Russula diversity in NE Scotland

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A Citizen-science Project

The Grampian Fungus Group (GFG) has been recording fungi across NE Scotland for nearly 25 years and over that time an impressive list of *Russula* records has been compiled, but there are relatively few recent records for many species. To update our list and improve our identification skills we joined forces with Dr Andy Taylor from the James Hutton Institute (JHI) to embark on a project matching field mycology with DNA sequencing. Grampian Fungus Group members collected and processed specimens during 2019 and 2020, collecting systematic data (based on Geoffrey Kibby's recording sheet, 2012) and photographs, and then drying for DNA sequencing by JHI. In addition, species records from 2021 and 2022, some without DNA verification have also been included.

Our Approach

Specimens for the project were collected on Group and individual forays and were placed individually in plastic pots to minimise contamination; surrounding tree species were recorded in the field. For each specimen, size, colours and textures were recorded, and extent of cap cuticle peel. Taste and smell, and reactions to guaiac and ferrous sulphate, and when relevant potassium hydroxide, were noted. Cap cuticle cell structure was examined in the thin tissue at the edge of a peeled section, stained with Buyck's cresyl blue (*Russula* formulation) and, when judged necessary to aid identification, with carbol fuchsin to highlight fuchsinophile granules. Finally, a section of the cap was set overnight for a spore print to both assess spore colour but also to provide spores for staining with Melzer's solution and examination of spore ornamentation. Remaining cap sections were then dried in a domestic fruit drier for 10 hours at 40°C, following which gill material was separated and stored in plastic tubes ready for DNA extraction. Gill samples from 2019 and 2020 were sequenced at JHI using the whole of the ITS region. Some specimens from 2021 and 2022 were sequenced by Aberystwyth University under the BMS Sequencing Grant after DNA extraction and amplification using the Group's Bento Lab.

Over the two earlier seasons, we dried 171 specimens. After sequencing, we had 140 good sequences and 31 short sequences, some of which were too short to allow definitive identification. An additional 16 whole ITS sequences were obtained from specimens collected in 2021 and 2022. To help determine species identities we copied each sequence into the analysis tool of the UNITE sequence database (https://unite.ut.ee/). This tool produces a list of 'matches' between the query sequence and sequences in the database and gives a measure of how good the matches are, and the length of the coverage between the query sequence and the reference. For most of our specimens we obtained a probable identification, but for some the matches were not as good, with two or more species being possible; these specimens require further assessment. We also checked sequences against those in GenBank as the two databases hold some different sequences, but we cautiously made comparisons only with sequences from voucher specimens (not root or soil extractions) from published research.

Russula Habitats in NE Scotland

The Grampian region is dominated by peats, non-calcareous gleys and brown soils with very little basic soil, leading to mainly acidic woodland types. These include naturally derived birch (*Betula* spp.) and Scots pine (*Pinus sylvestris*) woodlands, and commercial plantations of Scots pine, spruce (*Picea* spp.), larch (*Larix* spp.) and other conifers. However, at highest elevations dwarf Sub-Arctic scrub, comprising a range of willows (*Salix* spp.), occurs, and in lower areas pockets of mixed deciduous woodland with oaks (*Quercus* spp.), aspen (*Populus tremula*), alder (*Alnus glutinosa*), willows, Wych elm (*Ulmus glabra*), hazel (*Corylus avellana*) and beech (*Fagus sylvatica*) are found over brown and alluvial soils. In addition, parkland associated with castles, country houses and urban areas provide a wider range of tree species, including lime (*Tilia* spp.). Along the coasts, conifer plantations occur in some areas, mainly of various pine species (*Pinus* spp.), and mixed scrub is found in dune slacks, which includes willow, birch, hazel and alder. With the exception of high elevation dwarf woodland, all of these habitats were explored for the project.

Russula Species

From the DNA sequences we confirmed records for 54 species of *Russula* (Table 1), with an additional six species identified from macro and micro characteristics (denoted as non-sequenced in Table 1) giving a total over the four years of 60 species. Notable records for our region are described briefly below.

A comparison between species determinations from morphology and chemical tests with those from DNA determination revealed that about 74% (108 of 146 specimens) of morphological determinations were correct, demonstrating the effectiveness of the main keys we used (Kibby 2012 and Knudsen & Vesterholt 2012) and the importance of collecting good data on microscopic features, including effective use of carbol fuchsin for staining pileocystidia in the cap cuticle. Species determinations for a further 12 specimens based on morphological characteristics were not conclusive, and specimens with short DNA sequences (<150 bp) were excluded. However, there were some interesting errors in our determinations and findings, and some of these are described below within the species notes and under 'Difficulties with *Russula* Identifications'.

Notable Species Records for NE Scotland

Russula violaceoincarnata Knudsen & T. Borgen (Fig. 1)

A single specimen[1] was found growing under silver birch (*Betula pendula*) at Wood of Delgaty, Turriff (NJ7550) on 31 August 2019. The specimen wasn't identified prior to DNA sequencing and no voucher material has survived, but this represents only the second British record for this species following the collection reported by Mario Tortelli (2020) in Abernethy. A specimen collected from birch woodland in the Forest of Birse (NO5891) on 18/09/2021, yielded a moderate length ITS sequence that also matched this species.

Russula vinososordida Ruots. & Vauras (Fig. 2)

Two specimens were collected from under birch at two different locations; Muir of Dinnet, Aboyne (NO4499) on 24 August 2019[2], and Morrone Birkwood, Braemar (NO1490) on 17 August 2020[3]. There are four other confirmed British records for this species in the FRDBI (accessed 27/08/23), but it could be more frequent in Scotland due to its association with birch and possible confusion with *R. vinosa*.

Russula renidens Ruots. *et al.*

A specimen was collected from under birch at Haughton Country Park, Alford (NJ5616) on 14 September 2019, but tentatively identified as *R. persicina* due to near-adnate cuticle peel and very acrid taste. The DNA sequence from the specimen was a good match for *R. renidens* in UNITE (UDB015975, *Russula renidens*, Estonia, collected by Jukka Vauras, 2001, with length 660bp, coverage 11-660 and similarity 97%). There are just six other certain records and three likely records in the FRDBI (last 50 years) for this species in the UK.

Russula intermedia P. Karst

Three specimens from two locations were recorded for this distinctive birch associated species; Crathes Castle, Banchory (NO7396), 26 August 2020, and Dinnet, Aboyne (NO4698), 12 September 2020. Another specimen was collected from Haughton Country Park, Alford (NJ5616), on 2 September 2021, again from under birch, but not sequenced. The spores of this species are globose to sub-globose and reticulated, which is

[1] This specimen matched UNITE sequence UDB016635, *Russula violaceoincarnata*, Finland (collected by Katri Kokkonen and Jukka Vauras, 2007), with length 556bp, coverage 12-556, and similarity 99%.

[2] Specimen matched UNITE sequence UDB011301, *Russula vinososordida*, Estonia (collected by Jukka Vauras, 2011), with length 671bp, coverage 4-671 and similarity 99%.

[3] This specimen also matched UNITE sequence UDB011301, with length 632bp, coverage 1-632 and similarity 99%.

unusual in *Russula* and makes identification relatively easy (Fig. 3). There are 31 other records in the FRDBI (last 50 years), with notable clusters in Perthshire, Deeside and Cumbria, but it's possible that this is an under-recorded species in northern birch woods and it could be much more widespread.

Russula pelargonia Niolle
A challenging species to separate from *R. violacea* and both are rare in the UK making it difficult to develop familiarity. Kibby (2017) suggests that the main differences between the species are slow, dark blue guaiac reaction, pileocystidia 1-2 septate and partially reticulated spores in *R. pelargonia* versus rapid azure blue guaiac, pileocystidia 0-4 septate and spores with isolated warts in *R. violacea*. A specimen was collected from under aspen at Muir of Dinnet, August 2019. Its reaction to guaiac was rapidly mid green-blue, it had long, cylindrical, non-septate pileocystidia and long spore warts with some connectives. Unsurprisingly, whilst we considered this specimen to be more likely to be *R. pelargonia*, our identification was uncertain and it was good to get confirmation via DNA sequencing (matching UDB016031, *Russula pelargonia*, Finland, collected by Jukka Vauras, 2000, with length 653bp, coverage 14-653, similarity 100%). A collection of three specimens from under birch in Haughton Country Park, Alford, on 02/09/2021, was unusual in being large, robust and brown, but having a strong pelargonium smell, and were not identified from morphology. Their spores had isolated warts and all had strong, rapid reactions to guaiac, which suggested *R. violacea*. A good sequence was obtained from one of these specimens, which was also very similar to the above reference sequence UDB016031 (length 652bp, coverage 10-659, 98.6%). Specimens closely matching the morphology of this species were also found in 2022 from aspen woodland at Crathie, Ballater (NO2694), and in 2023 from a different area of birch woodland at Haughton Country Park, Alford, but neither has been sequenced. Whilst there are nearly 50 records in the FRDBI (last 50 years), there were just seven in Scotland prior to these four GFG records.

Russula amethystina Quél.
We now have records of this species from five locations in the region, with three confirmed through DNA analysis, all associated with spruce. The species is very like *R. turci*, which also occurs in the region under Scots pine. We found that spore ornamentation wasn't sufficiently and consistently different between the two species to be reliable for separation. The reaction to guaiac in our *R. amethystina* specimens was more consistently darker blue than in *R. turci*, in which some specimens were completely negative, but this was again inconsistent (Sarnari 2005 suggests neither species has a strong reaction to guaiac; Kibby, 2017, suggests the opposite guaiac reactions). The only consistent characteristics appeared to be habitat association and a tendency for cap colour in *R.*

Fig. 1. *Russula violaceoincarnata* growing in association with *Betula*, Delgaty, Turriff, Scotland, August 2019. Photograph © Toni Watt.

Fig. 2. *Russula vinososordida* growing in association with *Betula*, Morrone Birkwood, Braemar, Scotland, August 2020. Photograph © Helen Baker.

amethystina to be purple-toned and in *R. turci* to be red-brown. There are only five other 'certain' records for *R. amethystina* in the FRDBI (last 50 years), and one of these is a more recent record for one location, but it's possible that this species may be more common in the UK in mature spruce forests and mixed woodland where mature spruce is present.

Russula curtipes F.H. Møller & Jul. Schaeff.
A beech associate, this distinctive species was found in seven locations, five confirmed through DNA analysis. There are just seventeen other Scottish records in the FRDBI (50 years), and only one from NE Scotland prior to the GFG records, but the species is much more frequent in England. It is very likely that this is an under-recorded species in Scotland.

Difficulties with *Russula* identifications

It is well known that whilst cap and stem colour can be a very useful characters for identification of *Russula*, not only do some species show a wide range of cap colours, not all of which are shown in the literature, but cap pigments can wash out. Taste and smell can also be useful additional characters, but both can vary within species and some recorders will be limited by their own sensory ability. However, having at least a reliable taste for a specimen can help place it within a particular section of the genus and narrow identification possibilities.

Spore colour determined from a good spore print is one of the most useful, perhaps critical, characters for identification and appeared to vary little within the species where we had several specimens for comparison. This is where collecting 'good' specimens is important; immature (cap not fully extended) or over-mature specimens will not give good spore prints. Spore ornamentation (observed at x1000 under oil immersion) is also an important character. However, some species, for example *Russula integra*, can have very variable spore ornamentation, which might not be fully described in keys. Some species descriptions, for example those by Romagnesi (1967) and Sarnari (1998 & 2005), include illustrations of spore variations and are extremely useful reference resources. Observation of cap cuticle cell structure is extremely helpful and staining of cap cuticle preparations with carbol fuchsin (CF) or sulpho-vanillin can be essential. CF is not easy to use because of the need to wash with hydrochloric acid, but is accessible and with practice is incredibly useful. Some of our early collections were not stained with CF and this limited species determinations and caused some errors in identifications.

One of the outcomes from the DNA sequencing has been to improve our knowledge of several difficult 'pairings' of commoner species:
Russula puellaris and *R. versicolor* are very similar species and share yellowing in the stem typical of the subsection *Puellarinae*. Kibby (2017) suggests that size, taste, spore ornamentation and the pileocystidia are useful ways to separate these species. Of six specimens collected, we identified just one as *R. puellaris*,

Fig. 3. *Russula intermedia* spores. Photograph © Helen Baker.

Fig. 4. *Russula pelargonia* found growing in association with *Betula*, Haughton Country Park, Alford, Scotland, September 2021. Stem blue with guaiac staining on left and pink with iron sulphate to right. Photograph © Helen Baker.

Fig. 5. *Russula pelargonia* in association with *Betula*, Haughton Country Park, Alford, Scotland, August 2023. © Helen Baker.

Fig. 6. *Russula amethystina* group growing in association with *Picea*, Bin Forest, Huntly, Scotland, August 2020. Photograph © Helen Baker.

based on 0-1 septate, clavate pileocystidia and mild taste, but this proved erroneous and all were *R. versicolor* on DNA analysis. There are plenty of records for these species in FRDBI and NBN Atlas, but *R. puellaris* appears twice as common as *R. versicolor*; DNA analysis in our region suggests that this may not be the case, but a larger sample would be helpful, collected over several seasons, to be sure of comparative frequency.

Another interesting pairing is *R. nitida* and *R. robertii*, which are very similar. We collected five specimens of *R. nitida* and just one of *R. robertii*; from this small sample the consistent differences were spore colour and ornamentation, and habitat, with *R. robertii* probably occurring only in very wet birch woodland, such as on the edges of mires. Stem colour of *R. nitida* varied from wholly white to fully pink so a white or slightly pink-flushed stem wasn't reliably indicative of *R. robertii*. In addition, the pileocystidia in *R. nitida* were variable with some having few septa and thus more like those observed and described for *R. robertii*.

Twenty-three of our sequences were determined as *R. integra* using UNITE and most were initially identified as this highly variable species, but several were misidentified. Three of them were identified as *R. melitodes* (2) and *R. romellii*

(1) primarily on an ecological basis as all three were associated with beech. Interestingly, the genetic variation in all 23 sequences was extremely small. The three specimens from beech woodland raise an interesting possibility that *R. integra* is not just a pine/conifer specialist.

Eight of our sequences matched *R. aquosa*, although three were short (<150 bp compared with >600 bp for most specimens), but we had misidentified three of them: one as *R. emetica*, another as *R. sylvestris* and the third as *R. fragilis*. One of the causes of confusion for these similar species within the sub-section *Russula* related to habitat and all three misidentifications were of specimens collected from relatively dry mixed woodland with birch present, suggesting *R. aquosa* is not restricted to wet habitats. One feature mentioned in keys is taste, but our specimens ranged from mild to very acrid, which led to some of the misidentifications. Spore colour was off-white (Romagnesi code Ib-IIa or B in Kibby 2012) in all but one specimen, which had a white (Ia or A) spore print; this might be a useful character to help separate *R. aquosa* from very similar species within the sub-section *Russula*. *R. aquosa* is, however, typically a dusky pink colour giving it a certain look, which with increased familiarity helps separation from washed out similar species.

Section *Xerampelinae* taxonomy is well known to be challenging and DNA sequences in our collection provided some surprises, including finding apparent *Russula amoenoides* in association with Scots pine and birch/willow, and *R. xerampelina* lacking any red colouring in the stem, and with dark red-brown cap colours superficially resembling *R. favrei*. We now have three ITS sequences from apparent *R. amoenoides*, but it is possible that these are a different species and more work needs to be done to assess the phylogenetic relationships between our specimens and others within the section. Amongst specimens collected in deciduous woodland, there seemed little consistency in identification characteristics, as shown by Adamčik *et al.* (2016). Of seven specimens, five matched *R. nuoljae* and two, with only moderate length sequences (c. 320–390 bp), had matches with both *R. nuoljae* and *R. clavipes* sequences in UNITE. One of these latter specimens had all micro-characteristics consistent with *R. nuoljae*, as described by Adamčik *et al.* (2016), and was associated with *Betula*, whilst the other had spore ornamentation like *R. clavipes*. The keying out of *Xerampelinae* on morphological characters remains problematic, but the key in Adamčik *et al.* (2016) provides the best approach, critically requiring careful processing of the cap cuticle to observe cell morphology at different locations in the pileus. However, our sequences suggest that *R. nuoljae* is frequent or common in our region, most closely associated with birch, including in lowland woods. It was first recognised as British in 2020, after a collection from Abernethy was sequenced, and is illustrated among the addenda in Kibby, Vol. 4 (2023).

Missing Species and Future Recording

Historical records for Grampian are available from several sources: the FRDBI and the North-East Scotland Biological Recording Centre (NESBReC) are the primary sources (GFG records are provided to NESBReC after verification), but additional information is available from the NBN Atlas, and from publications. Exploration of these records shows that another 28 species of *Russula* have been recorded in the region, although not all records are verified, which suggests that about half of all British species may be present.

The project fulfilled its aims to improve knowledge of *Russula* species in our region and increase confidence in processing and identification, although there is still much to learn. In future, we hope to use DNA sequencing in a selective way to confirm identification of interesting specimens for which the morphological approach leaves significant doubt, but this will depend on funding availability.

Table 1. A full list of *Russula* species recorded between 2019 and 2021 in the Grampian region (ns = no DNA sequence), arranged according to the classification adopted by Sarnari (1998).

Subgenus *Compactae*
Section *Compactae*
Russula nigricans
Russula anthracina
Russula albonigra
Russula adusta
Russula densifolia

Section *Lactarioides*
Russula chloroides

Subgenus *Ingratula*
Section *Ingratae*
Russula foetens
Russula illota
Russula laurocerasi
Russula recondita
Russula amoenolens
Russula fellea

Subgenus *Heterophyllidia*
Section *Heterophyllae*
Subsection *Cyanoxanthinae*
Russula cyanoxantha
Subsection *Heterophyllae*
Russula vesca
Subsection *Griseinae*
Russula parazurea
Russula ionochlora
Russula grisea
Russula medullata (ns)
Russula aeruginea

Subgenus *Russula*
Section *Russula*
Subsection *Russula*
Russula atropurpurea
Russual aquosa
Russula fragilis
Russula laccata
Russula betularum
Russula emetica
Russula mairei
Subsection *Violaceinae*
Russula pelargonia
Subsection *Sardoninae*
Russula sanguinaria
Russula sardonia (including forma *viridis* and forma *mellina*)
Russula queletii
Russula gracillima
Russula renidens
Subsection Urentes
Russula badia (ns)
Russula intermedia
Section *Viscidinae*
Russula ochroleuca
Section *Polychromae*
Subsection *Xerampelinae**
Russula xerampelina
Russula cf *amoenoides*
Russula graveolens
Russula nuoljae
Russula clavipes
Subsection Integriforminae
Russula decolorans
Russula vinososordida
Russula paludosa
Russula romellii
Russula curtipes
Russula velenovskyi
Russula violaceoincarnata
Section *Paraincrustatae*
Subsection *Integrae*
Russula integra
Section *Tenellae*
Subsection *Puellarinae*
Russula versicolor
Subsection *Laricinae*
Russula cessans
Subsection *Betulinae*
Russula brunneoviolacea
Russula robertii
Russula nitida
Section *Amethystinae*
Subsection *Amethystinae*
Russula turci
Russula amethystina
Subsection *Chamaeleontinae*
Russula acetolens (ns)
Russula risigallina (ns)
Subsection *Integroidinae*
Russula vinosa (ns)
Russula claroflava (ns)
Russula caerulea

**Xerampelinae* – see text.

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