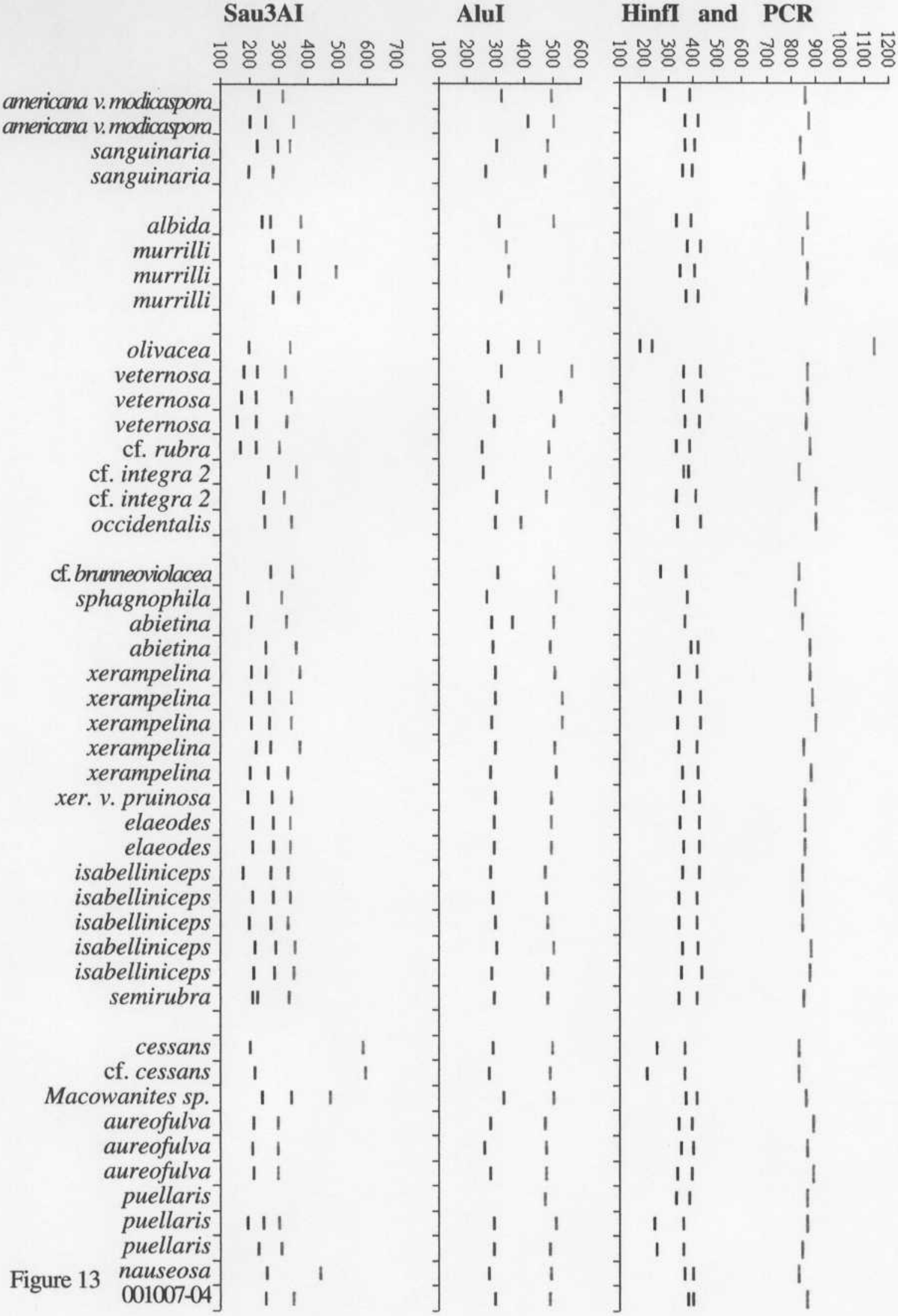
queletii
queletii
queletii
queletii
queletii
cavipes
pelargonia

Figure 12


Sau3AI
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1000
1100
1200
americana v. modicaspora
americana v. modicaspora
sanguinaria
sanguinaria
albida
murrilli
murrilli
murrilli
olivacea
veternosa
veternosa
veternosa
cf. rubra
cf. integra 2
cf. integra 2
occidentalis
cf. brunneoviolacea
sphagnophila
abietina
abietina
xerampelina
xerampelina
xerampelina
xerampelina
xerampelina
xer. v. pruinosa
elaeodes
elaeodes
isabelliniceps
isabelliniceps
isabelliniceps
isabelliniceps
isabelliniceps
semirubra
cessans
cf. cessans
Macowanites sp.
aureofulva
aureofulva
aureofulva
puellaris
puellaris
puellaris
nauseosa
001007-04

Figure 13

DNA Analyses Part 2: Generating a phylogenetic tree from previously published sequence data as a basis for establishing the relative taxonomic importance of morphological characters.

Introduction

Classification within the genus *Russula* has in recent years increasingly recognised the importance of microscopic characters such as specialised hyphal structures in the epicutis and the ornamentation of the spores. The work of Romagnesi (1967, 1985) set a standard in classification of Russulas that has formed the basis of subsequent monographs. In more recent works, Romagnesi (1987) upgraded the supersections to subgenera. Bon (1988) returned to the more traditional two subgenera of *Compactae* and *Russula* but otherwise closely followed Romagnesi's classification. Sarnari (1998) maintained six subgenera, but combined most of Romagnesi's subgenera of *Tenellae, Polychromidia, Coccinula and Insidiosula* into *Russula* based on which characters he felt were more important taxonomically.

One of the problems with such a diverse genus is that although many small groups of species may be defined easily by a set of distinctive characteristics, the relationship between groups has not always been clear. Added to this problem are a few species that seem difficult to place with confidence since they possess characters in common with several groups, and these in particular can be confusing to identify. Attempts to clarify the infrageneric relationships of *Russula* by phylogenetic analysis of DNA sequences have been made by Eberardt (2002), Henkel *et al.* (2000) Miller *et al.* (2001), Miller and Buyck (2002), and Shimono *et al.* (2004). The upper clades of these phylogenies in particular show little structure analogous to the classifications of Sarnari (1998) and Romagnesi (1985). The reasons for this may be because little actual evolution has taken place in this piece of DNA within these upper clades, or because the analysis methods used are inadequate for the degree of variation present in the ITS region. Some of the morphological characters were mapped onto these phylogenies to assess their taxonomic correspondence. Eberhardt (2002) mapped taste, spore colour, cap colour, discolouration of flesh, four classes of epicutal hyphae and cystidia, SV reaction, host, and two classes of mycorrhizal type onto a tree of 26 species but found that only the mycorrhizal type corresponded to sequence data. Shimono *et al.* (2004) mapped spore colour and amyloidity of the suprahilar patch onto the LSU-DNA neighbour-joining tree of 95 *Russulaceae* (including 50 *Russula* species) and found spore colour to be related to the degree of apomorphy, and amyloidity to be useful at higher taxonomic levels within the genus. Miller and Buyck (2002), mapped spore colour, taste and presence of incrustations onto their most parsimonious tree, finding general trends with some exceptions from base to top of the tree, and correspondence to clades quite good for the last two features. Morphological and molecular data have also been integrated in estimating phylogenies (Lutzoni and Vilgalys 1995).

DNA sequences hold considerably more evolutionary information than morphological characters, but differences at variable regions of the sequence are of less importance functionally and phylogenetically than those in conserved regions, so the topography of the final tree depends to some extent on the weightings applied to different substitutions in different parts of the genome. Some published analyses have removed parts of the sequence that aligned ambiguously, for example Miller and Buyck (2002), Høiland and Holst-Jensen (2000), Peinter *et al.* (2001). Although these residues are viewed as 'noise' when the number of sequences is small, as new sequences are added to an analysis, more of this'noise' becomes 'signal'. When many sequences are aligned, fewer of these 'unique to one sequence' sites occur, as pairs and groups with similarities in these sites are more likely. Thus, the more (related) sequences that are used in an alignment, the more sites are phylogenetically useful, and the more nuances of relationships between taxa are uncovered. Miadlikowska *et al.* (2003) formulated a coding system for hypervariable regions and re-integrated them into the dataset, since these regions contained phylogenetically useful information. Phylogenies generated by different authors for the *Russulaceae* and the genus *Russula* using either the LSU or the ITS regions have shown only partial agreement in their topology according to the analysis methods used. Neighbour-joining analyses are distance-based methods of inferring phylogenies, in which a match or mismatch at a site between pairs of sequences is scored. The method is quick but involves very little qualitative assessment of the data. Maximum parsimony analysis is a character-based method that finds the tree topology requiring the fewest changes to explain the data, but there may be several different, equally parsimonious trees. Maximum likelihood again is character based and looks among all possible trees for one that maximises the probability of observing the data by comparing one tree against the next closely related tree and moving on to the better one. The search suffers the risk of returning a suboptimal tree if that tree is "surrounded" by lesser trees, effectively blocking further progress. Eberhardt (2002) used maximum likelihood for her analysis of 62 *Russula* and *Lactarius* LSU-DNA sequences, but as this method requires long computing times it is used less often than the other two methods.

As the recognition of useful taxonomic characters rests upon a clear, unambiguous phylogeny, consideration was given to analytical methods other than those mentioned above, which purport to give a more realistic model of the evolutionary tree. One maximum likelihood-based method is Bayesian analysis, which has the power of maximum-likelihood but reduces the pitfall of getting stuck at a suboptimal tree.

Bayesian theory is named after its originator, the Reverend David Bayes (1702-1761) , an English minister and mathematician. His theorem is summed up by von Baeyer (2003) as the new probability (the posterior probability) that something is true is based on the prior probability multiplied by a factor, greater or less than one, which is derived from new information. In a sense, the algorithm "learns" from experience. In Bayesian analyses of phylogenetic trees, any given proposed tree begins with an estimated probability that it is true based on the information to-date. One or more of the nodes are perturbed by a given amount (a tuning parameter) and the new tree examined for likelihood. If it is an improvement (i.e., fewer evolutionary steps are required), the factor to be multiplied with the prior probability, the likelihood, will be >1 and the new tree accepted together with the new probability, the posterior probability. This process may continue until the probability reaches a steady state beyond which no further improvements occur, termed convergence. This chain of successive tree searches with the updating of probability as more information is discovered is referred to as the Markov chain Monte Carlo (MCMC) (Huelsenbeck *et al.* 2001). Archibald *et al.* (2003) and von Baeyer (2003) give a non-technical explanation of Bayesian analysis methods, Huelsenbeck and Ronquist, (2001), Huelsenbeck *et al.* (2002) and Larget and Simon (1999) give a fuller, technical explanation of Bayesian methods and the MCMC chain as it pertains to phylogenetic inferences.

One of the main advantages of Bayesian phylogenetic inference methods is that they are computationally more efficient than standard maximum likelihood with bootstrapping methods, such that sets of more than 100 sequences can be analysed in several hours rather than days or weeks. When the number of cycles is sufficient to reach convergence, all trees sampled after the convergence point (when the log-log-likelihood values reach a plateau) can be used towards a consensus tree. Bootstrapping is not necessary, since the tree sampling from along the converged log-log-likelihood plateau performs a similar statistical function; in the consensus tree the branch-node scores indicate the number of trees in which the species leafwards of the node occurred, and posterior probabilities can be assigned to these figures. The difference between bootstrapping and tree-sampling is that in the former, a subset of the alignment data is sampled for each bootstrap cycle, whereas the MCMC chain always uses the full data, making it more sensitive to errors in the alignment since they would be incorporated into every cycle (Huelsenbeck *et al.* 2002).

Two currently available programs that run Bayesian analyses are MrBayes (Huelsenbeck and Ronquist, 2001), which can run several (usually four) MCMC chains simultaneously, and BAMBE (Simon and Larget, 2000), which runs one Metropolis-Hastings MCMC chain but which samples from a wider area. Hall (2001) gives a brief but useful summary of the MrBayes program with explanations on the output files and on determining parameters and the length of the run; information also relevant to the BAMBE program. Both programs can be run on an ordinary PC, but longer runs take several hours and stability can be a problem. The Pasteur Institute currently runs a BAMBE server on the Internet which accepts aligned sequence files, runs a Bayesian analysis according to user-defined parameters and returns the results within a few days.

A Bayesian phylogenetic analysis of previously published sequences was decided upon in an attempt to obtain a clearly defined clade structure of mainly temperate zone Russulas that may subsequently be used as a framework to place local species related to those in the analysis. Morphological characters to be mapped onto the tree included those represented in some form in all species and which were expected to form a gradient from basal to upper clades. Other characters were examined for clade specificity.

Methods

Sequence selection and treatment

A search of the Genbank database returned 365 entries for the ITS region of *Russula* species and the EMBL-EBI database returned 213 entries. These two organisations collaborate with one another so many of the entries are identical, however, the two searches together returned a few sequences not found by searching only one site. Some of these sequences were downloaded and opened in a sequence editing program (BioEdit, Hall 1999). Sequences were trimmed at the 5' end to a common starting motif (CATTAT), and at the 3' end to a common ending motif (CATATCAAT). Shorter sequences which were missing data from either end were supplemented by data from 18 S or 28 S sequences of the same species when available. Sequences without either ITS1 or ITS2 were not used in the final analysis.

An initial alignment was conducted with ClustalW (Thompson *et al.* 1994), running on the EMBL-EBI site, using a gap open penalty of 10, a gap extension penalty of 0.05 and equal weighting for all nucleotides. The alignment was returned to the BioEdit program where duplicate sequences were removed and the two sequences of *Russula olivacea* had the large insertion of around 250bp in ITS1 trimmed, leaving a short remnant of 4nt. Treatment of long insertions that do not appear in the rest of the sequences being aligned have been excluded in the cases of *Boletus edulis* by Shimono *et al.* (2004) and of *R. olivaceae* by Eberhardt (2002). Miller (2002) coded the insertion as a fifth base.

The remaining sequences were re-aligned with ClustalX (Thompson *et al.* 1997) using a gap opening penalty of 25 and a gap extension penalty of 0.05 for pairwise and multiple alignment parameters. A low gap extension penalty was chosen because in the variable regions insertions were often of repeated units such as multiples of A, T, or CT. These short repeats may be due to separate single events at a site, but were more likely a single insertion of multiple residues at a single site. A higher gap opening penalty reduced spurious gaps, but penalties higher than 25 for the whole sequence tended to result in misalignment. Poorly aligned sections were realigned using a lower gap opening penalty for those residues only. The alignment was transferred to BioEdit and fine-tuned by hand. One part of the alignment in ITS2 was very difficult to align satisfactorily because several different homologous sequence patterns were seen in that region. An initial neighbour-joining analysis with 1000 bootstraps for several variations of this alignment were compared, including one of the hypervariable section only, and it was found to give a clade structure similar to that derived from the whole sequence. As gap-rich regions can hold useful information (Miadlikowska *et al.* 2003), this part of the sequence was retained but not recoded.

The dataset still contained two or more non-identical sequences for nine of the species, and in the comparison of the aforementioned neighbour-joining trees, those consistently paired on a terminal branch had only one representative retained, preference being given to the one with fewest ambiguous characters and those derived from basidiomata rather than ectomycorrhizae. The two *R. olivacea,* two *R. xerampelina* and two *R. vesca* were retained to check constancy throughout the analyses. Two *R. amethystina* were retained, as these fell into quite different clades and were only 88.8% similar. *R. pallidispora* and *R. littoralis* were identical to one another, as were *R. cremoricolor (*AJ277910) and *R. raoultii* 2 (AY061712) and the first of each pair was retained. There remained 108 *Russula* sequences and a *Gymnomyces gilkeyii* which were re-aligned and fine-tuned as previously described, but with a gap-extension penalty of 5. The two outgroup sequences of *Albatrellus flettii* and *Gloeocystidiellum aculeatum* were added to the alignment.

The final alignment had 870 sites, of which 647 were unique. Sites 1 to 6, 286 to 445 (the 5.8S subunit) and 824 to 870 (the start of the 28S subunit) were highly conserved. Sequence similarities ranged from 71% to 98% between pairs of Russulas and from 47 to 74% for pairs with an outgroup.

Bayesian analysis

The aligned sequences were categorised so that the parameter values would develop independently during analysis for differently conserved parts of the sequences. The three categories were; one for ITS1, the second for all conserved sites in the 5.8 S subunit and the beginning of the 28S subunit, and a third for ITS2. The alignment was next subjected to a number of short pre-analysis runs in the BAMBE program following the procedures outlined in the program documentation (Simon and Larget, 2000). The aim of these preliminary runs was to determine the starting and tuning parameters and an appropriate number of cycles and the burn-in. The burn-in cycles are perturbed by a global algorithm, in which a change can affect the topography of the whole tree. These initial burn-in trees were discarded from further analyses since they were at a coarse stage of resolution, the main-run trees begin at the last burn-in topology with perturbation confined to a local algorithm which only affects the topography of one branch-pair at a time, thus refining the resolution of the trees until they converge within a narrow range of likelihoods.

Huelsenbeck *et al.* (2002) comment that Bayesian analysis methods are sensitive to chosen prior distributions unless the MCMC chain is run long enough. To see if this was a problem, two sets of 600,000 cycles with a 3,000 cycle burn-in were run, each from a different seed number and starting the first using the BAMBE program default tuning parameters and the second using the calculated tuning parameters and using the final kappa and theta values from the first run as initial values for the second. These returned log-log likelihoods of -17602 and -17594 respectively with a plateau reached about half way through the cycles, indicating that prior conditions were not biasing the outcome. The overall tree topologies, assessed visually, were very similar. It should be mentioned that the likelihood plateau does not reach total stasis, but rather the values generally move up and down within a range of about 25-40 points, so a stable, trendless range is considered a plateau (Simon and Larget, 2000).

Hall (2001) recommends a run of about five times the number required to reach convergence, which called for a run of 1,500,000 cycles with local tuning with a burn-in of 300,000 with global tuning, and starting with the following empirically determined parameters: initial kappa = 3.8855, 5.1502, 3.7229; initial theta = 1.2423, 0.0429, 1.3414; global tune = 0.01, kappa tune = 0.16, theta tune = 3000, pi tune = 6000, local tune = 0.19. The HKY85 with molecular clock likelihood model was used (Hasegawa *et al.* 1985), with parameter updating every cycle, a tune interval of 200 cycles, and tree sampling every 200. This run was well into the plateau by cycle 300,000, but the first 1,500 trees sampled up to this point were discarded out of a total of 7,500 trees sampled from the full run. The final log-log likelihood was -17578.6, with the plateau range stabilizing about 20 points higher than that of the short runs. The last 6,000 trees were summarized using Consense in the PHYLIP package version 3.6 (Felsenstein 1989, 2004) after minor reformatting of the topology file, and using the extended majority rule method to generate the consensus tree. Unfortunately the BAMBE program's own summarize function could not deal with files of 111 taxa, and branch lengths were stored only for the initial and final trees and not with each tree in the topology file. As the branch lengths on the consensus tree do not relate to evolutionary distance, this and the final tree should be considered in concert. Trees were visualized in TreeView (Page, 1996).

Re-alignment and analysis of upper clades

A subset consisting of 54 Russulas forming the upper clades of the majority rule tree, which showed a few lower scoring branches, were realigned along with *Albatrellus flettii* as previously described. The alignment was then stripped of all fully conserved sites, leaving only variable sites, the intention being to exaggerate the differences to help resolve weakly resolved clade structures derived from the full data. The final alignment was of 530 sites of which 481 were unique. The analysis procedure on this alignment was the same as that for the full dataset, with the exception that all sites were treated as one category. In four short preliminary runs of 600,000 the final log-log-likelihoods ranged only between -7929 and -7943, and that of the 1,500,000 cycle run was -7938.8. The empirically determined initial parameters for the 1,500,000 cycle run were: initial kappa = 4.2070, initial theta=1, global tune = 0.01, kappa tune = 0.25, theta tune = 2000, pi tune = 4000 and local tune = 0.1900, burn-in, sampling, and consensus analysis as for the full set.

The sequence details and parameters used in the Bayesian analysis for the *Russula* phylogenetic analyses are detailed in appendix 2.

Mapping characters

Characters and character states, mostly of microscopic characters, were tabulated in clade order (Table 6). This table does not include the major macroscopic group – defining suites of morphological characters by which the lower clades in particular are differentiated. These are mentioned in the text where necessary but the reader is referred to monographs of *Russula* for full circumscriptions of taxonomic groups. The categorization of pileocystidial shape, tips, and septation is shown in figure 23 in Appendix 3. Pileocystidia are commonly included in species descriptions, but have not previously been categorised in a way suitable for determining the taxonomic position of a species.

Most of the character information was found in Bon (1988), Romagnesi (1985), Sarnari (1998) and Shaffer (1964, 1970, 1972). Not all the information was available, so some fields have been left blank. In the pileocystidia columns, for clarity, zeros have been filled in only for those species with no pileocystidia, blanks in other rows do mean missing data if there is at least one category filled in. The epicutis terminal hyphae column contains information on features noted in the literature, and which is of varying taxonomic value: for instance the vacuolar pigment mentioned for *Russula emetica* is not unique, and most species have this to some degree, but it was very noticeable to the original author.

Results

Trees

The final tree of the Bayesian analysis of 111 *Russula* and outgroup taxa is shown in two halves in figures 14 and 15 and in radial format in figure 19, both of which express the branch length in terms of substitutions per site. This tree is one of more than 1,200,000 probable trees created after the algorithm converged, each of which has some variation in branching order and length. The radial format of the final tree (fig. 19) very clearly shows the separation between clades, and when this figure was adapted for a lecture by colour coding the branches according to taste of species, and adding the spore colours, the clade specificity of these characters was striking. Unfortunately the cap colours (not shown here) were rarely clade-specific. The clades' relationship to the classification of Sarnari (1998) has been indicated on the radial tree in place of the species names, and shows good correlation with taxonomic divisions in all but the lower left clades. The extended majority rule consensus tree is shown in two halves in figures 16 and 17 and includes scores for the frequency among the trees in which the species to the right of the node appeared, expressed as a percentage. Ten main clades have also been indicated, with 7, 8, 9 and 10 corresponding to the clades of mixed taxonomy at the lower left of the radial tree.

The consensus tree of the minimised sequences of the 54 upper clade Russulas and an *Albatrellus fletti* as outgroup is shown in figure 18, and also bears node scores.

The full tree showed high support, 70-100%, for most of the major clades and terminal clades. Scores as low as 40% were found within clade 10 and in the branching between clades 8, 9 and 10, probably due to the inclusion of *R. maculata*, whose taxonomic position differed in several preliminary analyses. The minimalised alignment tree had the same suite of species in most of the terminal clades but these were grouped differently, with very low scores for these groupings. The *R. persicina* to *R. lepida* clade now included *R. maculata* which again lowered all the branch scores. *Russula olivacea* appeared basal to the clade containing *R. xerampelina,* with 99% of trees having this configuration. All told, this minimalizing method did not clarify relationships, since the tree topologies evidently were more variable.

Clade and character relationships

Spores: The amyloid reaction of the suprahilar patch is a major clade-specific trait, dividing the genus into two clade groups: 1, 3 and 4 (inamyloid or very weakly amyloid), from 2 and 5 to 10, in which this patch is strongly amyloid. The size of spores was considered (not shown in Table 6) but was found to be more or less species-specific, with the exception that clades 8d, 9 and 10a contained many species with comparatively large spores of 10 microns or more in length.

Spore colour: In general there was a gradient with palest colours in the lower clades and darkest in the upper clades. Spore colour was sub-clade specific for clade 5, dividing the lower two sub-clades from the upper one. In clades 5c to 10 spore colour was a within-clade, species-specific character.

Spore ornamentation: In general wart height increased from lower to upper clades but with numerous exceptions. The degree of reticulation divided clade 4 and 5 into subclades, while in other clades this character was species-specific.

Pileocystidia: Overall, there was a gradient from bottom to top of the phylogram with more capitate-strangulate cystidia (type 2) at the base of the tree and fewer occurrences towards the top. Septation and long, cylindrical cystidia with obtuse tips (type 5) increased towards the top of the phylogram. Most species had two or three cystidial types but the co-occurrence of types 2 and 5 were most frequent in clade 5.

Type 1, tapering and not or barely SV+, was clade-specific for clade 3b. The same shape but without the same SV reactions occurred in *R. melitodes* but this species also had other pileocystidial shapes.

Type 2, with capitate to strangulated tips, was loosely major-clade specific, occurring most often in clades 1 to 5 but also in clade 9. The occurrence of this type alone was species-specific within several clades.

Types 3 and 4, clavate with an obtuse tip, were not clade-specific, but a preponderance of type 3 in a cutis is more species-specific.

Type 5, long, cylindrical, with obtuse tips, was mainly clade-specific to clades 5 b,c and d and clade 10, this last clade all with multi-septate versions, a form more species-specific in clade 5. Occasional species-specific occurrences of non-septate to multi-septate type 5 pileocystidia were in other clades.

Type 6, with diverticulae, was clade-specific for clades 1b and 10.

Type 7, incrusted pileocystidia, was clade-specific for clades 7 and 8 but did not define a subclade. Within these clades, and for *R. insignis* in clade 3, this character was species-specific.

Incrustations in the epicutis, usually but not exclusively associated with fucshin positive primordial hyphae, is clade specific, occurring in clades 7 and 8, and in *R. camarophylla* of clade1, *R. pulverolenta* in clade 3 and *R. ochroleuca* of clade 5. The incrustations of *R. pulverolenta* are not analogous to those of clades 7 and 8, being pigmented and non-soluble in KOH.

Sulphovanillin reaction of pileocystidia: The basal clades 1 to 4 c generally had a null to weak reaction of the pileocystidia to sulphovanillin, appearing light grey or colourless, in some cases with small darker granules in the cytoplasm. Clade 4d of the *Heterophyllae* had weakly to positively reacting species. Clades 5 and 10 consisted of species with a positive SV reaction in which the pileocystidial contents stain purple to black. Clade 7 consisted of SV- negative species, but the tissues in general often stain deep pink or red in this reagent, a reaction not seen in the lower SV-negative clades. Clade 8 and 9 were mixed, with some negative or weakly staining species and some, particularly the peppery species in 8c, with a positive reaction. A positive SV reaction was frequently linked to a peppery taste, but these two characters could also be independent of one another.

Epicutal hyphae: The epicutal hyphae in most species in the upper clades tend to be interwoven, thin-walled, generative hyphae embedded in a gelatinous matrix, with tips often similar in shape to those of the pileocystidia, but smaller. Ampullate hyphal tips occurred in clades 8, 9 and 10: these have an inflated subapical cell and a tapered to capilliform apical cell, and sometimes this shape is formed by the terminal cell alone. Chains of inflated cells, or hyphae inflated between frequent septa so that they appear articulated like the leg of a crab, occurred mainly in the basal clades 1 and 4.

Macroscopic characters

Bruising: The tendency of the flesh to turn grey to black, sometimes with a red interphase, was clade-specific for clade 1b and species-specific within clade 8. Weaker greying reactions occurred in clade 5 and were species-specific.

Yellow bruising was common in clades 5e, 9 and 10 and was species-specific within these clades: after the yellow stage the bruised tissue turns brown in clade 9 species and in several others. Yellowing species occurred sporadically in clade 8.

Clades 2 , 3 and 4 were composed of species bruising brown (a more reddish brown in clade 3). This character was also found sporadically in clades 6-10 but rarely in clade5: it is species-specific for these clades.

The waterlogged effect, in which the tissues of the whole stipe appear greyish or yellow-grey as if waterlogged in age was common in clade 5a and b and (from personal observation,) in 10b.

Taste: While peppery and mild species were found throughout the genus, some clades were specific for one character state. Clade 5 contained entirely peppery species, clades 4, 7 and 9 normally mild species, and clade 8 predominantly mild species, in which case pepperiness was species-specific. Other clades were mixed for this character.

Odours: This property is subjective, since people's sensitivity to different aromatic components varies, and the age and condition of the basidioma affects the odours given off. Unfortunately the chemical compounds responsible for the odours are known in only a few cases. However, there were both clade-specific and phylogenetic gradient properties noticed in *Russula* odours. Fruity or coconut odours, sometimes mixed with pelargonium, were common in the upper clades from 5 to 10 and also in clade 2, with the most frequent notations of this odour occurring in clade 5. In fact, 75-80% of peppery species had odours noted in the literature, compared with 62-70% of mild species. The odour of shellfish (trimethylamine) was specific for the subclade containing *R. xerampelina*. Honey or gingerbread odours were found in clade 8 and once in clade 10, and the basal clades 1 and 4 contained cheese or wine-barrel odours. Clade 3, the *Ingratae*, has long been defined in part by strong complex odours and these include benzaldehyde (almond), spermatic or bleach-like, rubbery and Jerusalem artichoke smells, which are rarely found outside of this group and then much weaker. In the upper clades (5 – 10), stewed apple, coconut or pelargonium odours appear to be plesiomorphic and honey, iodoform and menthol a more advanced character.

Conclusions and Discussion

Most useful characters

The characters mapped onto the clade structure represent only a few of the many that are recorded and used in systematics, and some groups, particularly the basal clades, are sufficiently distinctive to be segregated on macroscopic characters alone. These distinguishing characters have not been included in Table 6, but the main ones are, in brief: Clades 1, 2 and 3 lack bright coloured pigments, 1 and 2 have hard but brittle flesh, and fairly regularly spaced free subgills between the gills, clade 1 bruises grey to black, some also with a red phase first. Clade 3 members have a glutinous cap cuticle with brown pigments associated with the cell walls, strongly striate to tuberculate cap margins, regular lenticular cavities in the stipe and usually strong complex odours encompassing spermatic, rubbery, benzaldehyde and other nuances. Clade 4 contains the *Heterophyllae* which are the only taxa in this lower half of the tree to possess red, yellow and blue pigments in their cuticle, non-cavitate stipe and usually firm but elastic flesh.

Members of this group can be confused with the upper clades without microscopic examination or chromatographic pigment analysis.

The upper clades have fewer clade-specific macroscopic characters which unambiguously place them in a group, and as can be seen, some clades incorporated species from several taxa. That the structure of the last three clades showed much less segregation and consistent branching order than other clades indicates a more recent separation between groups. This is particularly apparent in figure 13.

Those characters that determine the position of a species relative to the phylogeny presented here were either clade-specific, species specific, or distributed along a gradient. Obviously the specific characters can be used to reach an identification, at least of a clade. The gradient characters including spore colour, pileocystidial shape and septation and, to a lesser degree, the bruising reaction and odours, can be used to estimate the position and probable clade of a species that is less easy to identify and place in a traditional classification.

Ranking these characters in order of taxonomic usefulness is difficult because they tend to co-vary. However, following the major division with the amyloidity of the suprahilar patch, the pileocystidia and epicutal hyphal types showed a more consistent relationship to overall clade order than spore colour or taste. Spore ornamentation was generally the least clade-order related character of those considered.

Anomalous clade members

Some species had characters seemingly anomalous in relation to those in the rest of their clade. ***Russula ochroleuca*** fell amongst subsection *Russula* with *R. atropurpurea* and *R. viscida* where its position was well supported, appearing in 86% of trees, and in agreement with the findings of Eberhardt (2002) and Miller and Buyck (2002). This suggests that the incrustations (of yellow pigment) are of the same origin and type as is found in the *Ingratae* in species like *R. pulverulenta.* This species was placed in *Felleinae* in Bon (1987), then in subsection *Ochroleucinae* amongst other incrusted subsections in Bon (1988).

In this analysis *Russula viscida* was not in the same group as *Russula melliolens* in the *Melliontinae* of Romagnesi (1967). Sarnari (1998) created Section *Viscidinae* in subgenus *Russula* to house this species. The inclusion of *R. viscida* in the clade with *R. stuntzii* was only supported in 50% of trees and morphological characters such as dermatocystidia with capitate ends as well as the long, cylindrical, multi-septate "*Tenellae*" type pileocystidia do not clarify its position. Moreover, the position of *R. melliolens* amongst the *Tenellae* and *Urentinae* only occurred in 40% of trees, so the taxonomy of neither species was resolved.

In clade 8, *R. veternosa, R. firmula* and *R. californiensis* are peppery, non-incrusted species amid mild, incrusted species. This clade was well supported in both the minimised sequence analysis and the full analysis. *Russula californiensis* has the greying flesh and colouration of *R. decolorans* in this clade. *Russula firmula* and *R. veternosa* are in separate series within subsection *Urentes,* as is *R. maculata* (Sarnari 1998). This sub-clade should be investigated further, perhaps with more collections being sequenced and compared, before making any changes to the classification.

Russula messapica fell amongst the *Tenellae*, validating Sarnari's (1998) placement of this species near the *Tenellae*, but erecting a new section; the *Messapicae.* This veiled species was considered close to the *Ingratae* in Courtecuisse and Duhem (1995). *Russula solaris*, also in this clade, has been previously placed close to *R. raoultii* (Bon, 1988, Sarnari, 1998, Romagnesi, 1967), but its strong yellow bruising, diverticulate pileocystidia and yellow spores are in keeping with the *Tenellae* of clade 10.

General taxonomic observations and recommendations based on these analyses

The basal clades reflect the existing classification, beginning anticlockwise from the outgroup, subgenus *Compactae*, and its subdivision into *Archeinae, Compactae* and *Lactaroides* (fig. 19). There was 95% consensus of this clade structure, which is in contrast to that of Miller and Buyck (2002) who found the *Lactarioides* to be basal to the *Heterophyllidia* and *Ingratae,* and, in agreement with Shimono *et al*. (2004) the *Compactae* to be basal to the remaining upper *Russula* clades. The amyloid patch on the spores would support this position. Clearly the relative positions of these groups was sensitive to the analysis methods and choice of other taxa.

The clade of subgenus *Ingratae* was subdivided into the groups corresponding to series *Foetens* and *Pectinata*, suggesting these groups could be raised to section level.

The inclusion of *R. cyanoxantha* as basal to this clade rather than *Heterophyllidia* in agreement with Miller and Buyck (2002) is indicative of the evolutionary roots of the *Ingratae.* Its precise position may not be quite accurate in this analysis, in Shimono *et al.* (2004) the LSU showed a clade of *R. cyanoxantha* and *R. cutefracta* as basal to the *Ingratae, Heterophylla* and *Amoenula*, as did Eberhardt (2002).

Russula fellea allied with the *Russula* clade in this analysis and in that of Miller and Buyck (2002), and Eberhardt (2002) for both ITS and LSU rDNA, and Shimono (2004), a position recognised in Bon (1988) and supported by the morphological character of an amyloid suprahilar patch on the spores.

Clade 4 contained the *Heterophyllae* and showed the white-spored species branching off before the yellow-spored species, with a rough correspondence to the subsections.

In the upper clades, the first branch included the white and mostly lighter yellow spored peppery species, which contains the type for subgenus *Russula: R. emetica.* This well-defined clade included section *Russula* subsections *Consobrinae, Russula,* and *Sardoninae* but excluded *Violaceinae, Urentes* and *Rubrinae.* In Miller and Buyck's (2002) analysis, this clade was also seen, and included similar sub-clades but with some differences in the branching order. As this clade is so clear, the subgenus *Russula* should be redefined to exclude groups not in this clade in order to make it monophyletic.

A small clade of three species, *R. rosacea, R. violacea* and *R. aurata,* occurred in 98% of trees in the full analysis and 72% in the minimalised analysis. This found a parallel in the analysis of large subunit rDNA by Shimono *et al.* (2004), who found a small separate clade with *R. rosacea, R. aurea* (synonymous with *R. aurata*) and two of three *R. flavida*, all collections from Japan. Their collections of *R. violacea* and *R. sanguinea,* which is considered a synonym of *R. rosacea,* fell into the clade with the remaining members of subgenus *Russula*. These species do not seem to have morphological characters in common, so the clumping could be due to long branch attraction rather than a real relationship.

In recent classifications, the incrusted species are divided into two subgenera based on the presence or absence of pileocystidia. In agreement with Miller and Buyck (2002), this Bayesian analyses showed two mixed clades of incrusted species from both subgenera, indicating that in this group at least, the presence of pileocystidia was secondary to the possession of incrustations and/or primordial hyphae. The subgenus *Incrustatula* needs to be expanded to accommodate section *Paraincrustatae* Sarnari and incrusted species and groups from section *Polychromae* (Maire) Sarnari, presently in subgenus *Russula.* The Pacific northwestern species *R. occidentalis* was included in clade 8, which also held other grey-bruising species such as *R. decolorans.* An examination of cap cuticles of dried local collections, after staining with fuchsin and mounting in 5% HCl showed that they possessed incrusted hyphae, a character not mentioned in any of the descriptions. Thiers (1997) placed it in subsection *Decolorantinae* of section *Coccineae*, subgenus *Russula,* (following Romagnesi, 1967), a section which does contain other incrusted species.

The *Xerampelinae* which make up part of clade 9 have some plesiomorphic characters, the pileocystidia, normally very sparse, are generally aseptate, often with capitate ends, and SV negative. Type 5 pileocystidia are not found. The green reaction with $FeSO_4$ is also seen in *R. cyanoxantha* and close relatives. In Miller and Buyck's (2002) analysis this clade branched off earlier, becoming basal to the incrusted species and all clades above, corresponding to clades 7, 8 and 10 in the present analysis.

The grouping of *Russula olivacea* in the clade with *R. xerampelina* in the minimised sequence tree, as in Romagnesi's 1967 classification, and its grouping (in only 63% of trees), in the neighbouring clade with *R. caerulea,* basal to several species with incrusted pileal hyphae as in Sarnari's 1998 classification based on the presence of primordial hyphae, shows that either classification could be right.

Clade 10 brought together yellow-spored, often yellow bruising species with long multi-septate pileocystidia, also in some species diverticulate pileocystidia, which in this group had taxonomic precedence over taste.

Clades 7 to 10 did not fall conveniently into two distinct clades of incrusted and non-incrusted species and so any reclassification of these groups has to be either phylogenetically correct but counter-intuitive, or imperfect but usable. Perhaps the better alternative would be to continue investigations into these terminal clades in pursuit of confirmation or correction, then erect taxa to house them. Henkel *et al.* (2000) and Buyck (1995) comment on the need to make a more fully integrated phylogeny for the

Russulas by including the many diverse species found in the tropics, which have been relatively little studied compared with temperate zone Russulas. These may shed more light on the relative importance of various characters and clarify some of the clade structures.

Character variability - hypotheses on developmental processes.

Characters found in basal clades may reappear in upper clades and the genetic mechanisms governing the formation of a given character and its evolution are not fully understood. Continuous characters, such as spore colour, pepperiness and density of each pigment in the cuticle tend to be of a similar nature within a clade, but the exceptions give a clue as to how these characters develop. One hypothesis is of a system whereby a set of genes code for the default or plesiomorphic character, perhaps an aseptate form of pileocystidia with strangulated tip, with other genes modifying the growth and form of these as they develop. In any cap cuticle it is possible to find several cystidial types, although one usually predominates, indicating that several different versions of the modifying genes may exist in one thallus. Spore colours in the sister genus *Lactarius* range from white to light orange, indicating that the ability to produce some yellow pigment in the spore wall is a common ancestral quality in *Russulaceae*. With continuous characters the basic genes may code for continuous production of spore pigments until switched off by an environmental or genetic trigger, a state which happens much sooner in basal clades. In this case a non-functional gene would give rise to white spores as in *R. azurea, R. lilacea, R. rosea* and *R. lepidicolor* in an otherwise yellow-spored group. An alternative explanation would involve simply multiple functional copies of the spore pigment gene in darker spored species, in which case white-spored species are harder to explain. These two hypotheses are not mutually exclusive.

The sesquiterpenes responsible for pepperiness and carried in the laticifers can be detected in the immature stages of some mild species, and in the gills but not the trama of some variably peppery species. The inclusion in some groups of peppery and mild species prompted the hypothesis that the default condition is one of continuous production of the peppery compounds until the genes responsible are inhibited at some developmental stage by a controller gene or genes. Mild species occur when this critical stage is reached early or if the genes for the production of sesquiterpenes (and/or laticifers) are not functional. Pepperiness occurs frequently in *Lactarius* and so is probably plesiomorphic: completely mild species occur mostly in upper clades.

A general hypothesis regarding genetic control of cuticle pigments would have to assume separate control and production genes for each pigment, probably with similar mechanisms as those hypothesised above for spore and sesquiterpene production. Cap colours vary between species because the production of one or more colours is considerably inhibited in relation to the others. Further discussion of pigments is in the chapter on Chromatography.

Table 7 Taxonomically important characters of Russulas used in the phylogenetic analyses, organised by clade (following 7 pages). Not all the information is available or remarkable, as is reflected by the absence of information in a given category. Epicutal terminal hyphae are (correctly or not) assumed to be undifferentiated filamentous hyphae unless noted otherwise.

Abbreviations: Col = colour of spores in Romagnesi's 1967 code. Am. Ptch=amyloid suprahilar patch. Wart ht. = height of warts as follows: 1= small, 0.1 - 0.4 μm; 2 =medium, 0.4 - 0.9 μm; 3 =large, 0.7- ≤ 2 μm; Retic = amount of reticulation: A = warts isolated, B = warts connected 2 - 3, C = warts connected by a partial reticulum, D = complete reticulum, E = warts catenate forming ridges, both according to Woo's and Bon's keys, see Figure 30 in Appendix 3. SV = reaction to sulphovanillin where grey to black to purple is a positive reaction, colourless or pink is a null reaction. Inc. = fucshin positive acid resistant incrustations. Prim hyphae = primordial hyphae, also staining pink in fucshin. Picy = pileocystidia. Taste codes: p = peppery, pm = mature basidiomata usually peppery but mild forms occur, m = mild, mp = mature basidiomata are usually mild but immature stages or gills may be peppery, b = bitter. Bruising codes: y = yellow, ybr = yellow then brown, br = brown, rg = red then grey to black, g = grey or black, wl = the stipe becomes greyish or yellow-grey as if waterlogged but is not a true bruising reaction.

Clade	Species	Spores				Pileocystidia									Epicutal hyphae			Macroscopic characters		
						— shape —														
		Col.	Am. ptch	Wart ht	Retic.	1	2	3	4	5	6	7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
1a	*R. archaea*	Ia	0												0	0		m	br	
1a	*R. camarophylla*	Ia	0	1	A		2						0	0+	0	0	clavate end cells	m	ybr	none or cheese rind
1b	*R. densifolia*	Ia	0	1	B-C		2				6		0	0	0	0	inflated- articulated	p	bk	weak, fruity
1b	*R. adusta*	Ia	0	1	B-C		2			5	6		0	+	0	0		mp	bk	weak, wine barrels
1b	*R. nigricans*	Ia	0	1	B-C				4				0	0	0	0		p	bk	
2	*R. pallidospora*	IId	1	1-2	A-D		2						0	0,+	0	0		m,b	br	fruity
2	*R. chloroides*	Ib	1	1	B-C		2						0		0	0		p	br	fruity
2	*R. brevipes*	Iab	1	2,3	B-C										0	0		m-p	br	
2	*R. delica*	IIa	1	1-3	C-E		2						0	+	0	0		mp	br	fruity
3a	*R. cyanoxantha*	Ia	0	1	A		2						0	+	0	0		mp	br	
3b	*R. pectinatoides*	IId	0	2	A-B	1	2						0	0	0	0		p	rbr	rubber
3b	*R. amoenolens*	IIb	0	2	A-B	1							0	0	0	0		p	rbr	Jerusalem artichokes
3b	*R. pectinata*	IIb-d	0	2	B	1	2						0	0	0	0		p	rbr	burnt flesh, nauseous
3c	*R. pulverulenta*	IIc	0	2,3	B-C	0	0	0	0	0	0	0	0	0	+	0	warty yellow walls	mp	ybr	

Clade	Species	Spores				Pileocystidia — shape —									Epicutal hyphae			Macroscopic characters		
		Col.	Am. ptch	Wart ht	Retic.	1	2	3	4	5	6	7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
3c	*R. insignis*	IIa	0	2	C	1						7	0	0	0	0		m nauseous	rbr	weak-fruity
3c	*R. foetens*	IIbc	0	2	A-B		2						0	0	0	0		p	rbr	rubber, nausous
3c	*R. laurocerasi*	IIab	0	3	E		2		4	5			0	+	0	0		p	rbr	benzaldehyde
4a	*R. amoenicolor*	IIcd	0	2	C or E	0	0	0	0	0	0		0	0	0	0	articulated	m		Jerusalem artichokes
4a	*R. violeipes*	IIa	0	1	D-E	0	0	0	0	0	0		0	0	0	0	articulated	m		
4b	*R. mustelina*	IIb	0	1	A-E		2		4				0	0	0	0		m		weak, cheese
4b	*R. virescens*	Ib	0	1,2	A-D	0	0	0	0	0	0		0	0	0	0	inflated cells	m		weak, fruit or cheese
4b	*R. alboareolata*	Ia	0												0	0				
4c	*R. heterophylla*	Ia	0	1	A			3	4				0	0	0	0	inflated-articulated + thick walled hair	m		
4c	*R. vesca*	Ia	0	2	A			3	4				0	0	0	0	inflated-articulated + thick walled hair	m		
4d	*R. parazurea*	IIb	0	1	C		2		4				0	0,+	0	0	articulated	mp		weak, cheese
4d	*R. ilicis*	IId	0	1	B-C		2		4				0	+	0	0		mp		methyl salicilate, fruity
4d	*R. grisea*	IIc	0	1,2	B-C		2	3	4				0	+	0	0		m		
4d	*R. aeruginea*	IIb	0	1	B		2						0	0,+	0	0		mp		

Clade	Species	Spores				Pileocystidia — shape —									Epicutal hyphae			Macroscopic characters		
		Col.	Am. ptch	Wart ht	Retic.	1	2	3	4	5	6	7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
5a	*R. atropurpurea*	Ia	1	2	D		2		4				0	+	0	0		pm	ybr,wl g	apple
5a	*R. ochroleuca*	Iab	1	2,3	D		2						0	0	+	+	yellow walls	p	br	
5a	*R. viscida*	IIb	1	1	D,E		2	3		5			0-m	+	0		yellow picy & laticifers	mp		fruity
5a	*R. stuntzii*	Ib	1	2,3	B		2		4				0-1	+	0			p	wl	weak, fruity
5b	*R. fellea*	Iab	1	2,3	C		2		4				0-2		0		pigment on hyphal walls	p		apple, pelargonium
5b	*R. aquosa*	Ib	1	2	C		2	3					0	+	0			p	wl	weak, fruity
5b	*R. raoultii*	Ia	1	2	D			3	4				0	+	0			p	wl	coconut
5b	*R. cremoricolor*	Ia	1	1	B		2						0	+	0			p		
5b	*R. mairei*	Ia	1	1,2	C		2		4	5			0-3+	+	0			p		
5b	*R. fragilis*	Ib	-1	2	D		2	3	4				0-2	+	0			p	wl	amyl acetate, coconut
5b	*R. betularum*	Iab	1	2	D			3	4	5			0-4	+	0			p	wl	fruity
5b	*R. nana*	Ia	1	1	D		2		4	5			0-1		0			p	wl	fruity
5b	*R. bicolor*	Ia	1	1,2	A-B		2							+	0			p	wl	weak, fruity
5b	*R. emetica*	Iab	1	2,3	D		2		4	5			3+	+	0		red vacuolar pigment	p	wl	weak, fruity

Clade	Species	Spores				Pileocystidia									Epicutal hyphae			Macroscopic characters		
						— shape —														
		Col.	Am. ptch	Wart ht	Retic.	1	2	3	4	5	6	7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
5c	*R. helodes*	IId	1	2	D		2		4	5			0	+	0			p	g	
5c	*R. consobrina*	IIbc	1	2	D		2		4	5			0-m	+	0		vacuolar pigment	pm	gbr	weak, fruity
5d	*R. persicina*	IIbc	1	1,2,3	B		2		4	5			0	0,+	0			p	y	weak, fruity
5d	*R. gracillima*	IIab	1	2	A				4	5			0-2	+	0			pm		apple
5d	*R. exalbicans*	IIIb	1	2	B-E		2		4	5			0-1	+	0			p	wl	apple/plum
5e	*R. cavipes*	IIa	1	2	B-C		2						0-1	+	0			p	y	apple/plum
5e	*R. drimeia*	III	1	1	C		2						0	+	0			p	y	apple/plum
5e	*R. sardonia*	IId-IIIa	1	2	C		2						0	+	0			p	y	apple/plum
5e	*R. fuscorubroides*	IIc	1												0			p		apple/plum
5e	*R. queletii*	IId	1	2,3	A		2			5			0-1	+	0			p		apple/plum
6	*R. violacea*	IIab	1	2,3	A			3	4	5			1-4	+	0			p	ybr	apple, pelargonia
6	*R. rosacea*	IIIa	1												+			p	y	weak, fruity
6	*R. romellii*	IVd	1	2	C-E		2		4	5			0	(+)	0		long narrow pointed			weak, fruity
6	*R. aurata*	IIIcIVb	1	2,3	C or E	0	0	0	0	0	0	0	m	0	0			m	ybr	
7a	*R. amethystina*	IVa	1	2,3	B-C	0	0	0	0	0	0	0		0	+	+	pseudoparenchym. subc	m	br	

Clade	Species	Spores				Pileocystidia									Epicutal hyphae			Macroscopic characters		
						shape														
		Col.	Am. ptch	Wart ht	Retic.	1	2	3	4	5	6	7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
7a	*R. roseipes*	IVab	1	2	BC	0	2?	3?	0	0	0	7?	0	0	+	+				
7a	*R. turci*	IVa	1	2	DE	0	0	0	0	0	0	0		0	+	+	pigmented between sub and epicutis, infl. cells	m		iodoform at stipe base
7a	*R. paludosa*	IIIab	1	2,3	C				4			7	0-2	(+)	+			m		weak, fruity
7a	*R. postiana*		1												+					
7a	*R. risigallina*	IV	1												+					
7b	*R. rosea*	Ib	1	1	BC	0	0	0	0	0	0	0		red trama	+	+	inflated cells subcutis	m		none
7b	*R. azurea*	Ia	1					3					0-1	(+)	+	+	clavate end cells	m		none
7b	*R. lilacea*	Iab	1	2,3	A	0	0	0	0	0	0				+	+		m		weakly fruity
7b	*R. lepidicolor*	Ib	1	1	C,E				4				0	red trama	(+)	+		m	br	
7b	*R. aurantiaca*	IVc	1	3	A			3	4	5		7	0-2	0	+		capilliform hyphae	mp		fruit/apple
7b	*R. lepida*	IIa	1	1	ED				4	5		7	0	0	+	+		b, menthol		cedar wood, menthol
8a	*R. caerulea*	IVb	1	2	CD	0	0	0	0	0	0	0	0	red trama	+		infl cells, ampullate	m,b	g/br	
8a	*R. olivacea*	IVc	1	3	A	0	0	0	0	0	0	0	0	0	0	+		m	ybr	weakly fruity
8b	*R. melitodes*	IVb	1	3	AB	1		3	4				0-2	(+)	+	+		m	ybr	weak fruit, honey
8b	*R. decolorans*	IIcIIIa	1	2,3	B				4				0-2	+	0		picy numerous	m	g	weak honey, cheese

Clade	Species	Spores				Pileocystidia									Epicutal hyphae			Macroscopic characters		
						— shape —														
		Col.	Am. ptch	Wart ht	Retic.	1	2	3	4	5	6	7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
8c	*R. californiensis*	III	1	1	D		2						0		0			p	g	
8c	*R. veternosa*	IVb	1	3	A			3	4	5			m	+	0		inflated picy ends, numerous picy	p	(g)	honey, gingerbread
8c	*R. rubra*	IIIb	1	2	C		2		4			7	0-3	+	+			p,b	g-y	weak, honey
8c	*R. firmula*	IVd	1	1	AB		0	3	4	5			0-3	+	0			p	y	weak, apple
8d	*R. velenovskyi*	IIIab	1	2	B				4	5		7	0-m	+	+	+		m		none
8d	*R. integra*	IVc	1	3	A				4	5		7	0-1	+	+	##		m	br	iodoform, fruit, honey
8d	*R. integriformis*	III	1												+			m		none
8d	*R. claroflava*	IIIbc	1	3	BC	0	0	0	0	0	0	0	0	red hyphae	+	+		m	g	fruit
8d	*R. vinosa*	IIIab	1	2	A	0	0	0	0	0	0	0	0	0	+	+		m	g	weak, fruity
8d	*R. occidentalis*	IVa	1	3	A	0	0	0	0	0	0	0	0	0	+	+		m	rg	weak
9a	*R. nitida*	IIIbc	1	2,3	A				4	5			0-2	0-+	0			m	y	weak, fruity
9a	*R. sphagnophila*	IIcd	1	2	BC				4				0-2	+?	0			m	(y)	weak, fruity
9b	*R. amoenipes*	IIIb	1	2	C		2	3	4				0	0	0		ampullate & inflated ends	m	ybr	shellfish
9b	*R. pascua*	IIIc	1	3	A				4				0-2	0	0			m	ybr	shellfish
9b	*R. xerampelina*	IIIc	1	2,3	AB		2		4				0	0(+)	0		ampullate	mp	ybr	shellfish
10a	*R. maculata*	IVcd	1	3	BC		2	3	4	5			0-2	+	0		inflated parts of picy	pm	g,y,br	cedar, menthol

Clade	Species	Spores				Pileocystidia			Epicutal hyphae			Macroscopic characters		
		Col.	Am. ptch	Wart ht	Retic.	— shape — 1 2 3 4 5 6 7	# Septa	SV	Inc.	Prim. hyphae	Terminal hyphae	Taste	Bruising	Odours
10a	*R. melliolens*	IIab	1	1	D	5	m	+	0			m	ybr	honey, gingerbread
10a	*R. cuprea*	IVe	1	3+	A	4 5 6	m	+	0		ampullate, diverticulate	p		weak fruity
10a	*R. adulterina*	IVe	1	3+	A	2 4 6	0-2	+	0			p		stewed apples
10a	*R. curtipes*	IVa	1	2	CE	3 4 5	0-m		0		ampullate, hair	m	ybr	weak fruity
10a	*R. cessans*	IVbc	1	2	CD	0 4 5 6	m	+	0			m	wl	
10a	*R. laricina*	IVcd	1	2,3	BC	0 4 5 6	##	+	0			mp	wl	
10b	*R. melzeri*	IVb	1	2,3	A	3 4 5	m	+?	0			m		fruity
10b	*R. puellula*	IIbc	1	2	B	5	m	+	0			p	y	
10b	*R. puellaris*	IIc	1	2,3	AB	3 4	0-3	+	0			p	y	
10b	*R. solaris*	IId	1	3	B	3 4 5 6	0-m		0		globules of yellow pigment	p		vinegar
10b	*R. messapica*	IVc	1			5	m		+		incrust. red in KOH	m		quince
10b	*R. nauseosa*	IVb	1	3	AB	3 4	0-2	+	0			p		
10b	*R. odorata*	IVc	1	2,3	C	4 5	m	+	0			mp	y	fruity, pelargonia
10b	*R. versicolor*	IIIb	1	1	BC	4 5	2-3	+	0			p	y	

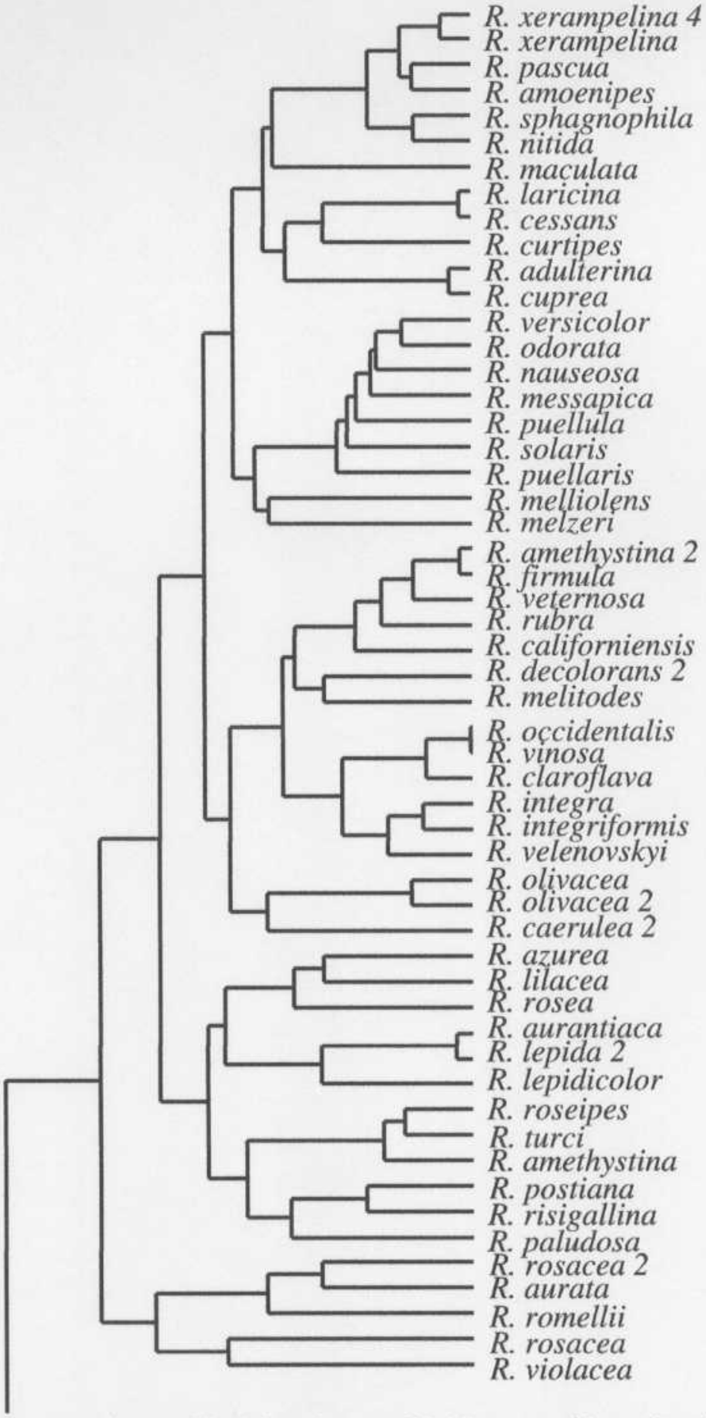

Figure 14 Bayesian maximum likelyhood tree of 111 taxa with molecular clock analysed using the HKY85 model (Haegawa, Kishino, and Yano, 1985), using Albatrellus flettii and Gloeocystidiellum aculeatum as outgroups. The tree is the final tree of 1500000 trees, with a log-log likelyhood of -17578.557772. Above are the upper clades and the figure is continued down to the lower clades in figure15 .

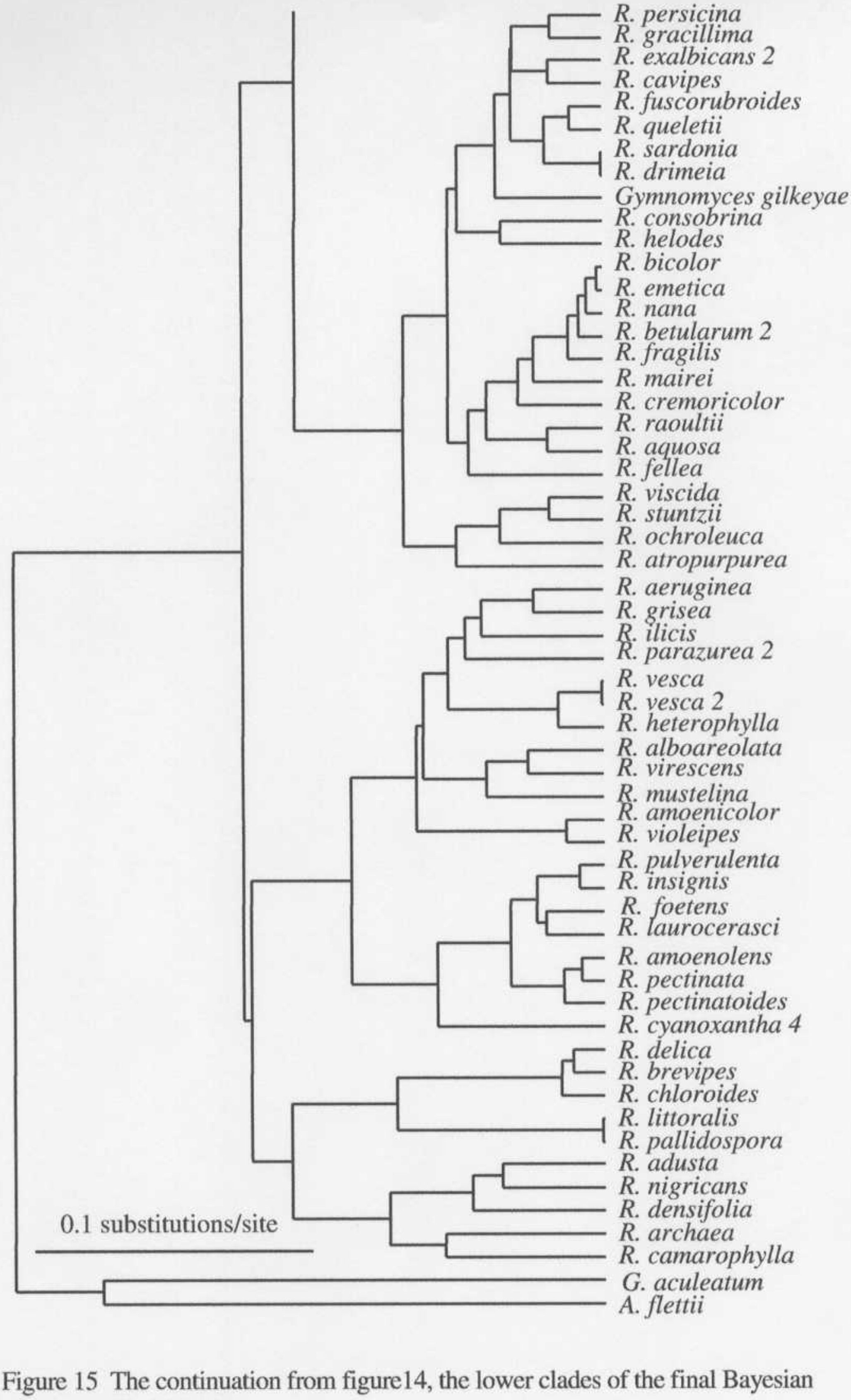

Figure 15 The continuation from figure14, the lower clades of the final Bayesian maximum likelyhood tree.

Figures 16 and 17 following pages. Extended majority rule consensus tree of the Bayesian maximum likelyhood with molecular clock analysis as outlined above. The numbers to the above left of the nodes indicate the percentage of times the group consisting of the species to the right of that node occurred among the trees, out of 6000 trees sampled at the rate of one in 200 from the 1200000 trees created after convergence. The terminal nodes are not marked except where the values were below 98%, heavy lines indicate values of 98-100%. Clades are marked and numbered, minor clades within each clade are given in Table 6.

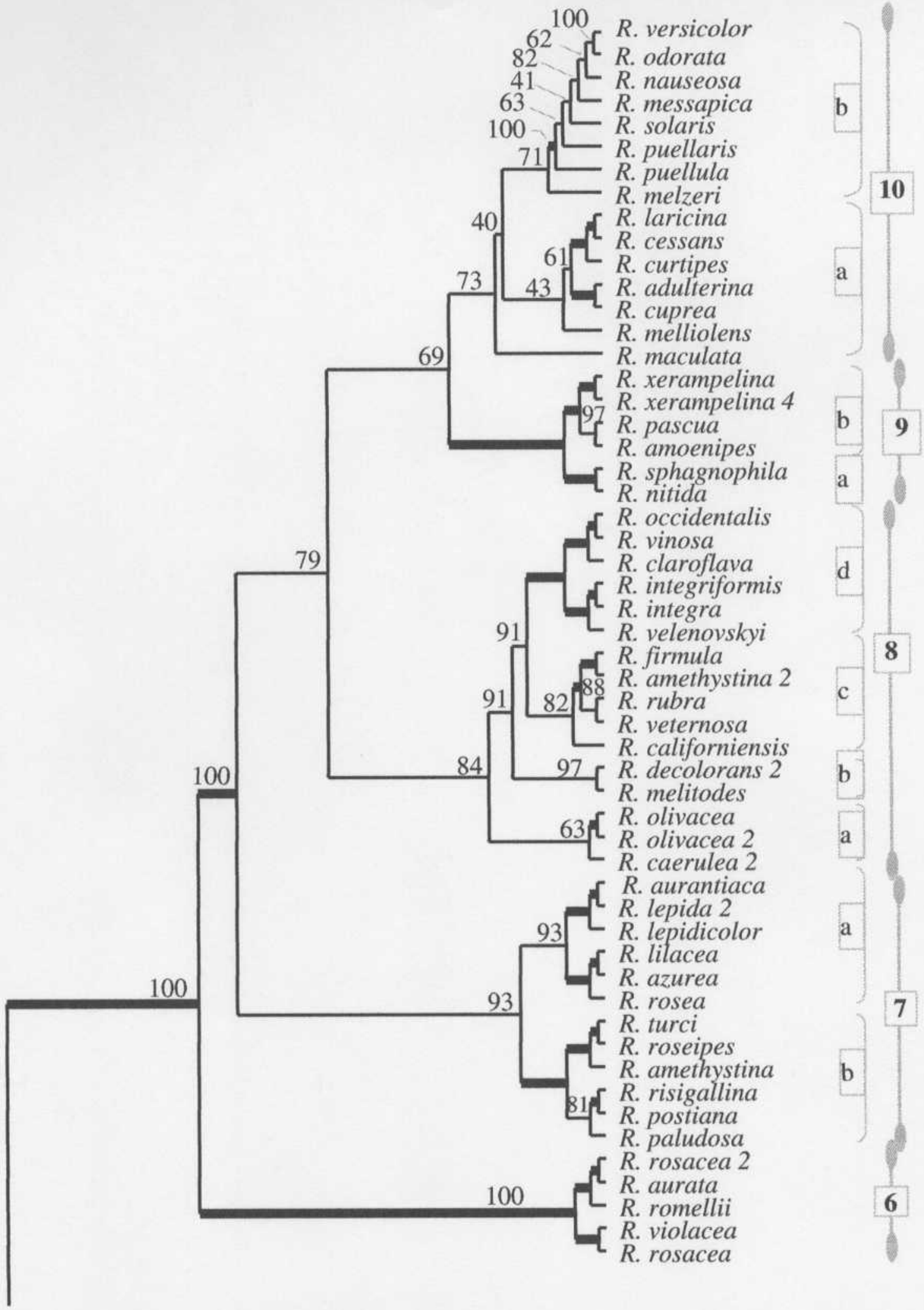

Figure 16 Extended majority rule tree, upper clades (numbered 6-10) of tree, the lower of which is continued downwards in figure17.

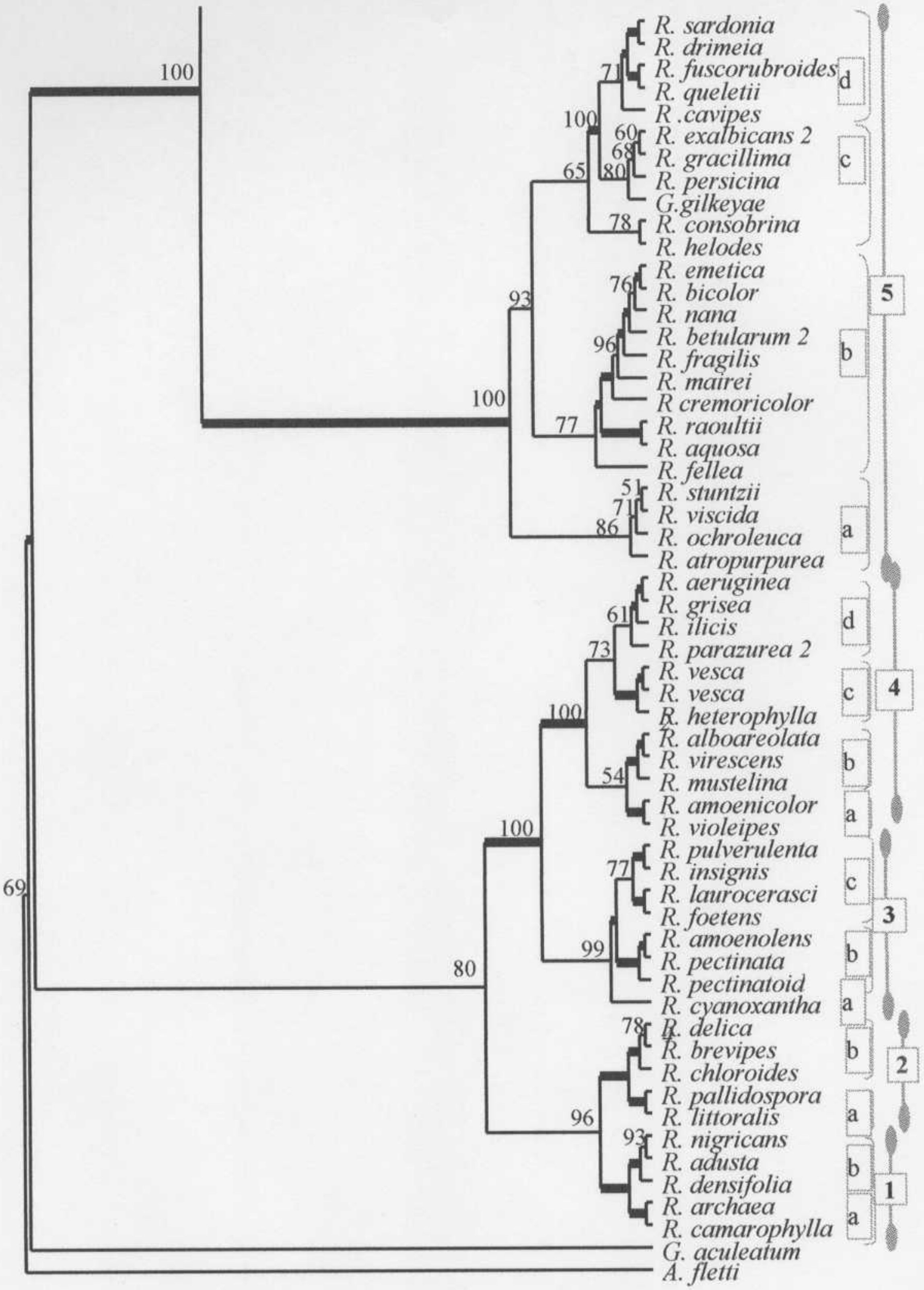

Figure 17 Lower clades (1-5) of extended majority rule tree, continued from figure 16. Full explanation of figure is given overleaf.

The numbers to the above left of the nodes indicate the percentage of times the group consisting of the species to the right of that fork occurred among the trees, out of 6000 trees.
The terminal nodes are not marked except where the values are below 98%,
heavy lines indicate values of 98-100%

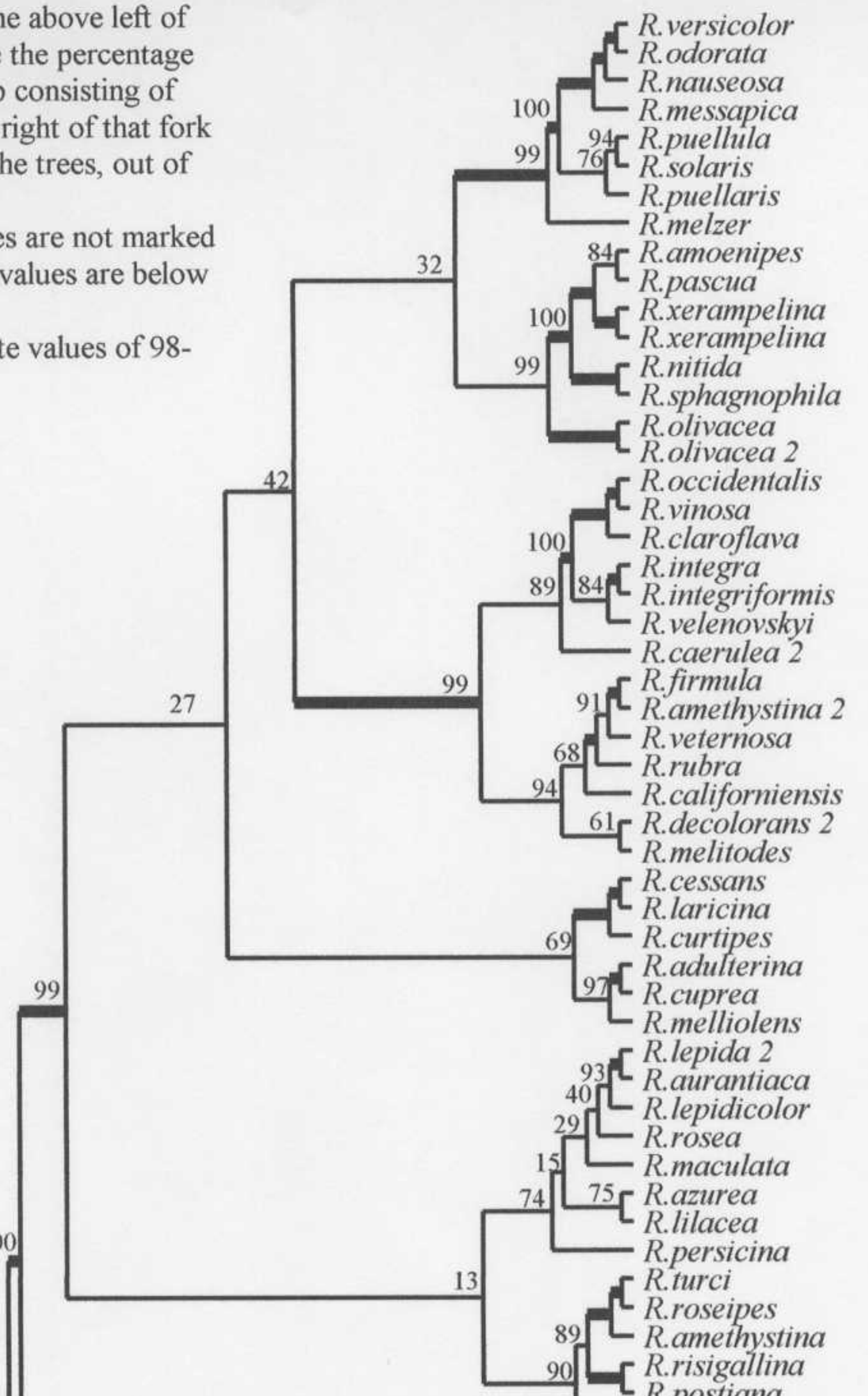

Figure 18 Extended majority rule consensus tree of 54 Russulas from the upper clades of the previous analysis plus an outgroup, re-analysed using sequence minimisation.

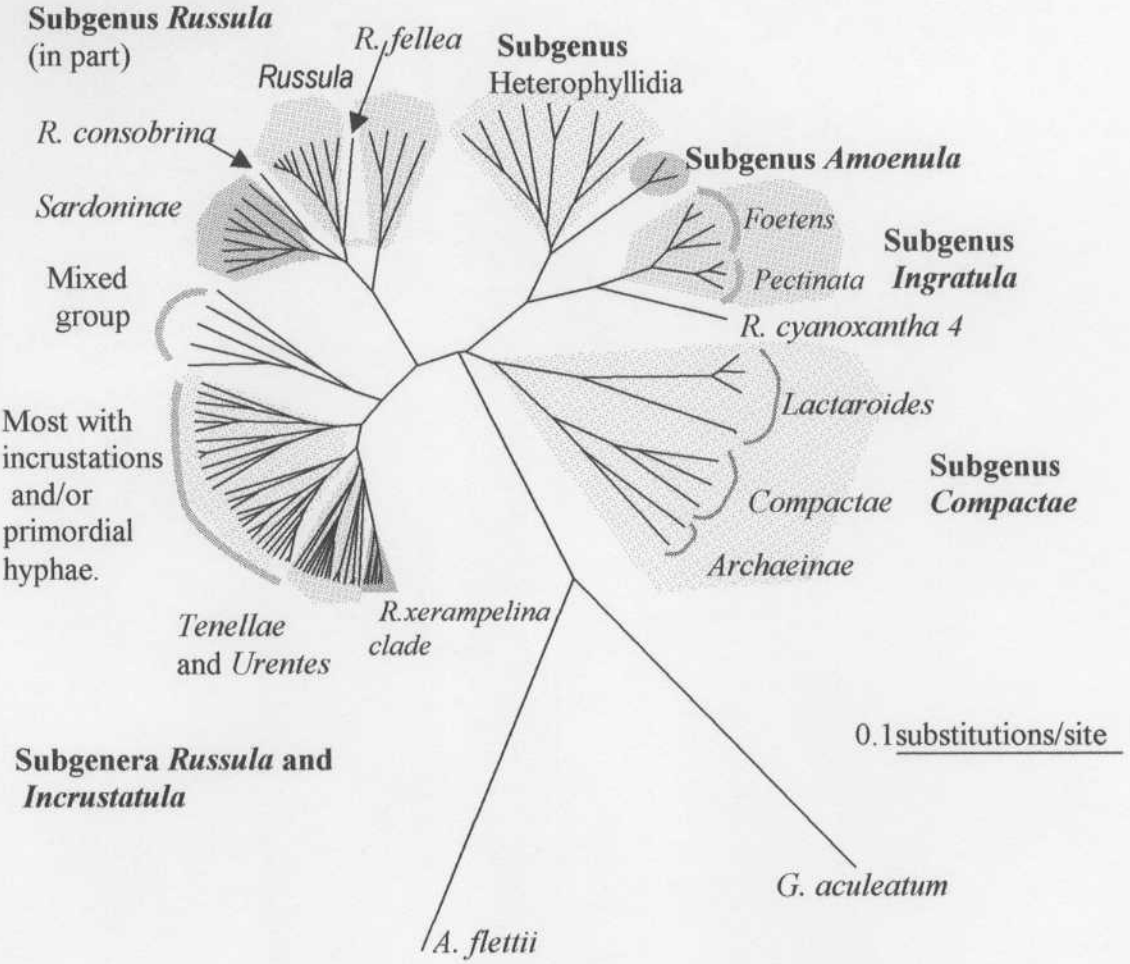

Figure 19 Unrooted Bayesian maximum likelyhood tree of 108 species of *Russula* and a *Gymnomyces gilkeyae*. *Albatrellus flettii* and *Gloeocystidiellum aculeatum* are the outgroup and are shown here with their branches truncated. This is the same tree as in figures 10 and 11 but this format shows more clearly the division within the genus of the subgenera *Russula* and *Incrustatula* on the left and the subgenera *Compactae*, *Ingratula, Amoenula* and *Heterophyllidia* on the right. The tree also shows that the subsections *Consobrinae, Sardoninae* and *Russula* which are currently in section *Russula* of subgenus *Russula* form a monophyletic group. The clades on the lower left include two groups in which most species have incrustations and/or primordial hyphae in the cap epicutis, and the terminal group of strongly coloured spores with no incrustations or primordial hyphae.

Chapter 4

Examination of character trends in Vancouver Island collections

Introduction

Russula taxonomists have considered characters such as relatively short, broad basidia as indicative of higher clades such as the *Tenellae,* clade 10. However many such character variations have not been examined for phylogenetically ordered gradations. In the previous chapter the characters and character states of each species were gleaned from the literature of many different authors, whose methods of measurement, degree of detail, and perceptions of what may be taxonomically useful, varied. The 229 collections of Russulas from Vancouver Island offered the opportunity to examine first-hand the various characters, to measure them, to investigate other characters that have not to date been systematically examined, and to verify or question the clade related gradient findings from chapter 3 part II.

Methods

Estimating clade positions of Vancouver Island species

From a consideration of the phylogenetic analysis in chapter 3 part 2, the Vancouver Island Russulas were assigned an estimated clade position based on their relationships to the species used in the analysis. Morphological characters, RFLPs of rDNA analyses (chapter 3 part 1), chromatographic analyses of cuticle pigments (chapter 2) and reference to monographs on Russulas, particularly those of Bon (1988), Romagnesi (1967, 1985) and Sarnari (1998), were used in determining relationships between species. Estimated clade positions are shown in the table below and in the descriptions (Appendix 3). Sequence data for two local species, *Russula crassotunicata* and *Russula farinipes* has become available since the completion of most of this dissertation. A Bayesian maximum likelyhood analysis using a smaller set of sequences suggests these species are basal to the other *Russula* clades, closer to the *Archea*, than to the *Ingratae* to which they have been traditionally allied. They were placed in a basal position for the analyses of characters using the MacClade program described below.

Evaluation and examination of characters of Vancouver Island collections

The following characters which could be expressed numerically were plotted against the clade position of each of 56 species to see if any trends were apparent: Spore print colour, recoded from 1 to 10 following the colour codes of Romagnesi (1967), where 1=Ia and 10=Ive. Spore shape, as the mean length to width ratio and its range (maximum L:W – minimum L:W); and maximum spore length of normal, mature spores, (not including the occasional oversized spores that normally occur in most spore prints, unless these comprise about 10% or more of the spores.) Maximum height of the spore ornamentation and degree of reticulation between warts, this latter based on Woo's' types (Appendix 3, figure 30), recoded numerically with type A=0, B=1, C=2, D=3 and E=4. These numbers actually relate closely to the number of connections the warts have to one another, with the exception of type E, in which the ornamentation is in the form of chains or ridges. Basidia dimensions, including minimum and maximum length and width of mature basidia.

While this provided an overall view of phylogenetically useful gradients, it was inappropriate to derive any significance from fitted trendlines. Characters showing trends were regressed against one another to see how they were correlated, and these regressions were examined for significance.

To examine trends further, character data were traced onto a phylogenetic tree using the MacClade program (version 4.03, Maddison and Maddison 1992), which calculates ancestral states for the branches. As few of the Vancouver Island species had sequences available with which to build a phylogenetic tree to use in the MacClade analyses, a hypothetical treewas created, organised as for the Bayesian majority rule tree (figs.16 and 17), and based on the estimated clade positions of each of 56 Vancouver Island species as described in the previous paragraph.

Characters examined in the MacClade program were treated as continuous in the case of basidia and spore dimensions, or categorical in the case of degree of spore reticulation. Spore colour, for convenience, is commonly treated in descriptions as categorical. It is actually a continuous character but has occasional occurrences of white-spored species appearing within clades of yellow-spored species (compare *Russula lilacea* with *R. aurantaica*, table 7: clade 7), suggesting that pigment loss takes fewer evolutionary steps than pigment gain. Using MacClade, the spore colour evolution was traced as a continuous character, although various asymmetrical step-matrices were also tried and compared. Continuous characters were also entered as categorical into the MacClade database in order to examine tree statistics, as the program does not include continuous characters for these statistics.

Pileocystidia of four shapes and incrusted primordial hyphae were either present or absent in a given species. Unfortunately a pure binary scoring for pileocystidia shapes would be incorrect since several species commonly have more than one shape, and the relative proportions of these can vary by collection and by age. As a compromise, a score of 1 was recorded when one pileocystidia shape only was found, 0.5 for each of two shapes found and 0.33 for each of three shapes found; no species had four shapes present. These figures and the original binary score were put through the following principal component analysis to see if the results differed. The pileocystidia binary figures were also among the characters used in the calculation of tree statistics in the MacClade program.

To examine how well a clade could be defined by a set of character measurements, a principal component analysis was done using the following microscopic characters: mean length to width ratio of spores, maximum spore length, spore ornamentation height, pileocystidia shape and presence of incrustations in the epicutis. This analysis of 54 Vancouver Island species used the Biplot 1.1 add-in software for Excel (Smith and Lipkovich 1999-2002). Data columns were centred and standardized for the singular value decomposition transformation, and 2 components (axis) were extracted, the transformed data being plotted using a row scaling adjustment factor of 1.228, automatically calculated by the Biplot software.

Results

Trends

Several clade related trends were found in Vancouver Island collections. Positive trends from lower to upper clades included spore colour (lighter to darker), the height of spore ornamentation, maximum spore length, and basidia width, (both minimum and maximum width). The basidia length showed very little trend, save for a slight reduction in clade 5. Decreasing trends were seen in mean spore L:W, spore L:W range (amount of variation), and the degree of reticulation on the spore walls. These characters were traced onto the phylogenetic tree in MacClade, which calculates and displays on the branches the ancestral states, all but the last two are shown in figs. 20 - 24. All characters showed scatter around the trendline, displayed in the variety of character states within a clade on the branch ends of the hypothesised evolutionary trees shown in figs. 20 - 24.

The Vancouver Island collections had no white-spored species above clade 6, and none with spores darker than shade 6 (Romagnesi IIIa) below clade 7 (fig. 20). There are localized trends within this overall picture, including a steep increase in spore colour from clade 5 to 7, confirming the usefulness of this character on several taxonomic levels. In the phylogenetic analysis in chapter 3 part II, clade 7 contains 3 white-spored species, but otherwise has the same overall light to dark trend, suggesting a loss of functionality of one or more pigment genes. With this in mind the reconstructed ancestral character states for clade 5, also with mixed pigmented and colourless spores, could be darker than is indicated in fig. 20.

The spore length to width ratio shows a reduction from lower to upper clades (fig. 21, top). The variability of the spore shape (not shown) closely follows the clade-related pattern of the L:W ratio, with the exception of clades 1b and 2 (the *Compactae* and *Lactaroides*), with variability like that of clades 6 upwards. Essentially spores are mostly ellipsoidal but quite variable in shape in the lower clades and mostly subglobose and less variable in the upper clades. Spore maximum length is generally higher in clades 7 to 10 than in lower clades (fig. 21 bottom). Two species with large spores, *R. olivacea* and *R. aureofulva*, in clades 8 and 10 respectively, may be biasing the calculated ancestral state, although other members of these taxonomic groups not included in this analysis have larger than average spores.

The spore ornamentation height shows some variation in the states at the branch tips, but this translates as a gradual trend in the ancestral states from lower to upper clades, as shown by the shade gradations between deeper nodes (fig. 22, top). The degree of reticulation between spore warts tends to be polymorphic for most species. Spores from several collections of a species, or often from a single piece of gill, may have quite a range of reticulation, although one category may predominate. (In the descriptions in Appendix 3 these ranges are given). This does not translate easily to the analysis mode in MacClade, in that polymorphisms are not calculated into the ancestral states. The resulting traced tree gives the illusion of much better defined states than is the reality. The chart (fig. 22, bottom) gives one a more realistic idea and shows a slight tendency for reticulated spores to be more frequent in the lower clades. This overall trend is perhaps less useful than the smaller trends such as the highly reticulate spores in clades 1-2 and 5a-b, and the frequency of none- to partially-reticulate spores in clades 3, 4 and 7-10. The occurrence of type E ornamentation –lines of fused warts forming ridges, is scattered throughout clades 1-8 and is peculiar to a species rather than showing a trend.

Basidial width at maturity showed a strong positive trend from lower to upper clades when plotted against species in clade order, with a slightly better line fit for the maximum width at which developed spores are seen. This trend is apparent in the reconstructed ancestral states traced onto the phylogenetic tree, which show mostly narrow basidia in clades 1-4, mid-sized ones in clades 5-7, and larger ones in clades 8-10 (fig. 23 top). Basidial length however, shows very little trend save that those in clades 5 and 6 are generally shorter than lower and higher clades.

The statistics for the tree used in figures 20-23 calculated by the MacClade program were: Treelength 208; minimum possible treelength 54; maximum possible treelength 257; consistency index 0.26; retention index 0.24; rescaled consistency index 0.06. These figures were derived from 12 unordered characters in category format with equal weight: Mean spore L:W, spore L:W range, maximum spore length, spore colour, spore reticulations, spores with ridge formations, basidia minimum width, four pileocystidia types, and presence of incrustations (none, traces or present).

Correlations between characters

There is little correlation between the basidia width and length (table 8), presumably because basidial length is a function of the age of a basidioma, and of the size and proliferation of the basidial support cells. Those basidia arising from lower in the hymenium naturally have a longer base than those arising nearer the surface, since they must all bear their spores at the surface. This suggests basidia can elongate from the base once the apex is differentiated into sterigmata and spores.

The basidial width and spore ornamentation height show a positive significant correlation of 0.61 ($\alpha = 0.05$, $P < 0.0001$; table 8 and fig. 24). Spore length to width ratio also shows a significant negative correlation of 0.33 with basidial width ($\alpha = 0.05$, $P = 0.001$, table 8 and fig. 25), and basidial width is up to 53% significantly positively correlated with maximum spore length and 56% with spore colour.

Correlations between characters regressed against one another are summarized in the following table.

Table 8 Regression analyses results

	vs	Correlation	R^2	P ($\alpha = 0.05$)
Basidia minimum width	Basidia minimum length	0.18	0.032	0.18
Basidia maximum width	Basidia maximum length	0.09	0.008	0.53
Basidia minimum width	Spore ornamentation height	0.61	0.38	<0.001
Basidia minimum width	Spore maximun length	0.53	0.28	<0.001
Basidia minimum width	Mean L:W spores	-0.33	0.11	0.013
Basidia minimum width	Spore colour	0.56	0.31	<0.001
Mean L:W spores	Spore ornamentation height	0.43	0.18	0.001

In the principal component analysis (fig. 26) clades 1 and 4 cluster mainly in the upper left quadrant influenced by having strangulate tips to the pileocystidia, ellipsoidal spores, and (negatively) by having smaller than average spore ornamentation and narrower basidia. Clade 3 clusters mostly in the lower left quadrant, where the pileocystidia are tapered and the spore L:W ratio has more influence than other spore or basidial dimensions. Two exceptions are *Russula crassotunicata, R. farinipes,* which cluster with others in clade 1; supporting the finding based on sequence information published since these analyses were completed, that these species branch off below those of other *Russula* clades. Most of clade 5 cluster along the y axis, having close to average dimensions in spores and basidia, but some having strangulate pileocystidia tips (above the x axis) and others with obtuse pileocystidia tips (below the x axis). The three clade 6 species are above the x axis where they are split between strangulate and diverticulate pileocystidia. Clades 7-10 are in or close to the lower right quadrant, influenced mainly by the obtuse pileocystidia tips, incrustations and larger spore and basidium dimensions. The fact that those species do not cluster with other clade members shows that although there are general trends which are quite reliable indicators of clade position, there are also frequent exceptions in one or more characters.

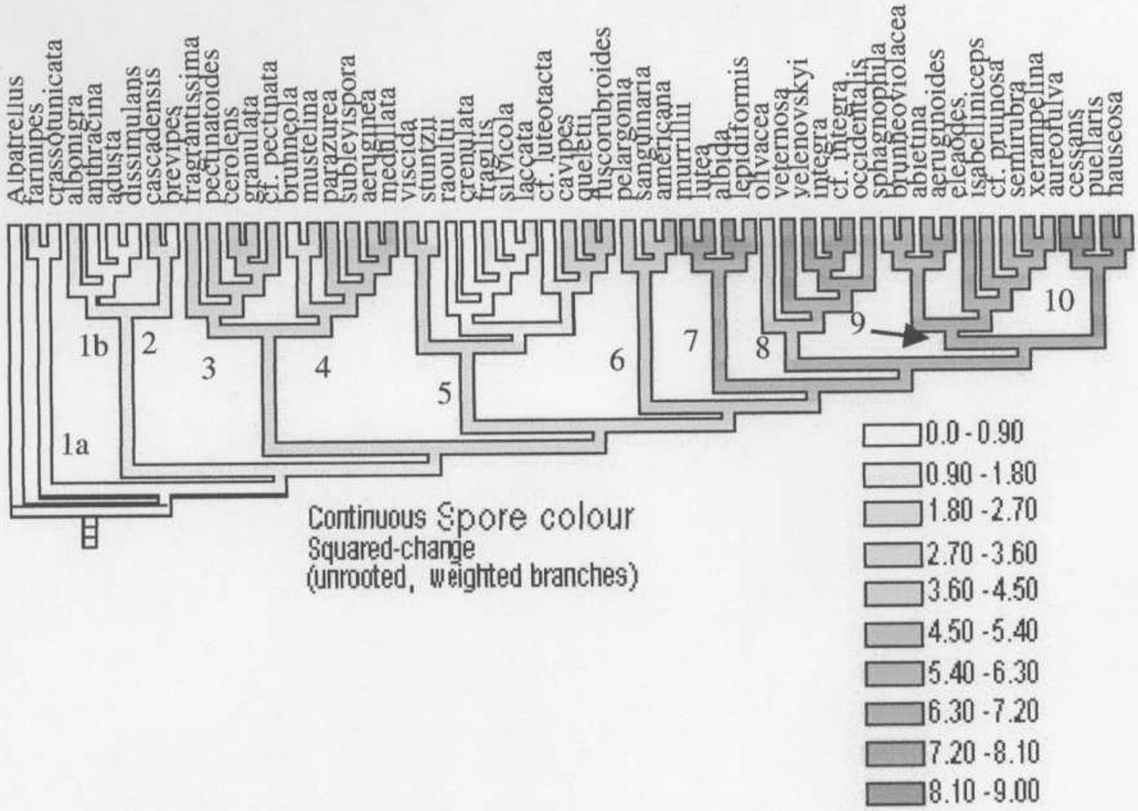

Figure 20 Spore colour of Vancouver Island collections with hypothesised ancestral states traced onto a phylogenetic tree. Colours are those of Romagnesi, coded 0 for white spores to 9 for deep ochraceous yellow spores, the colour scale is approximate. Character treated as continuous, clades are numbered adjacent to the supporting branch.

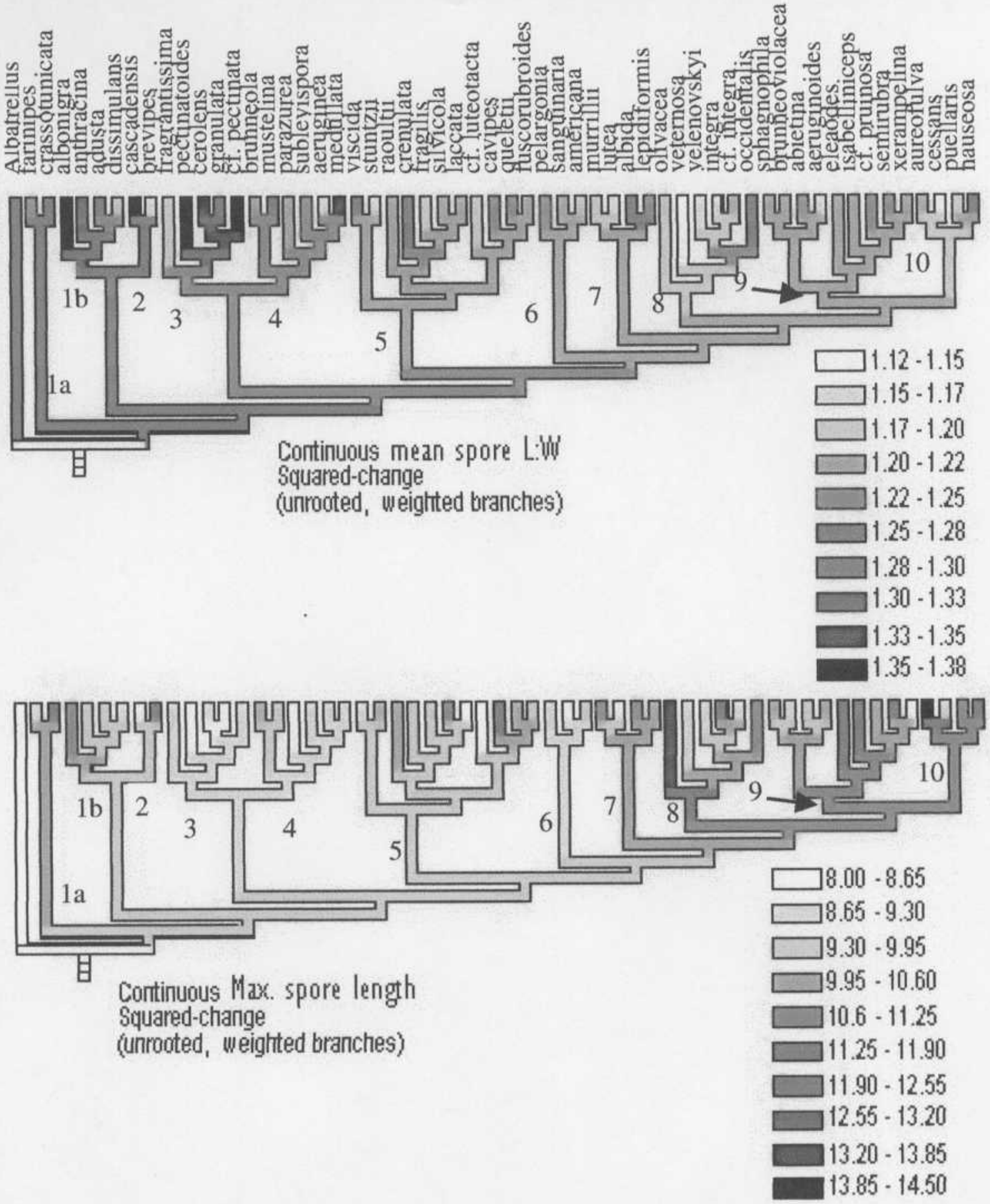

Figure 21 Spore shape and size with calculated ancestral states traced onto a hypothetical phylogenetic tree. Top: mean L:W ratios of spores showing a trend towards more globose spores in the upper clades; bottom, maximum length of (normal sized) spores, showing an increase in clade 7 and above. Albatrellus flettii, the outgroup on the left, has spores under 4.8μm long. Clades are numbered adjacent to the supporting branch. Both characters are treated as continuous, dimensions are in μm.

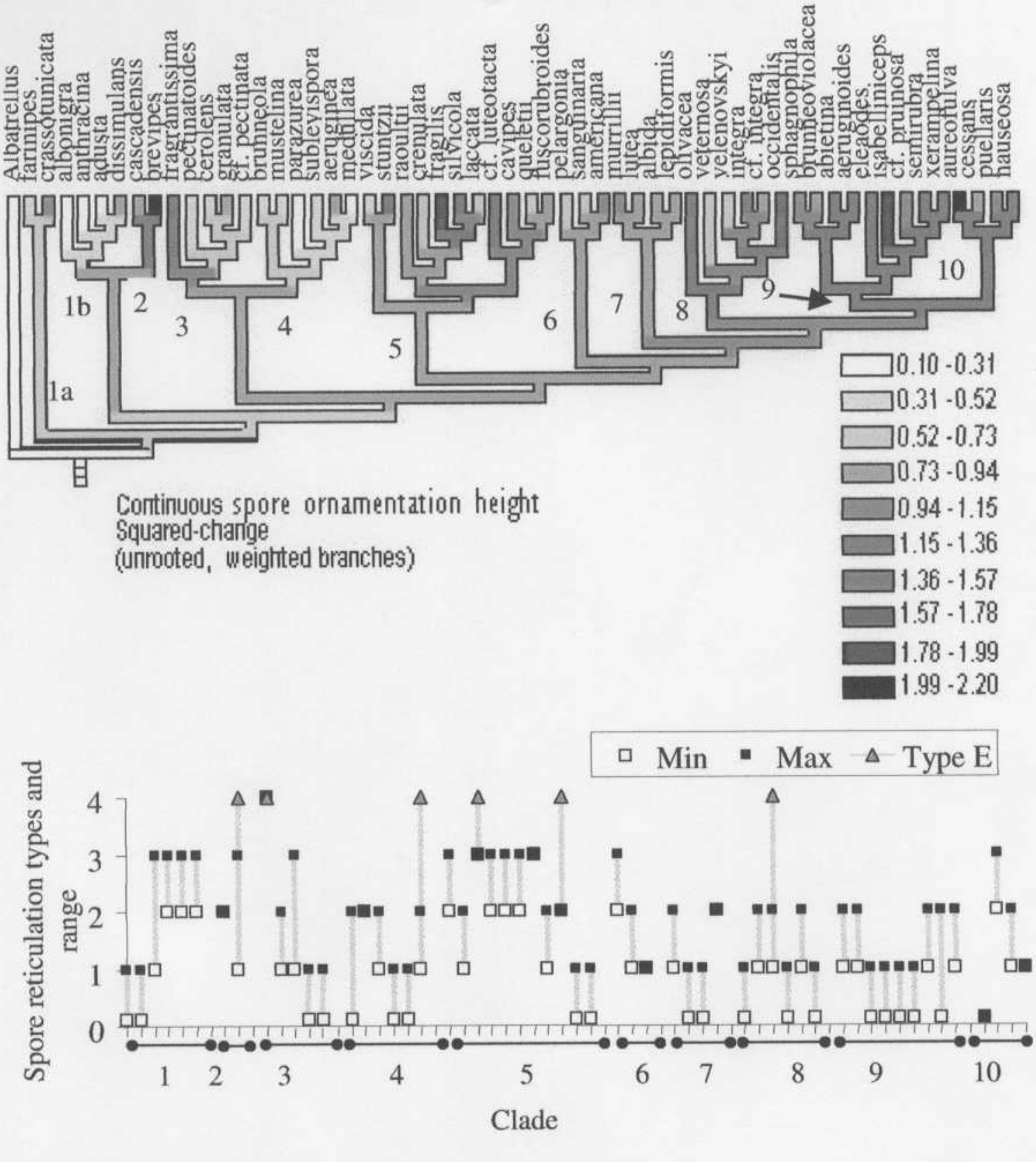

Figure 22 Spore ornamentation and its hypothesised ancestral states: Top, maximum height of the spore ornamentation in µm traced onto phylogenetic tree, showing an overall increase towards higher clades; bottom, amount of reticulation between warts on the spore wall. Most Russulas have a range of ornamentation, i.e. they are polymorphic and this is better portrayed by plotting the range against the species. The numerical divisions on the y axis approximate the number of connections between one wart and its neighbours, with the exception of 4, which stands for type E of the Woo spore ornamentation coding. This coding system also translates on this figure as 0=type A, 1=type B, 2=type C and 3=type D. Species are in the same order for both diagrams.

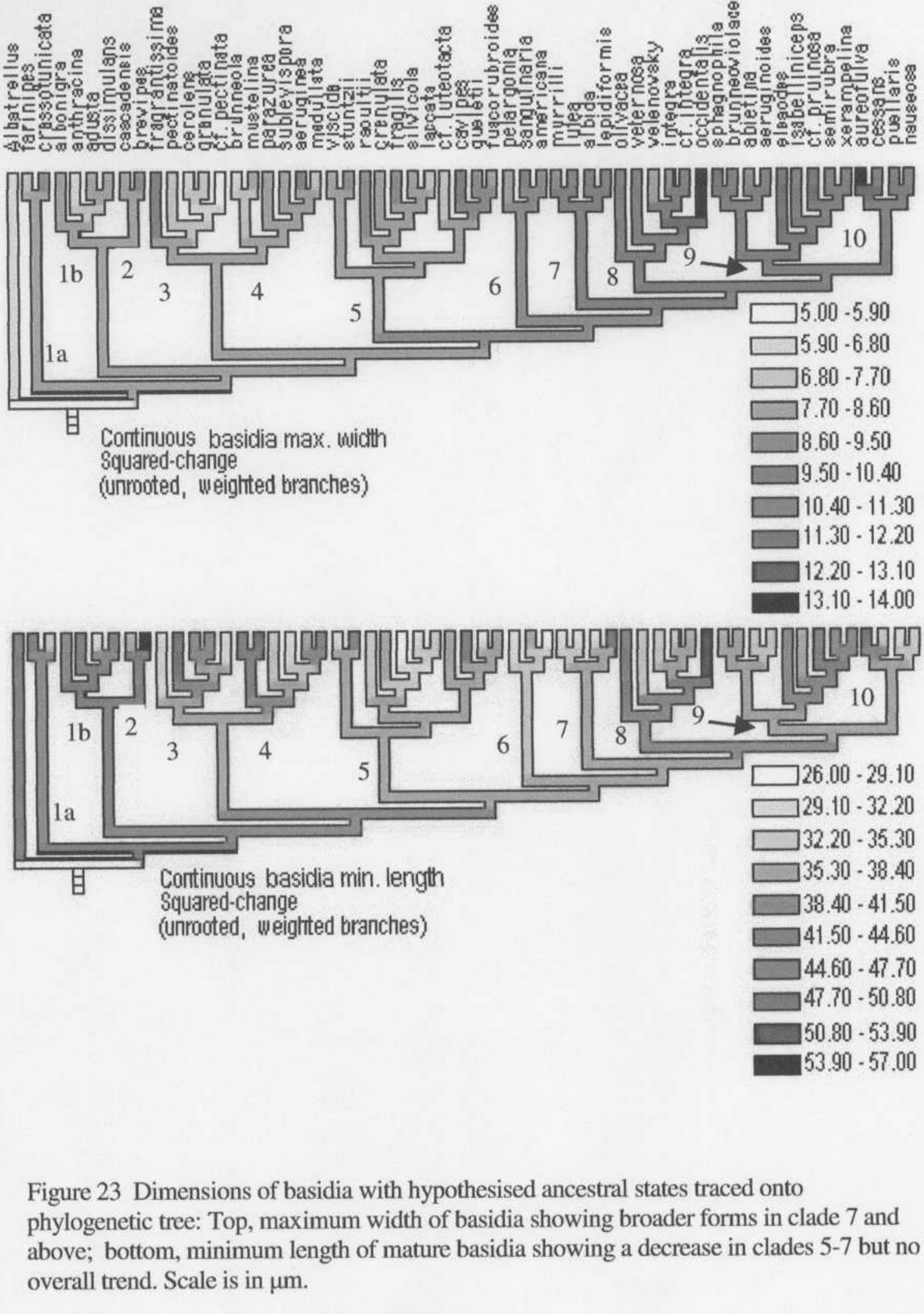

Figure 23 Dimensions of basidia with hypothesised ancestral states traced onto phylogenetic tree: Top, maximum width of basidia showing broader forms in clade 7 and above; bottom, minimum length of mature basidia showing a decrease in clades 5-7 but no overall trend. Scale is in μm.

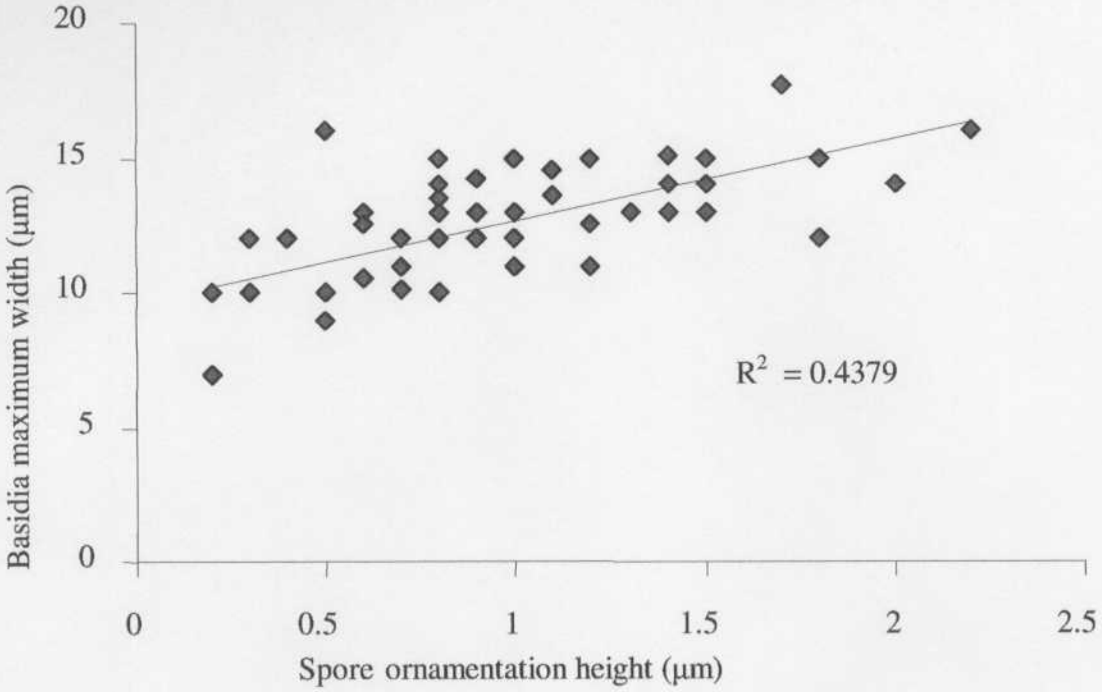

Figure 24 Basidia maximum width regressed against spore ornamentation height: they are 66% correlated ($\alpha = 0.05$, $P= 0.3^{-7}$).

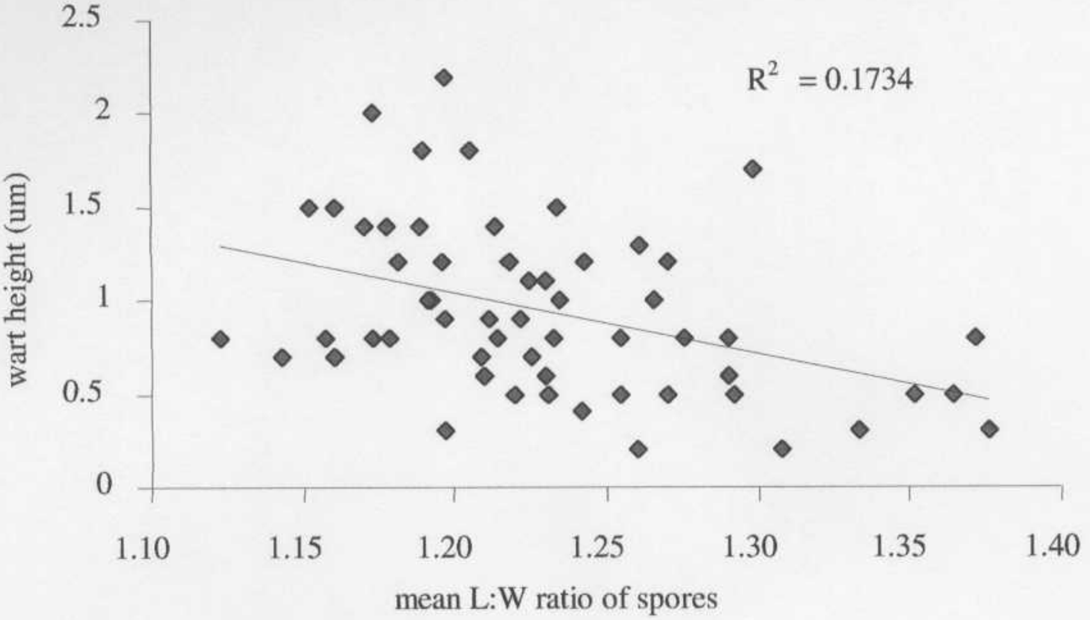

Figure 25 Spore wart height regressed against the mean length to width ratio of spores: they are 43% correlated ($\alpha = 0.05$, $P = 0.001$).

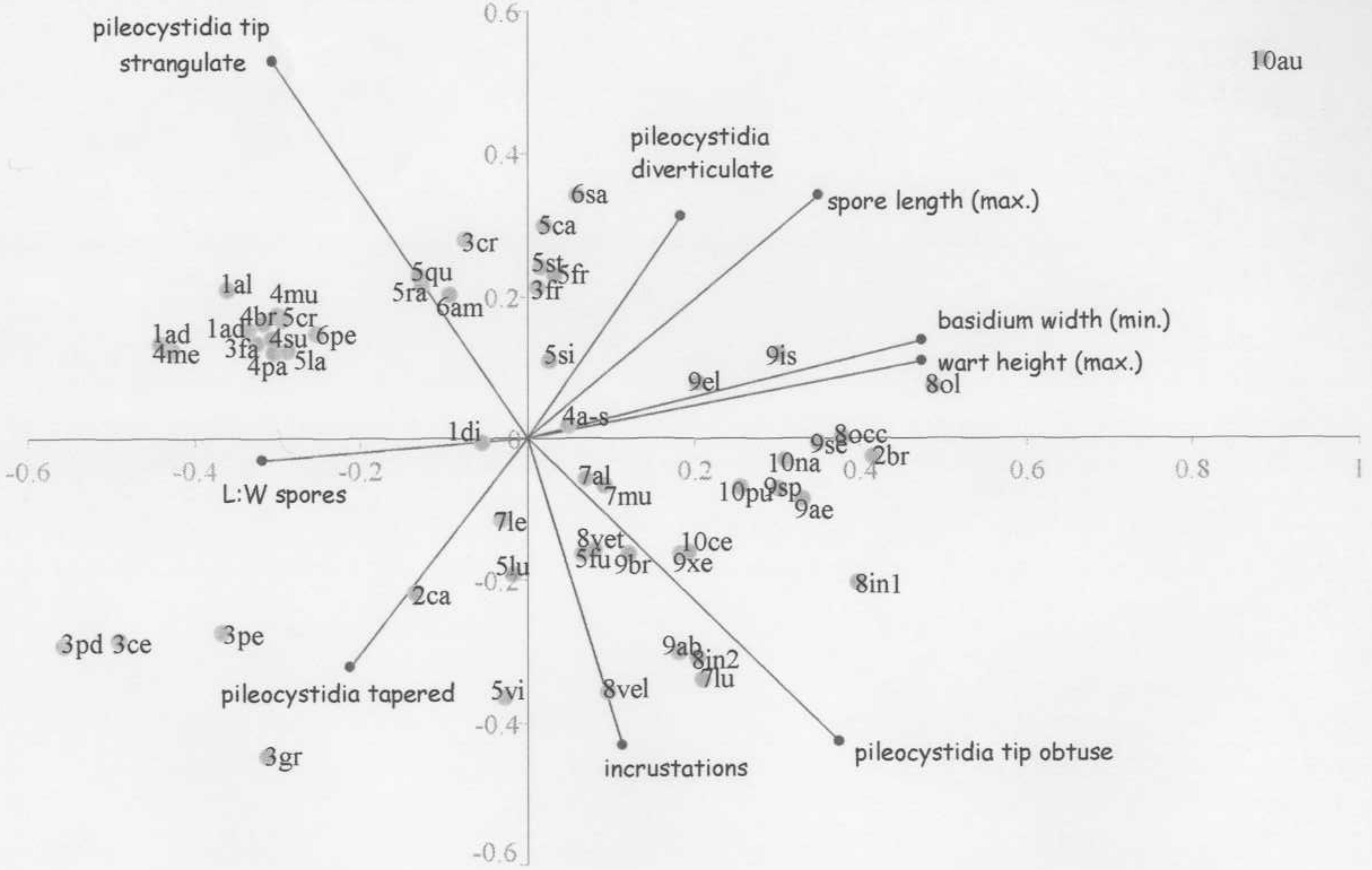

Figure 26 Principal component analysis of 54 *Russula* species from Vancouver Island, (each represented by a code). The species are arranged in space according to the influence of the following components: wart height, basidium width, spore length, length to width ratio of spores, shapes of pileocystidia – strangulate tips, tapered, obtuse tips, with diverticulae. Eigenvalues and key to codes on the following two

Summary of values computed in the principle component analysis in fig. 26

Singular and eigenvalues for the SVD (U LAMBDA V')

Singular values	Eigen values	Cumulative % of Eigenvalues	Eigen values	Cumulative % of Eigenvalues
1.7367	3.01613	0.33513	3.01613	0.33513
1.27927	1.63652	0.51696	1.63652	0.51696
Sum of eigenvalues	9		9	

Table 9 Key to codes representing species used in the principal component analysis in figure 26. Codes consist of the clade number followed by 2-3 digits of the species epithet.

Code	**Species**	**Clade**	**Code**	**Species**	**Clade**
1ad	*R. adusta*	1b	5qu	*R. queletii*	5e
1ad	*R. anthracina*		5fu	*R* .cf. *fuscorubriodes*	
1al	*R. albonigra*		5ca	*R. cavipes*	
1di	*R. dissimulans*		6pe	*R. pelargonia*	6
2br	*R. brevipes*	2b	6sa	*R. sanguinea*	
2ca	*R. cascadensis*		6am	*R. americana* var *modicaspora*	
3ce	*R. cerolens*	3b	7lu	*R. lutea*	7a
3pe	*R. pectinatoides*		7mu	*R. murrillii*	
3pd	*R.* cf.*pectinata*		7al	*R. albida*	7b
3gr	*R. granulata*		7le	*R. lepidiformis*	
3fr	*R. fragrantissima*	3c (or 1a)	8ol	*R. olivacea*	8
3pa	*R. pallescens*		8vet	*R. veternosa*	8c
3cr	*R. crassotunicata*	3d	8vel	*R. velenovskyi*	8d
4mu	*R. mustelina*	4b	8in1	*R.* cf. *integra* 1	
4br	*R. brunneola*		8in2	*R.* cf. *integra* 2	
4pa	*R. parazurea*	4d	8occ	*R. occidentalis*	
4me	*R. medulatta*		9sp	*R. sphagnophila*	9a
4su	*R.* cf. *sublevispora*		9br	*R. brunneoviolacea*	
4a-s	*R. aeruginea*		9ab	*R. abietina*	

Code	Species	Clade
5vi	*R. viscida*	5a
5st	*R. stuntzii*	
5ra	*R. raoultii*	5b
5cr	*R. crenulata*	
5fr	*R. fragilis*	
5la	*R. laccata*	
5si	*R. silvicola*	
5lu	*R. luteotacta*	5d
9ae	*R. aeruginoides* nom prov	
9xe	*R. xerampelina*	9b
9se	*R. semirubra*	
9is	*R. isabelliniceps*	
9el	*R. elaeodes*	
10au	*R. aureofulva*	10a
10ce	*R. cessans*	
10pu	*R. puellaris*	10b
10na	*R. nauseosa*	

Conclusions

The best indicators of phylogenetic position amongst the characters assessed above are, in order: basidium width, spore colour, spore ornamentation height, spore L:W, spore length and the maximum amount of reticulation on the spores. Basidium length is a poor predictor of phylogenetic position. These numerically expressible characters are in addition to the qualitative characters such as odours, taste, gill arrangement and amyloidity of the spore suprahilar patch, that are described in the keys to Vancouver Island Russulas that follow in Appendix 3, and to those previously discussed. Another microscopic character that appears to be linked to the phylogenetic branching order is average pileocystidia septation, which tends to increase in upper clades.

One character that is linked to clade, but not forming an overall trend, is the texture of the basidioma. Some are firm and elastic, others are firm and brittle, while others are quite soft and fragile at an equivalent growth stage, however this does not translate to easily measurable characters of the trama such as the relative amounts of sphaerocytes to generative hyphae. One may possibly measure the amount of force needed to break a certain thickness of flesh, but even this would have to take into account weather conditions, age of the basidioma and the amount of fungivorous arthropod infestation.

One of the issues brought up by the phylogenetic analyses in chapter 3 part II was the apparent mismatch between clade 8 and the current taxonomic system, where species with incrustations in the cap cutis were aligned with those without, and the fact that clades 7 and 8 have primordial hyphae but clades 9 and 10 are considered to lack this trait. By examining first-hand all the collections from these groups, I found hyphae in the epicutis of some of the *Tenellae* (clade 10) that show similarities to primordial hyphae, but are not encrusted (see *R. puellaris* and *R. nauseosa*), and occasional traces of incrustations in the epicutis of *R. veternosa*. This finding supports the phylogenetic analyses and indicates that the upper clades 7-10 could legitimately be placed together within subgenus *Incrustatae*.

The results of the character analyses of Vancouver Island collections supports, clarifies, and adds to those findings made from the literature in chapter 3 part II.

Taken together, microscopic characters alone can be used to narrow down an identification to within two or three clades, as shown by the principal component analysis. The spore colour narrows this down further and in theory at least, the additional use of macroscopic and habitat characters would narrow the possibilities down to one or two species.

Chapter 5

Summary

Russula species found in Vancouver Island coastal forests

The first question addressed in this thesis was "Are there are more species of *Russula* to be found on Vancouver Island than are currently recorded from local foray data and from published accounts of Pacific Northwest collections, and what are the characteristics and habitats of local forms of recorded and newly observed species?"

Sixty-one species and varieties of *Russula* have been described below (Appendix 3), 60 of which are represented by 229 collections from Vancouver Island. Thirty-one of these species are among the 78 (non-sequestrate) *Russula* species previously reported from the Pacific Northwest (Woo, 1988, Gibson and Gibson, 2004), confirming their occurrence in this region. There are two new species: *Russula auruginoides* nom. prov. and *R. aureofulva* nom. prov.; two new varieties: *R. americana* var. *modicaspora* nom. prov., and *R. fragilis* var. *mitis* nom. prov. and a proposed raising to species status of *Russula xerampelina* var. *isabelliniceps*, renamed *Russula isabelliniceps.* New records for the Pacific Northwest include 11 species: *Russula albida, R. anthracina* var. *insipida, R.*cf. *luteotacta, R.* cf. *pruinosa, R.* cf. *subvelispora, R.*cf. *fuscorubroides, R. laccata, R. lepidiformis, R. medullata, R. nauseosa* and *R. velenovskyi* and one variety: *R. queletii* cf. var. *flavovirens.* Some of these names derive from European descriptions and in some cases these lacked sufficient detail to make a confirmed identification; in others the Vancouver Island collections varied slightly from the European species. These issues are discussed in the notes section of each of the descriptions in Appendix 3.

The descriptions of Vancouver Island material contain new information and illustrations that will enable other taxonomists of the genus to judge the veracity of the identifications and compare them with their own collections more critically. It serves as a partial monograph to local species, building on those of Grund (1965) and Woo (1998). In addition, a further 48 species are included in the key, since these have been reported from the Pacific Northwest in a variety of publications and foray lists (Gibson 2003, Woo 1998,). Some of these have not been confirmed, a few could be erroneous, but their inclusion in the key widens the scope of possible identifications of a given collection. Several Vancouver Island collections have not been satisfactorily identified to date, and are not included in these descriptions. Such problems are common here where studies of the local fungal flora have barely begun.

Habitat specificity

Of the 60 Vancouver Island species 42 were found in the coastal Douglas-fir moist maritime biogeoclimatic zone (CDFmm), of which 26 were only found in that zone, 3 in stands which also included pines or pine-madrone, 3 in garry oak stands, and one with willows bordering a lake. Not counting the latter 7 species, there were 6 species found only in old-growth Douglas-fir forest, 13 found only in regeneration forest, and 10 species found in both ages of forest.

A total of 34 species were found in the coastal western hemlock very wet hypermaritime zone (CWHvh1), of which 18 were found only in that zone. The CWHvh1 zone has four categories in this thesis, two are age classes of western hemlock forest, a third, the spruce fringe, is a strip of forest along the seafront that is particularly rich in Sitka spruce, many of the trees are stunted and small, but the sites have not generally been logged. The seafront dunes are somewhat similar but with more shore pine, salal and kinnickkinnick and a loose, sandy substrate. The western hemlock old growth, regeneration forest, spruce fringe and dunes had, respectively, 26, 19, 17 and 8 *Russula* species, of which 3, 1, 1 and 2 were found only in that one habitat. Just 5 species were found in all CWHvh1 habitats.

Present in both zones were 16 species, which most likely have western hemlock as a mycorrhizal host and/or red alder or ericaceous plants, since these too are in both zones. These species have a tolerance of a relatively wide moisture range. Ubiquitous in all habitats except garry-oak stands were *Russula brevipes, R. brevipes* var. *acrior* and *R. fragilis.* These species have a wide distribution throughout North America and may have a range of host trees, although locally *R. fragilis* has consistently been observed with western hemlock in its vicinity, sometimes merely an understory seedling.

Although many species are occasionally found on rotten wood, simply because it is a major part of the forest floor, especially in old growth forest, 5 species are regularly if not always on rotten wood: *Russula stuntzii, R. raoultii, R. fragilis, R. fragilis* var. *mitis* and *R. silvicola. Russula stuntzii* is found on wood in an advanced stage of decay, the others can colonize wood that retains some integrity. *R. aeruginoides* was found just once, on wood, which may or may not be its required substrate. The table below summarizes the main habitats for each species.

The habitat is not useful in defining any clades, at least not with the resolution the 61 Vancouver Island species and those used in the phylogenetic analyses can provide. Of interest are the fact that 5 of the 6 species on woody debris were in clades 5a and b, all representatives of clade 1were found in regeneration forests, not old-growth, and those of clade 3 were all found less than 2km, and in most cases less than 1km from the sea shore.

Table 9 Summary of biogeoclimatic zones and habitats of Vancouver Island Russulas

	Zone	**CDFmm**				**CWHvh1 zone**				**Specific habitats**
Clade	Species	DF1	DF2	Garry oak	Other hosts	WH1	WH2	SF	D	
1a	*R. farinipes*					WH1				
1a	*R. crassotunicata*					WH1		SF	D	
1b	*R. adusta*		DF2							
1b	*R. anthracina* var. *insipida*		DF2							
1b	*R. albonigra*		DF2							
1b	*R. dissimulans*		DF2				WH2			
2b	*R. brevipes*	DF1	DF2			WH1	WH2	SF	D	
2b	*R. brevipes* var. *acrior*	DF1	DF2			WH1	WH2	SF	D	
2b	*R. cascadensis*		DF2			WH1				
3b	*R. cerolens*		DF2							
3b	*R. pectinatoides*	DF1	DF2							
3b	*R. cf.pectinata*								D	
3b	*R. granulata*	DF1								
3c	*R. fragrantissima*					WH1	WH2	SF	D	
4a	*R. smithii**					WH1				
4b	*R. mustelina*		DF2							
4b	*R. brunneola*					WH1	WH2	SF		
4d	*R. parazurea*		DF2							
4d	*R. medullata*								D	
4d	*R.* cf. *subvelispora*		DF2							
4d	***R. aeruginea***		DF2			WH1	WH2	SF	D	
5a	*R. viscida*				Madrone -pine					
5a	*R. stuntzii*	DF1	DF2			WH1				On woody debris
5b	*R. raoultii*	DF1	DF2			WH1	WH2			On woody debris
5b	*R. crenulata*			Oaks						
5b	*R. fragilis*	DF1	DF2			WH1	WH2	SF	D	On woody

	Zone	**CDFmm**				**CWHvh1 zone**				**Specific habitats**
Clade	Species	DF1	DF2	Garry oak	Other hosts	WH1	WH2	SF	D	
										debris
5b	*R. fragilis* var. *mitis*					WH1	WH2	SF		On woody debris
5b	*R. laccata*				Willow					
5b	*R. silvicola*		DF2			WH1		SF		On woody debris
5d	*R. luteotacta*						WH2			Alders
5e	*R. queletii*					WH1		SF		
5e	*R. queletii* cf. var. *flavovirens*							SF		
5e	*R. fuscorubroides*					WH1	WH2			
5e	*R. cavipes*	DF1								
6	*R. pelargonia*		DF2							
6	*R. sanguinea*				Pine		WH2			sometimes in bogs
6	*R. americana* var. *modicaspora*	DF1				WH1	WH2			wet seeps
7a	*R. lutea*			Oaks						
7a	*R. murrillii*	DF1	DF2			WH1	WH2			
7b	*R. albida*	DF1								
7b	*R. lepidiformis*			Oak?						
8a	*R. olivacea*	DF1								
8c	*R. veternosa*		DF2							
8d	*R. velenovskyi*				Madrone /pine					
8d	*R. integra* 1					WH1		SF		
8d	*R. integra* 2		DF2							
8d	*R. occidentalis*	DF1	DF2			WH1	WH2			
9a	*R. sphagnophila*					WH1		SF		
9a	*R. brunneoviolacea*		DF2							
9a	*R. abietina*	DF1	DF2							
9a	*R. aeruginoides*					WH1				On woody debris

	Zone	**CDFmm**				**CWHvh1 zone**				**Specific habitats**
Clade	Species	DF1	DF2	Garry oak	Other hosts	WH1	WH2	SF	D	
9b	*R. xerampelina*	DF1	DF2				WH2 ?			
9b	*R. semirubra*		DF2			WH1				
9b	*R. isabelliniceps*					WH1	WH2	SF		
9b	*R.* cf. *pruinosa*	DF1								
9b	*R. elaeodes*	DF1				WH1	WH2	SF		
10a	*R. aureofulva*	DF1								
10a	*R. cessans*		DF2							
10b	*R. puellaris*					WH1		SF		
10b	*R. nauseosa*						WH2	SF		

Abbreviations used in the table above: DF1= old growth Douglas-fir forest and mixed-age stands with veteran trees, DF2. = regeneration Douglas-fir forest, WH1=old growth western hemlock forest, WH2 =regeneration western hemlock forest, SF = spruce fringe, D =dunes, **Russula smithii* was not yet found on Vancouver Island.

Evaluation of the various tools of identification

The second question addressed in this thesis asks "While traditional identifications rest on morphological characters, can the RFLPs of an amplified piece of DNA, specifically the ITS region of the rDNA gene be usefully compared with virtual restrictions of published sequence data to confirm an identification, and can paper chromatography of cuticle pigments be employed in the identification of taxonomic groups?"

Both the RFLP's of the ITS region of rDNA and the chromatography methods were able to place collections into major clades, but identifying individual species requires the macroscopic and in most cases the microscopic investigation of morphology. What is useful about all these methods is that they support one another in the majority of cases, providing confirmation of the identification. Where there is ambiguity in morphological characters, such as in immature basidiomata or difficulty matching all characters to a given description, then the RFLP data or the chromatography data or both supply additional information towards a resolution. Examples from this study include an immature basidioma, in which the RFLP analysis showed a pattern closer to that of *R. xerampelina* rather than its close relative *R. isabelliniceps.*

The chromatographs showed that the new species *R. aeruginoides,* was closer to the *Sphagnophilae* in clade 9 than it was to the *Laricinae* in clade 10, because its magenta pigments were more like those of the *Xerampelinae* of clade 9. Not all the collections yielded sufficient pigment to run a chromatograph. Pale, faded specimens do not make good candidates for this analysis and it is difficult to get a clean separation of pigments in those with a very viscid cutis. Attempts to precipitate the viscid substance with alcohols failed because the pigment co-precipitates. In conclusion, neither the RFLP patterns of ITS rDNA nor the chromatographs can substitute for an examination of morphological characters; their most useful role is in pre-screening of multiple collections and support or clarification of other data.

Evaluation of morphological characters in relation to phylogenetic relationships and clade structure

The final question this thesis addresses is: "How does the suite of morphological characters used in traditional classification within the genus *Russula* relate to the clade structure within a phylogenetic tree based on DNA sequences, and can a more efficient identification key be based on those characters sorted by the taxonomic level at which each is useful?"

Morphological characters and clade structure

There are both continuous and discontinuous characters following the branching order of the phylogenetic tree. There is a general increase in spore colour, the height of spore ornamentation, and basidium width from basal to upper clades, and a decrease in the length to width ratio of spores and also in the amount of variation in this character. As long as it is borne in mind that there are variations about this trend, a set of these characters will quickly give an approximate position along the phylogenetic tree. The charts of these characters in chapter 4 are useful for this. Continuous characters are more difficult to use in a key, which necessarily breaks species down into groups, but are very useful in placing new species, especially when these have reduced epicutis characters as in *R. olivacea* group. Discontinuous characters are the ones most useful in keys, particularly for those seeking to familiarize themselves with the genus.

The keys given in Appendix 3 for Vancouver Island Russulas and those reported from the Pacific Northwest take two approaches; the polychotomous key assumes a relative unfamiliarity with the genus and divides groups of species by more obvious and initially macroscopic characters. These follow phylogenetic order in the major clade divisions and those of the finer divisions that can be unambiguously segregated by the relative novice. Other species are keyed out as groups of superficially similar species. The synoptic keys divide species up by each character individually, beginning with macroscopic characters and working down to microscopic ones. This type of key lends itself to computerized searches which tend to be more useful for those with more familiarity with the genus, since they can concentrate only on relevant characters and thereby shortcut through higher clade divisions. The synoptic keys are not sorted into a taxonomically based order in which they should be consulted; while this would be easy to do, it would make finding a given character unwieldy, and would not be relevant anyway were these keys on computer and accessed by search terms.

Microscopically the primary division is based on the amyloid reaction of the suprahilar patch on the spores, splitting clades 1, 3 and 4 from 5-10, with clade 2. The keys to the Vancouver Island Russulas (Appendix 3) use several macroscopic characters in the earlier queries which further divide the two primary clades. The type of character that defines one higher level clade differs from that which defines another. In the polychotomous keys clades 1 and 2 are defined by the gill arrangement, and distinguished from one another by bruising reactions. Clade 3 is defined by a number of characters including the appearance of the cap and the odour. Clade 4 is defined mostly by microscopic characters (e.g. spores, epicutis) but also a particular stature and feel, which is easier to demonstrate by touch than it is to put into words or photographs. This clade also differs in cutis pigments from similarly coloured species in higher clades. Clades 5 – 10 can be defined macroscopically by cutis pigments, and separated by a combination of spore colour, taste, bruising reactions, pileocystidia and/or primordial hyphae and the tendency or otherwise of the cap margin to become striate. Clade 9b is defined by its reaction with $FeSO_4$ and cuticular structure). Cap colour is useful but plays a much less important role in *Russula* taxonomy than it does in many other agaric genera, yet it is often the first thing people look for in a key.

Taxonomic observations and recommendations

Amongst the microscopic characters the epicutis structures and their chemical reactions have been used taxonomically for at least 40 years to group species, and remain among the primary tools for this. Primordial hyphae, which were considered to be confined to mild tasting species in clades 7 and 8, also occurred in peppery species, and in clades 9 and 10 stuctures appearing similar to non-incrusted primordial hyphae were occasionally seen. The colour, shape and ornamentation height of spores, and particularly the width of mature basidia show continua from basal to upper clades that allow a fairly accurate placement of query species within the phylogeny. However the basidium length is less important than has been previously assumed, save that clades 5 and 6 have generally the shortest basidia.

Within clades the basidial maximum length may be important, but this requires further examination with a larger sample size. Spore ornamentation, particularly the degree of reticulation, and again, spore colour are useful in conjunction with macroscopic characters.

In view of the fact that a relatively few species of *Russula*, taken mostly from the northern temperate zones of Europe and North America were phylogenetically analyzed, it would not be prudent to make major taxonomic revisions until a wider sampling of Russulas has been similarly analyzed, both through DNA and morphological studies. Having said that, all the data in this study point to narrowing the scope of subgenus *Russula* sensu Sarnari (1998) to include only section *Russula* subsections *Consobrinae* Sarnari, *Russula* (Romagn.) Sarnari, and *Sardoninae* (Singer) Sarnari.

The subgenus *Incrustatae* (Romagn.) Sarnari might then be expanded (and perhaps renamed) to house those species that have incrusted or none-incrusted primordial hyphae and broad basidia. Since clades 7-10 do not show high level phylogenetic divisions, this would entail their inclusion in the expanded subgenus. Ostensibly subgenus *Incrustatae* amended would include sections *Messapica* Sarnari, *Paraincrustatae* Sarnari, *Polychromae* (Maire) Sarnari (except for subsection *Auratinae* Bon), section *Tenellae,* and subsections *Rubrinae* (Melzer and Zvara) Singer and *Urentes* Maire; all currently in subgenus *Russula.* Those groups represented by species forming clade 6 should undergo further study to verify and understand their position.

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Appendix 1

Table 11 Excerpt 1 from Gluchoff 1969; Etude chimiotaxinomique des pigments des Russules: Propriétés spectrales et chromatographiques des Pigments de Russules

The first part of Table II dealing with the visible and UV colours:

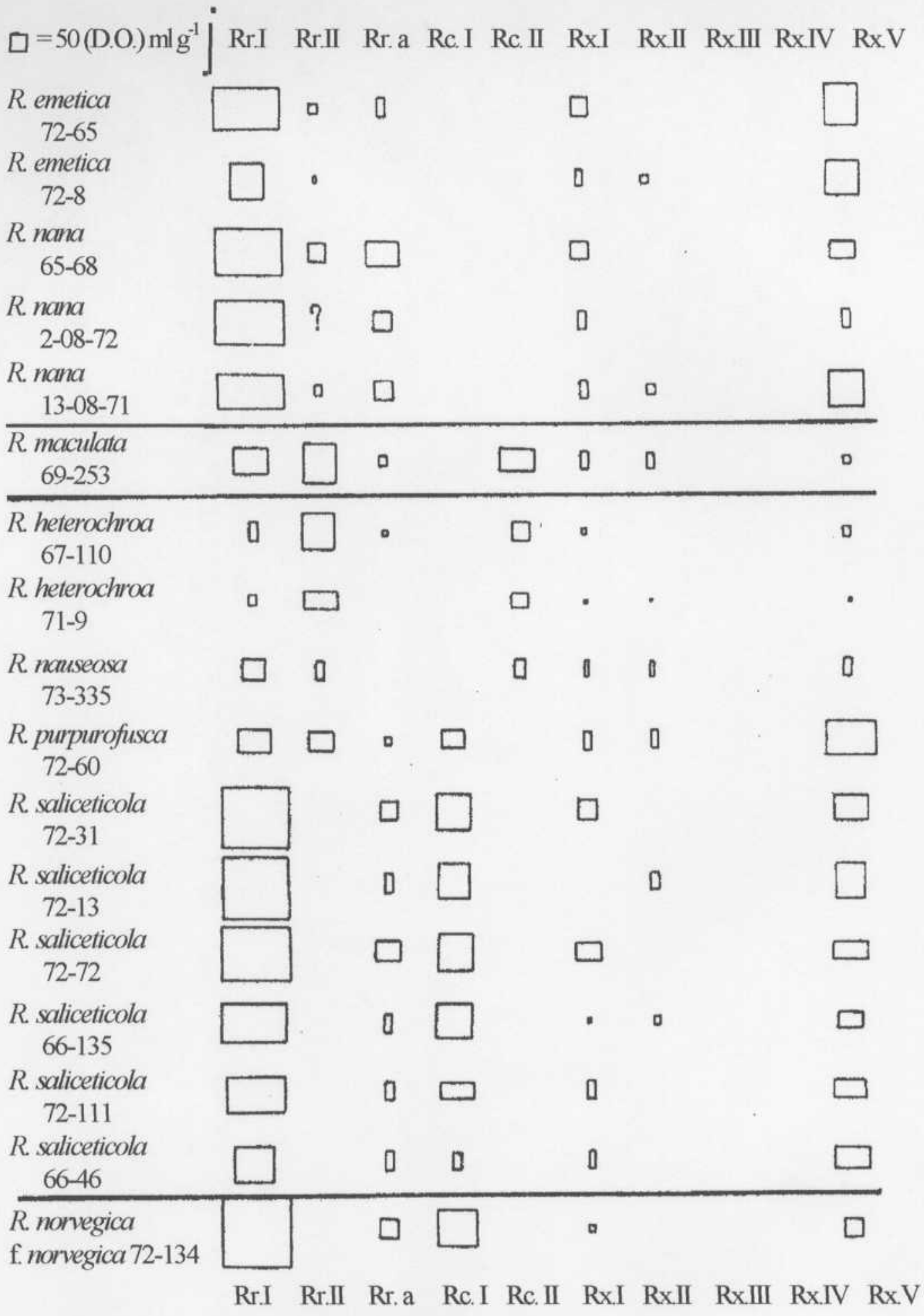
COMPOSES	COULEUR		
	visible	UV	+NH_4OH
Russularhodines			
Rr I	rose-orangé	orangé	rose-violacé (vis.)
Rr.II	rose-violacé	orangé	
Rr.a	rose-orangé	orangé	
Rr. *d'Heterophyllae*	rose-maUVe	orangé	
Russulacyanines			
Rc.I	bleu-vert	rien	rien
Rc.II	bleu	rien	rien
Rc. *d'Heterophyllae*	bleu-violet	orangé?	
Russulaxanthines			
Rx.I ("aurataxanthine")	jaune-or	ocre	
Rx.II	jaune	jaune-citrin	jaune+ pale
Rx.III	jaune-citrin	jaune-vert	bleu-vert (UV)
Rx.IV	jaune-citrin	jaune-vert	bleu-vert (UV)
Rx.V	jaune-citrin	jaune-vert	J.+pâle(vis.)verdâtre(UV)
Rx.VI	jaune	jaune-vert	
Rx. *d'Heterophyllae*	jaune	jaune-or	

Table 12 Excerpt 2 from Gluchoff 1969; Etude chimiotaxinomique des pigments des Russules.

	Rr.I	Rr.II	Rr.a	Rc.I	Rc.II	Rx.I	Rx.II	Rx.III	Rx.IV	Rx.V	Rx.VI	V.I.
PIPERINAE												
Lepidinae												
R. lepida	++	(+)	(+)	?	-	+	?	-	-	(+)UV	-	++
Emeticinae												
R. emetica (gp.)	++	(+)UV	?	-	-	(+)	+UV	-	-	+	-	++
R. Mairei	+++	(+)	(+)	-	-		++UV	-	-	(+)	-	++
Persicinae												
R. luteotacta	++	(+)	(+)	-	-	+UV		-	-	++UV	-	++
Atropurpurinae												
R. Atropurpurea	(+)	++	?	-	+	+UV	(+)UV	-	-	(+)UV	-	+++
R. fragilis	+	++UV		(+)	-	(+)UV	(+)UV	-	-	(+)	-	+++
Sardoninae												
R. sanguinea	+	(+)UV	(+)	(+)	-	+UV	(+)UV	-	-	(+)	-	+++
R. sardonia	+	+	(+)	+	(+)	(+)UV	(+)UV	-	-	(+)	-	+++
R. cavipes	+	(+)UV		+	-		(+)UV	-	-	+	-	+++
R. queletii	++	?	(+)	+	(+)	?	(+)UV	-	-	++	-	+++
R. badia	++	+	(+)	+	(+)	(+)UV	(+)UV	-	-	++	-	+++
INCRUSTATAE												
Roseinae												
R. rosea	++	?	(+)UV	-	(+)	?	+UV	-	-	++	-	+++
R. minutula	++			-	-		+UV	-	-	++UV	-	+
R. pseudointegra	+++	?	(+)UV	-	-	?	(+)UV	-	-	(+)UV	-	+
Lilacinae												
R. lilacea	+	?		+	?		(+)UV	-	-	+	-	++
Amethystinae												
R. turci	++	(+)UV	(+)UV	+	(+)	+UV	(+)UV	-	-	++UV	-	+++
Chamaeleontinae												

	Rr.I	Rr.II	Rr.a	Rc.I	Rc.II	Rx.I	Rx.II	Rx.III	Rx.IV	Rx.V	Rx.VI	V.I.
R. chamaeleontina	(+)UV	(+)		-	-	(+)		++	+	+UV	-	+
R. vitellina	-	-		-	-	(+)		+	?	(+)UV	-	+
TENELLAE												
Sphagnophilae												
R. brunneoviolacea	+++	(+)UV	(+)	+	+		(+)UV	-	-	(+)	-	+++
POLYCHROMAE												
Melliolentinae												
R. melliolens	(+)	(+)		-	(+)		(+)UV	–	-	+	-	++
Viridantinae												
R. erythropoda	+++	(+)UV	(+)	+	(+)		(+)UV	-	-	(+)	-	+++
Integroidinae												
R. claroflava	-	(+)	-	-	-	(+)		++	+	+UV	-	+
Integrinae												
R. integra	+	(+)UV		+	(+)	(+)UV	(+)UV	-	-	(+)	-	+++
COCCINAE												
Decolorantinae												
R. decolorans	(+)	(+)UV		(+)	-	(+)	(+)	-	-	++	-	++
Paludosinae												
R. paludosa	++	(+)		(+)	?	?	(+)UV	-	-	+	-	+++
Laetinae												
R. aurata	+	(+)UV	?	-	(+)	++	+	-	-	++	-	++
INSIDIOSAE												
Urentinae												
R. adulterina	++	(+)UV		+	-	(+)UV	(+)UV	-	-	+	-	+++
INGRATAE												
Felleinae												
R. fellea	-	-		-	-	-		-	-	-	-	-
R. consobrina	-	(+)UV		-	-	-		-	-	-	-	-
R. ochroleuca	-	-		-	-	?		+++	+	+UV	-	++

	Rr.I	Rr.II	Rr.a	Rc.I	Rc.II	Rx.I	Rx.II	Rx.III	Rx.IV	Rx.V	Rx.VI	V.I.
Foetentinae												
R. pectinatoides	-	-		-	-	-		-	-	-	-	-
R. laurocerasi	-	-		-	-	-		-	-	-	-	-
HETEROPHYLLAE												
Virescentinae												
R. amoena	(+)	++		-	+	-	(+)UV	-	-	(+)UV	-	++
R. violeipes	-	(+)UV		-	(+)	-	+UV	-	-	?	-	+++
Indolentinae												
R. cyanoxantha	-	-		-	-	-		-	-	-	-	(+)
Griseinae												
R. parazurea	-	-		-	-	-		-	-		+	?
Heterophyllinae												
R. vesca	-	-		-	-	-		-	-		+	?



Russules alpines: contenu pigmentaire de la cuticule piléique.

Rr.I, II, a: russularhodines I, II, a.

Rc.I, II: russulacyanines I, II.

Rx.I, II, III, IV, V: russulaxanthines I, II, III, IV, V.

Figure 27 Pigment analysis of cuticles of alpine Russulas, reproduced from Gluchoff 1975.

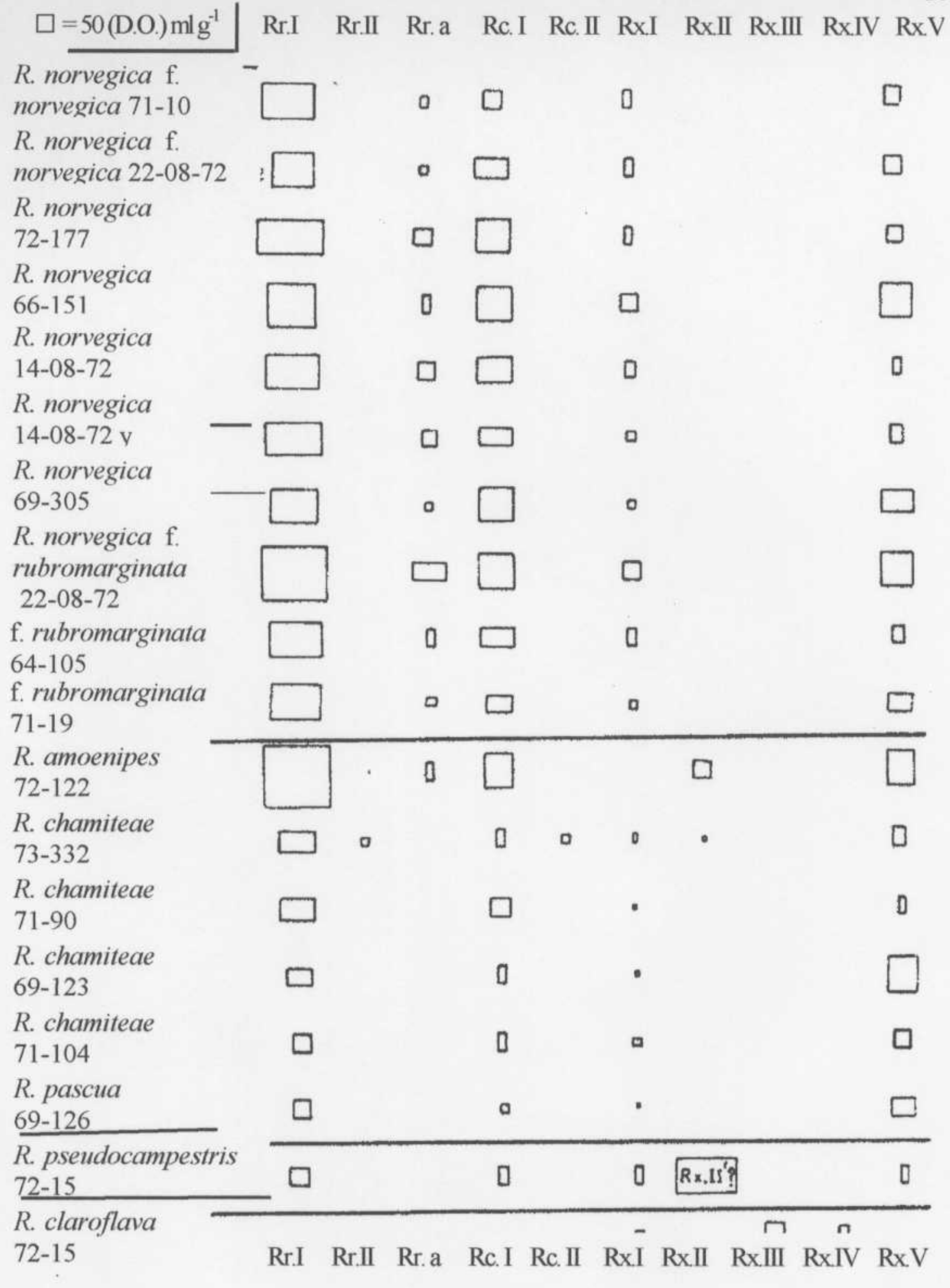


FIG. 2.

Figure 28 Pigment analysis of cuticles of alpine *Russulas,* reproduced from Gluchoff 1975.

Appendix 2

Table 13 Sequences downloaded from GenBank and EMBL-EBI and adapted for phylogenetic and RFLP analyses.

FP = final phylogenetic analyses, P = used in earlier phylogenetic analyses but omitted from the final tree, R = RFLP analyses. The remainder of the sequences not used for these analyses were used in initial alignments and as supplemental data.

Species	Accession number	Author	Origin	Analyses	
Russula adulterina	AY061651	Miller, S.L. and Buyck, B.	Europe	FP	
R. adusta	AY061652	Miller, S.L. and Buyck, B.	Europe	FP	R
R. aeruginea	AF418612	Eberhardt,U.	Europe	FP	
R. aeruginea 2	DQ421999	Eberhardt,U.	Europe		R
R. alboareolata	AF345247	Teaumroong, N., Manassila, M., Boonkerd, N. and Rodtong, S.	Thailand	FP	
R. albonigra	DQ422029	Eberhardt,U.	Europe		R
R. amethystina	AF418640	Eberhardt, U.	Europe	FP	R
R. amethystina 2	AY061653	Miller, S.L. and Buyck, B.	Europe	FP	
R. amoenicolor	AY061655	Miller, S.L. and Buyck, B.	Europe	FP	
R. amoenipes	AY061656	Miller, S.L. and Buyck, B.	Europe	FP	
R. amoenolens	AF418615	Eberhardt, U.	Europe	FP	R
R. amoenolens 2	DQ822824	Peay, K.G., Bruns, T.D., Kennedy, P.G., Bergemann, S.E. and Garbelotto, M.	U.S.A. (CA)		R
R. aquosa	AY061657	Miller, S.L. and Buyck, B.	Europe	FP	
R. archaea	AY061737	Miller, S.L. and Buyck, B.	Africa	FP	
R. atropurpurea	AF418618	Eberhardt, U.	Europe	FP	
R. atropurpurea 2	AY061654	Miller, S.L. and Buyck, B.	U.S.A.	P	
R. aurantiaca	AY061658	Miller, S.L. and Buyck, B.	Europe	FP	
R. aurantiaca 2	AF506427	Larsson,E. and Larsson, K.-H.	Sweden	P	
R. aurata	AY061659	Miller, S.L. and Buyck, B.	Europe	FP	
R. azurea	AY061660	Miller, S.L. and Buyck, B.	Europe	FP	

Species	Accession number	Author	Origin	Analyses	
R. betularum	AY061729	Miller, S.L. and Buyck, B.	Europe	P	R
R. betularum ITS2	AJ534937	Tedersoo, L., Hallenberg, N., Larsson, K.H. and Koljalg, U.	Europe (Estonia)	FP	R
R. bicolor	AY750161	Cline, E.	U.S.A. (WA)	FP	
R. brevipes	AF349714	Bidartondo, M.I. and Bruns, T.D.	U.S.A.	FP	
R. brevipes ITS 2	DQ367912	Durall, D.M., Gamiet, S., Simard, S.W., Kudrna, L. and Sakakibara, S.M.	Canada (B.C.)		R
R. brevipes var. *acrior*	EF411133	Morris, M.H., Smith, M.E., Rizzo, D.M., Rejmanek, M. and Bledsoe, C.S.	U.S.A. (CA)		R
R. brunneoviolacea ITS1	AM113956	Kjoller, R.	Europe (Denmark)		R
R. caerulea 1	AF418633	Eberhardt, U.	Europe	FP	
R. caerulea 2	AY061661	Miller, S.L. and Buyck, B	Europe	P	
R. californiensis	AY245542	Davis, R.M. and Wolfe,C.R.	U.S.A. (CA)	FP	
R. camarophylla	AY061662	Miller, S.L. and Buyck, B.	Europe	FP	
R. cavipes	AF418623	Eberhardt, U.	Europe	FP	R
R. cessans	AY061730	Miller, S.L. and Buyck, B.	U.S.A.	FP	R
R. chloroides	AY061663	Miller, S.L. and Buyck, B	Europe	FP	R
R. chloroides 2	AF418604	Eberhardt, U.	Europe	P	
R. claroflava	AY061665	Miller, S.L. and Buyck, B.	Europe	FP	
R. consobrina	AY061666	Miller, S.L. and Buyck, B.	Europe	FP	
R. crassotunicata	EU057119	Wright, S.H.A., Carruthers, E., Lim, S. and Berbee, M.L.	Canada (B.C.)		R
R. cf. *crassotunicata*	DQ384580	Berbee, M.L., Wright, S.H.A., Sihota, N.J.J. and Lim, S.	Canada (B.C.)		R
R. cremoricolor 1	AJ277910	Redecker, D., Szaro, T.M., Bowman, R. and Bruns, T.D.	U.S.A. (CA)	FP	
R. cremoricolor 2	AJ277911	Redecker, D., Szaro, T.M., Bowman, R. and Bruns, T.D.	U.S.A. (CA)	P	
R. cuprea	AY061667	Miller, S.L. and Buyck, B.	Europe	FP	

Species	Accession number	Author	Origin	Analyses
R. curtipes	AY061668	Miller, S.L. and Buyck, B.	Europe	FP
R. cyanoxantha	AF418608	Eberhardt, U	Europe	P
R. cyanoxantha 2	AY061669	Miller, S.L. and Buyck, B.	Europe	P
R. cyanoxantha 3	AF291361	Weiss, M. and Oberwinkler, F.	Germany	P
R. cyanoxantha 4	AF345251	Teaumroong, N., Manassila, M., Boonkerd, N. and Rodtong, S.	Thailand	FP
R. cyanoxantha 5	DQ422033	Eberhardt, U.	Europe	R
R. decolorans	AY061670	Miller, S.L. and Buyck, B.	Europe	P
R. decolorans 2	AF418637	Eberhardt, U.	Europe	FP
R. decolorans 3	AY194601	Fransson, P.	Europe (Sweden)	P
R. decolorans 4	DQ367913	Durall, D.M., Gamiet, S., Simard, S.W., Kudrna, L. and Sakakibara, S.M.	Canada (B.C.)	R
R. delica 1	AF418605	Eberhardt, U.	Europe	FP
R. delica 2	AF096987	Marin, M., Ibarra, M., Garcia,L. and Ferrer, S.	Europe (Spain)	P
R. delica 3	AF345250	Teaumroong, N., Manassila, M., Boonkerd, N. and Rodtong, S.	Thailand	P
R. delica 4	AY061671	Miller, S.L. and Buyck, B.	Europe	P
R. aff. *delica*	DQ422005	Eberhardt, U.	Europe	R
R. densifolia	AF418606	Eberhardt, U.	Europe	FP
R. drimeia	AY061672	Miller, S.L. and Buyck, B.	Europe	FP
R. emetica	DQ421997	Eberhardt, U.	Europe	R
R. emetica 1	AY061673	Miller, S.L. and Buyck, B.	Europe	FP
R. emetica 2	AF418619	Eberhardt, U.	Europe	P
R. cf. emetica 3	AY228350	Sheldrake, M., Berbee, M.L., Inderbitzin, P. and Fischer, A.L.	Canada (B.C.)	P
R. cf. emetica 4	AY228360	Tso, A., Berbee, M.L., Inderbitzin, P. and Fischer, A.L.	Canada (B.C.)	P
R. exalbicans	AY293156	Binder, M., Hibbett, D.S., Larsson, K.-H., Larsson,E., Langer, E. and Langer, G.	U.S.A.	P

Species	Accession number	Author	Origin	Analyses	
R. exalbicans 2	AY061674	Miller, S.L. and Buyck, B.	Europe	FP	
R. farinipes	AY061675	Miller, S.L. and Buyck, B.	Europe	FP	
R. farinipes 2	DQ421983	Eberhardt, U.	Europe		R
R. fellea	AF418616	Eberhardt, U.	Europe	FP	
R. fellea 2	AY061676	Miller, S.L. and Buyck, B.	Europe	P	
R. firmula	AF418631	Eberhardt, U.	Europe	FP	
R. firmula 2	DQ422017	Eberhardt, U.	Europe	R	
R. foetens	AY061677	Miller, S.L. and Buyck, B	Europe	FP	
R. foetens 2	AF418613	Eberhardt, U.	Europe	P	
R. foetens 3	AF230895	Calonge, F.D. and Martin, M.P.	Europe (Spain)	P	
R.* cf. *foetens	DQ422023	Eberhardt, U.	Europe		R
R. fragilis	AF230897	Calonge, F.D. and Martin, M.P.	Europe (Spain)	FP	
R. fragilis 2	DQ367914	Durall, D.M., Gamiet, S., Simard, S.W., Kudrna, L. and Sakakibara, S.M.	B.C., Canada		R
R. aff. *fragilis*	AF335443	Berbee, M.L., Inderbitzin, P. and Zhang, G	Canada (B.C.)	P	R
R. fuscorubroides	AF418624	Eberhardt, U.	Europe	FP	
R. gracillima	AY061678	Miller, S.L. and Buyck, B.	Europe	FP	R
R. gracillima 2	DQ422004	Eberhardt,U.	Europe		R
R. grisea	AY061679	Miller, S.L. and Buyck, B.	Europe	FP	
R. grisea 2	DQ422030	Eberhardt, U.	Europe		R
R. helodes	AY061680	Miller, S.L. and Buyck, B.	Europe	P	
R. heterophylla	AF418609	Eberhardt, U	Europe	FP	
R. heterophylla 2	AY061681	Miller, S.L. and Buyck, B.	Europe	P	
R. heterophylla 3	DQ422006	Eberhardt, U.	Europe		R
R. ilicis	AY061682	Miller, S.L. and Buyck, B.	Europe	FP	
R. insignis	AY061700	Miller, S.L. and Buyck, B.	Europe	FP	
R. integra	AF230896	Martin, M.P. and Calonge, F.D.	Europe	FP	
R. integra 2	AF418636	Eberhardt, U.	Europe	P	

Species	Accession number	Author	Origin	Analyses	
R. integra 3	AY061683	Miller, S.L. and Buyck, B.	Europe	P	R
R. integriformis	AY061684	Miller, S.L. and Buyck, B.	Europe	FP	
R. laricina	AY061685	Miller, S.L. and Buyck, B.	Europe	FP	
R. laurocerasi 1	AY061735	Miller, S.L. and Buyck, B.	U.S.A.	FP	R
R. laurocerasi 2	AF418614	Eberhardt, U.	Europe	P	R
R. lepida	AF418641	Eberhardt, U.	Europe	P	
R. lepida 2	AY061686	Miller, S.L. and Buyck, B.	Europe	FP	
R. lepida 3	DQ422013	Eberhardt, U.	Europe		R
R. lepidicolor	AY061687	Miller, S.L. and Buyck, B.	Europe	FP	
R. lilacea	AY061731	Miller, S.L. and Buyck, B.	U.S.A.	FP	
R. littoralis	AY061702	Miller, S.L. and Buyck, B.	Europe	FP	
R. maculata	AY061688	Miller, S.L. and Buyck, B.	Europe	FP	
R. cf. maculata	DQ422015	Eberhardt, U.	Europe		R
R. mairei 1	AF418620	Eberhardt, U.	Europe	FP	
R. mairei 2	AF230899	Calonge, F.D. and Martin, M.P.	Europe (Spain)	P	
R. melitodes	AY061689	Miller, S.L. and Buyck, B.	Europe	FP	
R. melliolens	AY061690	Miller, S.L. and Buyck, B.	U.S.A.	FP	
R. melzeri	AY061691	Miller, S.L. and Buyck, B.	Europe	FP	
R. messapica	AY061692	Miller, S.L. and Buyck, B.	Europe	FP	
R. mustelina	AY061693	Miller, S.L. and Buyck, B.	Europe	FP	R
R. mustelina 28S	AY606967	Eberhardt, U. and Verbeken, A.	Europe		R
R. nana	AY061694	Miller, S.L. and Buyck, B.	Europe	FP	
R. nauseosa	AY061733	Miller, S.L. and Buyck, B.	U.S.A.	FP	R
R. nauseosa ITS2	AF506462	Larsson, E. and Larsson, K.-H	Europe (Sweden)		R
R. nigricans	AF418607	Eberhardt, U	Europe	FP	
R. nigricans 2	AY061695	Miller, S.L. and Buyck, B.	Europe	P	
R. nigricans 3 (partial ITS1)	AY228357	Gendron, R., Berbee, M.L., Inderbitzin, P. and Fischer, A.L.	Canada (B.C.)	P	
R. nigricans 4	DQ422010	Eberhardt, U.	Europe		R

Species	Accession number	Author	Origin	Analyses	
R. nigricans 5	DQ367915	Durall, D.M., Gamiet, S., Simard, S.W., Kudrna, L. and Sakakibara, S.M.	B.C., Canada		R
R. nitida	AY061696	Miller, S.L. and Buyck, B.	Europe	FP	
R. occidentalis ITSI and partial ITS2	AY534206	Horton, T.R., Molina, R. and Hood, K	U.S.A. (OR)	P	R
R. occidentalis ITS2	AY228349	Karst, J., Berbee, M.L., Inderbitzin, P. and Fischer, A.L.	Canada (B.C.)	FP	
R. ochroleuca	AY061697	Miller, S.L. and Buyck, B	Europe	FP	R
R. ochroleuca 2	AF418617	Eberhardt, U	Europe	P	
R. ochroleuca 28S	AF325313	Eberhardt, U.	Europe		R
R. ochroleuca ITS2	AY254880	Smit, E., Veenman, C. and Baar, J.	Europe (Holland)	P	R
R. odorata	AY061698	Miller, S.L. and Buyck, B.	Europe	FP	
R. olivacea 1	AF418635	Eberhardt, U.	Europe	FP	
R. olivacea 2	AF418634	Eberhardt, U.	Europe	FP	R
R. olivacea 3	AY061699	Miller, S.L. and Buyck, B.	Europe	P	R
R. pallescens	DQ421987	Eberhardt, U.	Europe		R
R. pallidospora	AY061701	Miller, S.L. and Buyck, B.	Europe	FP	
R. pallidospora	DQ422032	Eberhardt, U.	Europe		R
R. paludosa	AY061703	Miller, S.L. and Buyck, B.	Europe	FP	
R. parazurea	DQ422007	Eberhardt, U.	Europe		R
R. parazurea 2	AY061704	Miller, S.L. and Buyck, B.	Europe	FP	
R. parazurea 3	AF418611	Eberhardt, U.	Europe	P	
R. pascua	AY061705	Miller, S.L. and Buyck, B.	Europe	FP	
R. pectinata	AY061706	Miller, S.L. and Buyck, B.	Europe	FP	R
R. pectinatoides	AY061732	Miller, S.L. and Buyck, B.	U.S.A.	FP	
R. pectinatoides 2	DQ422026	Eberhardt, U.	Europe		R
R. persicina	AF506463	Larsson, E. and Larsson, K.-H.	Sweden	P	
R. persicina 2	AY061707	Miller, S.L. and Buyck, B.	Europe	FP	
R. persicina 3	DQ422019	Eberhardt, U.	Europe		R

Species	Accession number	Author	Origin	Analyses	
R. persicina ITS2	AF506463	Larsson, E. and Larsson, K.-H.	Europe		R
R. postiana	AF230898	Calonge, F.D. and Martin, M.P.	Europe (Spain)	FP	
R. pseudointegra	AY061708	Miller, S.L. and Buyck, B.	Europe	FP	
R. puellaris	AF418628	Eberhardt, U.	Europe	FP	
R. puellaris 2	AY061709	Miller, S.L. and Buyck, B.	Europe	P	R
R. puellula	AY061710	Miller, S.L. and Buyck, B.	Europe	FP	
R. pulverulenta	AY061736	Miller, S.L. and Buyck, B.	U.S.A.	FP	
R. queletii	AF418625	Eberhardt, U	Europe	FP	
R. queletii 2	AY061711	Miller, S.L. and Buyck, B.	Europe	P	
R. raoultii	AF418621	Eberhardt, U	Europe	FP	
R. raoultii 2	AY061712	Miller, S.L. and Buyck, B.	U.S.A.	P	R
R. risigallina	AY061713	Miller, S.L. and Buyck, B.	Europe	FP	
R. romellii	AY061714	Miller, S.L. and Buyck, B.	Europe	FP	
R. rosacea	AF096978	Marin, M., Ibarra, M., Garcia, L. and Ferrer, S.	Europe (Spain)	P	
R. rosacea 2	AF345249	Teaumroong, N., Manassila, M., Boonkerd, N. and Rodtong, S.	Thailand	FP	
R. rosea	AY061715	Miller, S.L. and Buyck, B.	Europe	FP	
R. roseipes	AY061716	Miller, S.L. and Buyck, B.	Europe	FP	
R. rubra	AY061717	Miller, S.L. and Buyck, B.	Europe	FP	
R. sanguinea	AY061718	Miller, S.L. and Buyck, B.	Europe	P	R
R. sardonia	AF418626	Eberhardt, U.	Europe	FP	
R. solaris	AF418627	Eberhardt, U.	Europe	FP	
R. sphagnophila	AY061719	Miller, S.L. and Buyck, B.	Europe	FP	R
R. sphagnophila ITS2	AF506464	Larsson,E. and Larsson, K.-H.	Europe (Sweden)	P	R
R. stuntzii	AY281091	Davis, R.M.	U.S.A. (CA)	FP	
R. turci	AY061720	Miller, S.L. and Buyck, B.	Europe	FP	R
R. turci 2	EF530935	Denis, M.W., Carruthers, E.K., Wright, S.H.A. and Berbee, M.L.	Canada (B.C.)		R

Species	Accession number	Author	Origin	Analyses	
R. velenovskyi	AY061721	Miller, S.L. and Buyck, B.	Europe	FP	
R. velenovskyi	AY061721	Miller, S.L. and Buyck, B.	Europe		R
R. velenovskyi ITS2	AJ966748	Tedersoo, L.; Suvi, T.; Larsson, E.; Koljalg, U.	Europe (Estonia)		R
R. versicolor	AY061722	Miller, S.L. and Buyck, B.	Europe	FP	
R. vesca	AF418610	Eberhardt, U.	Europe	FP	
R. vesca 2	AY061723	Miller, S.L. and Buyck, B.	Europe	P	
R. vesca 3	DQ422018	Eberhardt, U.	Europe		R
R. veternosa	AF418630	Eberhardt, U.	Europe	FP	R
R. vinosa	AF418638	Eberhardt, U	Europe (Estonia)	FP	
R. vinosa 2	AY061724	Miller, S.L. and Buyck, B.	Europe	P	R
R. vinosa ITS1	AJ534938	Tedersoo L., Hallenberg N., Larsson K.H., Koljalg U.	Europe (Estonia)		R
R. violacea	AY061725	Miller, S.L. and Buyck, B.	Europe	FP	R
R. violacea ITS2	AF506465	Larsson, E. and Larsson, K.-H.	Europe (Sweden) ITS2 only	P	R
R. violeipes	AY061726	Miller, S.L. and Buyck, B.	Europe	FP	
R. virescens	AY061727	Miller, S.L. and Buyck, B.	Europe	FP	
R. virescens 2	DQ422014	Eberhardt, U.	Europe		R
R. viscida	AY061728	Miller, S.L. and Buyck, B.	Europe	FP	
R. xerampelina 1	AF418632	Eberhardt, U.	Europe	FP	
R. xerampelina 2	AY061734	Miller, S.L. and Buyck, B.	Europe	P	
R. xerampelina 28S	AY534210	Horton, T.R., Molina, R. and Hood, K.	U.S.A. (OR)	P	
R. xerampelina 4	AF540385	Davis, R.M.	U.S.A. (CA)	FP	
R. xerampelina 5	DQ367916	Durall, D.M., Gamiet, S., Simard, S.W., Kudrna, L. and Sakakibara, S.M.	Canada, (B.C.)		R
R. xerampelina ITS2	AY228344	Fischer, A., Berbee, M.L., Inderbitzin, P. and Fischer, A.L.	Canada, (B.C.)	P	R

Species	Accession number	Author	Origin	Analyses
Albatrellus flettii	AY061738	Miller, S.L. and Buyck, B.	U.S.A.	FP
Gloeocystidiellum aculeatum	AY061739	Miller, S.L. and Buyck, B.	China	FP
Gymnomyces gilkeyae	AY239346	Whitbeck, K.L., Castellano, M.A. and Spatafora, J.W.	U.S.A. ?	FP
Martellia pila	AF230894	Calonge,F.D. and Martin, M.P.	Europe (Spain)	R

Table 14 *Russula* collections used for RFLP analyses in chapter 2 part I, in the order in which they appear in figures 12 and 13. Full descriptions of the species together with information on the origin and habitat of each collection appear in appendix 3, (except *R.* cf. *basifurcata* and *Macowanites* sp.).

Taxonomic group	**Species**	**Collection number**
Sg. *Compacta* S. *Compactae*	*R. albonigra*	BK010904-01
	R. dissimulans	CR010814-04
	R. dissimulans	SVIMS021027-RN
Sg. *Compacta* S. *Lactaroides*	*R. brevipes*	CR001105-br/a
	R. brevipes	CR001001-05
	R. cascadensis	CR001007-cas
Sg. *Ingratula* S. *Ingratae* Ss.*Foetentinae* s. *Pectinata*	*R. cerolens*	CR001121-01
	R. cerolens	CR021219-01
	R. cerolens	CR001007-01
	R. cf. *pectinata*	CR021016-14
	R. pectinatoides	CR001002-02
	R. granulata	CR001108-01
Sg. *Ingratula* S. *Ingratae* Ss.*Foetentinae* s. *Foetens*	*R. fragrantissima*	CR030927-01
	R. fragrantissima	CR030924-01
	R. fragrantissima	CR010814-05
Sg. *Ingratula* S. *Ingratae* Ss.*Farinipedes*	*R. farinipes*	SVIMS021020-02
Sg. *Heterophyllidia* S. *Heterophyllae* Ss. *Heterophyllae*	*R. mustelina*	OC020717-01
	R. mustelina	OC020717-01 (repeat)
	R. cf. *basifurcata*	CR001011-57
	R. brunneola	PJ010919-04 W
	R. brunneola	CR030924-02 PR
Sg. *Heterophyllidia* S. *Heterophyllae* Ss. *Griseinae*	*R. medullata*	CR021016-21
	R. aeruginea	CR021016-22
	R. cf. *sublevispora*	CR010909-03

Sg. *Ingratula* Ss. *Crassotunicatinae*	*R. crassotunicata*	CR011112-LB.RF.
	R. crassotunicata	CR030924-06
Sg. *Russula* S. *Russula* Ss. *Russula* s. *Atropurpurea*	*R. crenulata*	CR030304-01
	R. stuntzii	CR001024-01
	R. stuntzii	BT021101-01
	R. viscida	CR981114-06
	R. raoultii	CR021118-02
	R. raoultii	CR001127-04
	R. raoultii	CR001108-05
	R. fragilis	CR980825-01
	R. fragilis	CR981014-02
	R. fragilis	CR031026-01
	R. fragilis	CR031130-01
	R. fragilis	CR030924-03
	R. fragilis	CR021015-09
	R. laccata	OC030526-01
	R. silvicola	CR021016-10
	R. silvicola	CR021016-10 repeat
	R. silvicola	CR981202-05
	R. silvicola	CR001011-53a
	R. silvicola	CR981014-01
Sg. *Russula* S. *Russula* Ss. *Sardoninae* s. *Sardonia*	*R. fuscorubroides*	CR981013-07
	R. fuscorubroides	CR000919-02
	R. fuscorubroides	CR001012-23
	R. fuscorubroides	CR021015-21b
	R. queletii	CR981029-01b
	R. queletii	CR010919-05
	R. queletii	CR010919-05 repeat
	R. queletii	CR020927-04
	R. queletii cf. var. *flavovirans*	CR981029-02
	R. cavipes	CR021201-01

Sg. *Russula* S. *Russula* Ss. *Violaceinae*	*R. pelargonia*	CR981003-02
Sg. *Russula* S. *Russula* Ss. *Sardoninae* s. *Sanguinea*	*R. americana* var. *modicaspora*	CR981118-01
	R. americana var. *modicaspora*	CR001012-21
	R. sanguinaria	PJ981124-03
	R. sanguinaria	CR030110-01
Sg. *Russula* S. *Paraincrustatae* Ss. *Lepidinae*	*R. albida*	CR010516-01
Sg. *Incrustatula* S. *Amethystinae* Ss. *Amethystinae*	*R. murrillii*	CR001104-02
	R. murrillii	CR021015-13
	R. murrillii	CR011111-02
Sg. *Incrustatula* S. *Amethystinae* Ss. *Olivaceinae*	*R. olivacea*	CR021019-01
Sg. *Russula* S. *Russula* Ss. *Urentes*	*R. veternosa*	CR001029-Breit
	R. veternosa	CR001108-03
	R. veternosa	CR010909-01
Sg. *Russula* S. *Russula* Ss. *Rubrinae*	*R.* cf. *rubra*	CR001202-04
Sg. *Russula* S. *Paraincrustatae* Ss. *Integrae*	*R.* cf. *integra*	CR021027-04
	R. cf. *integra*	CR021028-01
Sg. *Incrustatula* S. *Amethystinae* Ss. *Integroidinae*	*R. occidentalis*	JJ021020-03
Sg. *Russula* S. *Tenellae* Ss. *Sphagnophilae*	*R.* cf. *brunneoviolacea*	CR981114-01
	R. sphagnophila	CR011030-03
	R. abietina	CR001104-01
	R. abietina	DG010902-01

Sg. *Russula* S. *Polychromae* Ss. *Xerampelinae*	*R. xerampelina*	CR001028 Breit
	R. xerampelina	CR001001-02
	R. xerampelina	CR001127-01
	R. xerampelina	CR001202-02
	R. xerampelina	CR010814-03
	*R. xer.*cf. *pruinosa*	PJ010922-02
	R. elaeodes	PJ010919-PJ
	R. elaeodes	CR001011-55
	R. isabelliniceps	CR000920-04
	R. isabelliniceps	PK021112-01
	R. isabelliniceps	JD021027-02
	R. isabelliniceps	CR030924-08
	R. isabelliniceps	CR030924-05
	R. semirubra	CR001007-02
Sg. *Russula* S. *Tenellae* Ss. *Laricinae*	*R. cessans*	CR981120-01
	R. cf. *cessans*	CR011020-03
	R. Macowanites sp.	CR030924-
	R. aureofulva	CR001108-06
	R. aureofulva	CR021009-01
	R. aureofulva	CR031101-01
Sg. *Tenellae* S. *Tenellae* Ss. *Puellarinae*	*R. puellaris*	CR021016-11
	R. puellaris	BK011030-BKrf
	R. puellaris	CR020927-03
	R. nauseosa	CR010814-01
	001007-04	CR001007-04

Output files of the Bambe program running from the Pasteur Institute server

Summary of sequence data of all 111 sequences in the alignment and the initial parameters selected.

```
Output generated by BAMBE version 2.03 Beta

Data Summary

 111    number of taxa
 870    total number of sites
 647    total number of unique sites


Number of constant sites

Category  A    G    C    T  Total
 1      2    3    3    4   12
 2     47   35   34   42  158
 3      8    8    6    9   31
Total   57   46   43   55  201


Overall  Count  Proportion
  A    16870   0.1747
  G    16843   0.1744
  C    18802   0.1947
  T    20552   0.2128
  -   23488   0.2432
  R       3  0.0000
  M       0   0.0000
  S       1  0.0000
  V       0  0.0000
  W       2   0.0000
  K       3  0.0000
  D       0  0.0000
  Y       6  0.0001
```

```
H       0    0.0000
B       0    0.0000

Category    A     G     C     T     -     other
 1   Pi 0.2145 0.2245 0.2835 0.2775
   Prop 0.1495 0.1565 0.1976 0.1934 0.3030 0.0001
 2   Pi 0.2813 0.2283 0.2506 0.2397
   Prop 0.2797 0.2270 0.2491 0.2383 0.0058 0.0001
 3   Pi 0.2028 0.2370 0.2423 0.3179
   Prop 0.1362 0.1592 0.1627 0.2135 0.3282 0.0002

Taxon      A      G      C      T      -     other
 1        0.1701 0.1770 0.2080 0.2103 0.2345 0.0000
 2        0.1701 0.1759 0.2034 0.2046 0.2460 0.0000
 3        0.1736 0.1805 0.2080 0.2080 0.2299 0.0000
 4        0.1701 0.1747 0.1977 0.2069 0.2506 0.0000
 5        0.1701 0.1747 0.1977 0.2069 0.2506 0.0000
 6        0.1759 0.1690 0.1874 0.2207 0.2471 0.0000
 7        0.1736 0.1690 0.1943 0.2161 0.2471 0.0000
 8        0.1724 0.1713 0.1839 0.2264 0.2460 0.0000
 9        0.1690 0.1690 0.1770 0.2333 0.2517 0.0000
10         0.1759 0.1667 0.1839 0.2241 0.2494 0.0000
11         0.1747 0.1724 0.1862 0.2172 0.2494 0.0000
12         0.1770 0.1655 0.1851 0.2253 0.2471 0.0000
13         0.1609 0.1563 0.1782 0.2138 0.2908 0.0000
14         0.1632 0.1575 0.1770 0.1966 0.3046 0.0011
15         0.1667 0.1690 0.1920 0.2184 0.2540 0.0000
16         0.1667 0.1690 0.1920 0.2184 0.2540 0.0000
17         0.1563 0.1759 0.1908 0.2011 0.2759 0.0000
18         0.1644 0.1713 0.2011 0.2161 0.2471 0.0000
19         0.1598 0.1690 0.1908 0.2092 0.2713 0.0000
```

20	0.1644	0.1632	0.1874	0.2218	0.2632	0.0000
21	0.1678	0.1701	0.1920	0.2103	0.2598	0.0000
22	0.1644	0.1713	0.2115	0.2092	0.2437	0.0000
23	0.1724	0.1540	0.1989	0.2000	0.2747	0.0000
24	0.1770	0.1621	0.1989	0.2184	0.2437	0.0000
25	0.1632	0.1678	0.1862	0.1920	0.2908	0.0000
26	0.1770	0.1736	0.1897	0.2207	0.2391	0.0000
27	0.1816	0.1770	0.1885	0.2241	0.2287	0.0000
28	0.1816	0.1816	0.1954	0.2218	0.2195	0.0000
29	0.1897	0.1793	0.1931	0.2264	0.2115	0.0000
30	0.1839	0.1908	0.2000	0.2195	0.2057	0.0000
31	0.1632	0.1667	0.2023	0.1897	0.2782	0.0000
32	0.1621	0.1644	0.2011	0.1862	0.2862	0.0000
33	0.1759	0.1782	0.1943	0.1989	0.2529	0.0000
34	0.1713	0.1759	0.1931	0.2069	0.2529	0.0000
35	0.1770	0.1793	0.1954	0.2195	0.2287	0.0000
36	0.1667	0.1782	0.1977	0.2000	0.2575	0.0000
37	0.1793	0.1713	0.2069	0.2057	0.2368	0.0000
38	0.1828	0.1770	0.1966	0.2172	0.2264	0.0000
39	0.1747	0.1701	0.2000	0.2046	0.2506	0.0000
40	0.1770	0.1713	0.2023	0.2092	0.2391	0.0011
41	0.1839	0.1805	0.2023	0.2184	0.2149	0.0000
42	0.1701	0.1701	0.1977	0.2057	0.2563	0.0000
43	0.1770	0.1770	0.2046	0.2057	0.2356	0.0000
44	0.1897	0.1759	0.1989	0.2230	0.2126	0.0000
45	0.1828	0.1805	0.1989	0.2069	0.2310	0.0000
46	0.1816	0.1770	0.2034	0.2069	0.2310	0.0000
47	0.1828	0.1793	0.1966	0.2126	0.2287	0.0000
48	0.1839	0.1793	0.1943	0.2195	0.2230	0.0000
49	0.1805	0.1805	0.2011	0.2080	0.2299	0.0000
50	0.1770	0.1724	0.1897	0.2138	0.2471	0.0000

51	0.1828	0.1816	0.1943	0.2230	0.2184	0.0000
52	0.1897	0.1805	0.1931	0.2207	0.2161	0.0000
53	0.1828	0.1816	0.1897	0.2172	0.2276	0.0011
54	0.1851	0.1736	0.1874	0.2299	0.2241	0.0000
55	0.1851	0.1724	0.1885	0.2299	0.2241	0.0000
56	0.1839	0.1713	0.1966	0.2253	0.2230	-0.0000
57	0.1862	0.1690	0.1862	0.2299	0.2287	0.0000
58	0.1966	0.1586	0.1862	0.2218	0.2368	0.0000
59	0.1862	0.1690	0.1920	0.2161	0.2368	0.0000
60	0.1782	0.1724	0.1954	0.2011	0.2529	0.0000
61	0.1874	0.1713	0.1759	0.2195	0.2460	0.0000
62	0.1897	0.1793	0.1851	0.2230	0.2230	0.0000
63	0.1851	0.1724	0.1782	0.2310	0.2333	0.0000
64	0.1966	0.1632	0.1678	0.2333	0.2391	0.0000
65	0.1770	0.1816	0.1966	0.2149	0.2299	0.0000
66	0.1874	0.1736	0.1954	0.2115	0.2322	0.0000
67	0.1851	0.1736	0.1862	0.2218	0.2333	0.0000
68	0.1816	0.1747	0.1920	0.2126	0.2333	0.0057
69	0.1828	0.1805	0.1943	0.2069	0.2356	0.0000
70	0.1770	0.1816	0.1885	0.2149	0.2356	0.0023
71	0.1874	0.1793	0.1989	0.2172	0.2172	0.0000
72	0.1908	0.1793	0.2115	0.2138	0.2034	0.0011
73	0.1851	0.1724	0.1954	0.2046	0.2425	0.0000
74	0.1793	0.1724	0.1931	0.2207	0.2345	0.0000
75	0.1851	0.1678	0.1897	0.2046	0.2529	0.0000
76	0.1644	0.1621	0.1943	0.1966	0.2828	0.0000
77	0.2000	0.1805	0.1862	0.2310	0.2023	0.0000
78	0.1862	0.1736	0.1897	0.2310	0.2195	0.0000
79	0.1747	0.1655	0.1885	0.2092	0.2621	0.0000
80	0.1655	0.1782	0.1977	0.2046	0.2540	0.0000
81	0.1713	0.1724	0.1874	0.2103	0.2586	0.0000

82	0.1678	0.1747	0.1989	0.2115	0.2471	0.0000
83	0.1506	0.1816	0.1931	0.2126	0.2621	0.0000
84	0.1667	0.1862	0.2046	0.2230	0.2195	0.0000
85	0.1678	0.1678	0.1989	0.1966	0.2690	0.0000
86	0.1667	0.1770	0.1897	0.2080	0.2586	0.0000
87	0.1736	0.1770	0.1908	0.2103	0.2483	0.0000
88	0.1736	0.1736	0.1943	0.2092	0.2494	0.0000
89	0.1759	0.1713	0.1931	0.2092	0.2506	0.0000
90	0.1678	0.1839	0.1966	0.2195	0.2322	0.0000
91	0.1678	0.1839	0.1977	0.2184	0.2322	0.0000
92	0.1644	0.1851	0.2023	0.2126	0.2356	0.0000
93	0.1667	0.1828	0.2011	0.2149	0.2345	0.0000
94	0.1713	0.1782	0.1954	0.2287	0.2264	0.0000
95	0.1736	0.1816	0.1977	0.2126	0.2345	0.0000
96	0.1713	0.1713	0.1874	0.2207	0.2471	0.0023
97	0.1632	0.1793	0.1966	0.2126	0.2483	0.0000
98	0.1632	0.1759	0.1908	0.2057	0.2644	0.0000
99	0.1701	0.1793	0.1920	0.2207	0.2379	0.0000
100	0.1609	0.1828	0.1931	0.2069	0.2563	0.0000
101	0.1736	0.1862	0.2011	0.2241	0.2138	0.0011
102	0.1609	0.1851	0.2046	0.2057	0.2437	0.0000
103	0.1678	0.1644	0.1931	0.2046	0.2701	0.0000
104	0.1747	0.1701	0.2000	0.2000	0.2552	0.0000
105	0.1644	0.1851	0.2241	0.1782	0.2483	0.0000
106	0.1644	0.1897	0.2207	0.1839	0.2414	0.0000
107	0.1678	0.1713	0.1989	0.2034	0.2586	0.0000
108	0.1678	0.1701	0.2057	0.2023	0.2540	0.0000
109	0.1690	0.1713	0.1931	0.2126	0.2529	0.0011
110	0.1701	0.1724	0.2023	0.2184	0.2368	0.0000
111	0.1598	0.1874	0.2000	0.1908	0.2621	0.0000

Run Settings:

```
seed=24922567
burn=300000
burn-algorithm=global
main-algorithm=local
cycles=1500000
sample-interval=200
parameter-update-interval=1
update-kappa=true
update-theta=true
update-pi=true
update-ttp=true
update-gamma=true
tune-interval=200
window-interval=200
molecular-clock=true
likelihood-model=HKY85
category-list=1^285,2^160,3^378,2*
single-kappa=false
initial-kappa=3.8855,5.1502,3.7229
initial-theta=1.2423,0.0429,1.3414
estimate-pi=true
data-file=allfri5.aln
outgroup=1
global-tune=0.0100
kappa-tune=0.1600
theta-tune=3000.0000
pi-tune=6000.0000
local-tune=0.1900
use-beta=false
max-initial-tree-height=0.1000
file-root=results
initial-tree-type=random
newick-format=true
```

Input Taxa names: (in alignment order)

1 delica
2 chloroides
3 brevipes
4 littoralis
5 pallidospora
6 pulverulenta
7 insignis
8 foetens
9 laurocerasci
10 amoenolens
11 pectinata
12 pectinatoid
13 4cyanoxantha
14 heterophylla
15 vesca
16 vesca 2
17 aeruginea
18 ilicis
19 parazurea 2
20 alboareolata
21 virescens
22 mustelina
23 amoenicolor
24 violeipes
25 grisea
26 melliolens
27 decolorans 2
28 melitodes
29 veternosa
30 rubra
31 laricina
32 cessans
33 curtipes
34 versicolor
35 solaris
36 nauseosa
37 messapica
38 puellula
39 odorata
40 puellaris
41 melzeri
42 adulterina
43 cuprea
44 maculata
45 sphagnophila
46 nitida
47 xerampelina 4
48 xerampelina
49 pascua
50 amoenipes
51 amethystina 2
52 firmula
53 californiensis
54 occidentalis
55 vinosa
56 claroflava
57 integra
58 integriformis
59 velenovskyi
60 azurea
61 lilacea
62 aurantiaca
63 lepida 2
64 lepidicolor
65 rosea
66 roseipes
67 turci
68 amethystina
69 postiana
70 risigallina
73 paludosa
74 caerulea 2
75 rosacea
76 violacea
77 rosacea 2
78 aurata
79 romellii
80 persicina
81 G. gilkeyae
82 consobrina
83 helodes
84 fellea
85 exalbicans 2
86 fuscorubroides
87 queletii
88 sardonia
89 drimeia
90 bicolor
91 emetica
92 nana
93 betularum 2
94 fragilis
95 mairei
96 cremoricolor
97 raoultii
98 aquosa
99 ochroleuca
100 viscida
101 stuntzii
102 atropurpurea
103 cavipes
104 gracillima
105 archaea
106 camarophylla
107 adusta
110 G. aculeatum
111 A. flettii

Summary of sequence data and the initial parameters selected of the top clade of 55 sequences which have been stripped of all identical positions in the alignment.

Output generated by BAMBE version 2.03 Beta

```
Data Summary
  55       number of taxa
 530        total number of sites
 481        total number of unique sites

Number of constant sites
Category  A    G    C    T  Total
 1     1    0    2    0    3
Total    1    0    2    0    3

Overall  Count  Proportion
  A     4794     0.1645
  G     4691     0.1609
  C     6261     0.2148
  T     6497     0.2229
  -    6897     0.2366
  R       2    0.0001
  M       0     0.0000
  S       1    0.0000
  V       0     0.0000
  W        1     0.0000
  K       1     0.0000
  D       0     0.0000
  Y       5     0.0002
  H       0     0.0000
  B       0     0.0000
Category    A     G     C     T     -     other
```

1 Pi 0.2155 0.2109 0.2815 0.2921

Prop 0.1645 0.1609 0.2148 0.2229 0.2366 0.0003

Taxon	A	G	C	T	-	other
1	0.1679	0.1698	0.2226	0.2113	0.2283	0.0000
2	0.1660	0.1642	0.2302	0.2113	0.2283	0.0000
3	0.1585	0.1566	0.2075	0.2226	0.2547	0.0000
4	0.1642	0.1698	0.2264	0.2132	0.2264	0.0000
5	0.1755	0.1679	0.2226	0.2283	0.2057	0.0000
6	0.1811	0.1679	0.2434	0.2226	0.1830	0.0019
7	0.1679	0.1717	0.2151	0.2377	0.2075	0.0000
8	0.1792	0.1698	0.2132	0.2340	0.2038	0.0000
9	0.1792	0.1679	0.2132	0.2434	0.1962	0.0000
10	0.1698	0.1868	0.2245	0.2321	0.1868	0.0000
11	0.1679	0.1717	0.2075	0.2283	0.2226	0.0019
12	0.1660	0.1642	0.2057	0.2396	0.2245	0.0000
13	0.1660	0.1717	0.2170	0.2358	0.2094	0.0000
14	0.1717	0.1585	0.2038	0.2491	0.2170	0.0000
15	0.1717	0.1566	0.2057	0.2491	0.2170	0.0000
16	0.1698	0.1547	0.2189	0.2415	0.2151	0.0000
17	0.1736	0.1509	0.2019	0.2491	0.2245	0.0000
18	0.1887	0.1358	0.2019	0.2358	0.2377	0.0000
19	0.1736	0.1509	0.2113	0.2264	0.2377	0.0000
20	0.1623	0.1566	0.2132	0.2340	0.2340	0.0000
21	0.1717	0.1566	0.2170	0.2075	0.2472	0.0000
22	0.1491	0.1623	0.2132	0.2113	0.2642	0.0000
23	0.1547	0.1528	0.2245	0.2075	0.2604	0.0000
24	0.1623	0.1547	0.2358	0.2094	0.2377	0.0000
25	0.1415	0.1660	0.2208	0.2000	0.2717	0.0000
26	0.1585	0.1679	0.2170	0.2321	0.2245	0.0000
27	0.1679	0.1642	0.2189	0.2283	0.2208	0.0000

28	0.1585	0.1547	0.2283	0.2151	0.2415	0.0019
29	0.1698	0.1698	0.2283	0.2302	0.2019	0.0000
30	0.1755	0.1585	0.2170	0.2189	0.2302	0.0000
31	0.1717	0.1585	0.2019	0.2358	0.2321	0.0000
32	0.1660	0.1604	0.2113	0.2208	0.2321	0.0094
33	0.1566	0.1660	0.2151	0.1981	0.2642	0.0000
34	0.1340	0.1434	0.2264	0.1774	0.3189	0.0000
35	0.1358	0.1472	0.2283	0.1830	0.3057	0.0000
36	0.1679	0.1698	0.2151	0.2113	0.2358	0.0000
37	0.1585	0.1717	0.2057	0.2245	0.2358	0.0038
38	0.1792	0.1679	0.2019	0.2358	0.2151	0.0000
39	0.1717	0.1566	0.1906	0.2491	0.2321	0.0000
40	0.1585	0.1642	0.2321	0.2094	0.2358	0.0000
41	0.1472	0.1528	0.2208	0.2094	0.2698	0.0000
42	0.1792	0.1623	0.2226	0.2377	0.1981	0.0000
43	0.1906	0.1415	0.1717	0.2547	0.2415	0.0000
44	0.1604	0.1566	0.2170	0.2019	0.2642	0.0000
45	0.1755	0.1547	0.1849	0.2321	0.2528	0.0000
46	0.1585	0.1717	0.2189	0.2245	0.2264	0.0000
47	0.1396	0.1660	0.2208	0.2075	0.2660	0.0000
48	0.1377	0.1396	0.2151	0.1943	0.3132	0.0000
49	0.1736	0.1585	0.2075	0.2509	0.2094	0.0000
50	0.1547	0.1453	0.2057	0.2151	0.2792	0.0000
51	0.1717	0.1491	0.2075	0.2075	0.2642	0.0000
52	0.1585	0.1585	0.2075	0.2340	0.2415	0.0000
53	0.1679	0.1679	0.2189	0.2208	0.2245	0.0000
54	0.1698	0.1679	0.2151	0.2321	0.2151	0.0000
55	0.1302	0.1811	0.2245	0.1849	0.2792	0.0000

Run settings:

```
seed=98452531
burn=30000
burn-algorithm=global
main-algorithm=local
cycles=1500000
sample-interval=200
parameter-update-interval=1
update-kappa=true
update-theta=true
update-pi=true
update-ttp=true
update-gamma=true
tune-interval=200
window-interval=200
molecular-clock=true
likelihood-model=HKY85
category-list=1*
single-kappa=false
initial-kappa=4.2070
initial-theta=1.0000
estimate-pi=true
data-file=allfri3topcladestrip.aln
outgroup=1
global-tune=0.0100
kappa-tune=0.2500
theta-tune=2000.0000
pi-tune=4000.0000
local-tune=0.1900
use-beta=false
max-initial-tree-height=0.1000
file-root=results
initial-tree-type=random
newick-format=true
```

Input Taxa names: (in alignment order)

1 sphagnophila
2 nitida
3 amoenipes
4 pascua
5 olivacea
6 olivacea 2
7 amethystina 2
8 firmula
9 veternosa
10 rubra
11 californiensis
12 decolorans 2
13 melitodes
14 occidental
15 vinosa
16 claroflava
17 integra
18 integriformis
19 velenovsky
20 caerulea 2
21 paludosa
22 versicolor
23 odorata
24 messapica
25 nauseosa
26 solaris
27 puellula
28 puellaris
29 melzeri
30 roseipes
31 turci
32 amethystina
33 curtipes
34 cessans
35 laricina
36 postiana
37 risigallina
38 aurantiaca
39 lepida 2
40 cuprea
41 adulterina
42 maculata
43 lepidicolor
44 azurea
45 lilacea
46 rosea
47 persicina
48 violacea
49 aurata
50 romellii
51 rosacea
52 melliolens
53 xerampelina 4
54 xerampelina
55 Albatrellus