Studies in *Gyromitra* II: cryptic speciation in the *Gyromitra gigas* species complex; rediscovery of *G. ussuriensis* and *G. americanigigas* sp. nov.

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Abstract

Taxa in the *Gyromitra gigas* species complex were previously studied and their taxonomy resolved. During ongoing studies in this group, cryptic speciation was discovered in *G. gigas*. Sequences of the ITS and LSU regions from 75 specimens were included in maximum likelihood and Bayesian phylogenetic analyses to establish species boundaries and resolve species relationships. Sequence similarity comparisons were also conducted between the two ribosomal markers and between the ITS1 and ITS2 regions. *Gyromitra gigas sensu stricto* and two additional species were discovered within the *G. gigas* clade. *Gyromitra ussuriensis* was rediscovered as a distinct taxon and removed from synonymy under *G. gigas*. It occurs in central and eastern Asia, whereas *G. gigas* occurs mostly in Europe but also extends into central Asia. A neotype is designated for *G. ussuriensis*. A new species, *Gyromitra americanigigas*, is described and illustrated from eastern North America. Although morphology and the LSU exhibited little variation among the three species, the ITS1 and ITS2 regions displayed similar interspecific sequence variability around 0.5–1%, which is sufficient for species identification at the molecular level.

Keywords Ascomycota · Fungi · ITS sequences · Pezizales · Molecular systematics · 1 new taxon · 1 new typification

Introduction

The *Gyromitra gigas* species complex consists of six taxa: *G. gigas* (Krombh.) Quéhl., *G. kphanspurensis* Jabeen & Khalid, *G. korfii* (Raitv.) Harmaja, *G. montana* Harmaja, *G. pseudogigas* X.C. Wang & W.Y. Zhuang, and *G. ticiniana* Littini. Type specimens have been designated and sequenced, taxonomy and associated nomenclature have been reconciled, and species concepts and biogeographical distributions have been resolved through molecular and morphological analyses for all six species in this complex (Krisai-Greilhuber et al. 2017; Carbone et al. 2018; Wang and Zhuang 2019; Miller et al. 2020).

*Gyromitra gigas* is a widespread taxon that is infrequently collected throughout Europe (Carbone et al. 2018), but has also been reported from China, Japan, North America, and Russia (MyCoPortal 2022). An epitype (MBT 383600) specimen has been designated from the Czech Republic (Carbone et al. 2018). It is reported to grow near or on rotten logs and old stumps in woods of *Abies*, *Betula*, *Carpinus*, *Picea*, *Populus tremula*, *Quercus*, and *Tilia* from mid-March to early May (Carbone et al. 2018). ITS sequence data has recently shown its distribution to be limited to Europe, with single reports from China and Russia (Miller et al. 2020). Although the European name *G. gigas* has been frequently used for North American material, this species does not occur in North America. Rather, *G. korfii* occurs throughout eastern North America, whereas *G. montana* occurs primarily in western North America and Canada (Miller et al. 2020).

*Gyromitra ussuriensis* was described in 1950 from the Ussurisky Nature Reserve (formerly known as the Suputinsky Nature Reserve) in Far East Russia (Vassiljeva 1950). It is infrequently collected and reported to grow on...
rotten logs and stumps of *Pinus koraiensis* and on dead trunks and stumps of *Betula costata* from late May to early June. It was compared to *G. gigas* because of its similar ascospores, but distinguished by the smaller hymenophore with a free edge, longer stem, and its growth on wood (Vassiljeva 1950). Since he found no morphological differences, Raitviir treated *G. ussuriensis* as a synonym of *G. gigas* (Raitviir 1970) where it remains today (Carbone et al. 2018, Index Fungorum).

During our ongoing taxonomic and systematic studies of *Gyromitra* Fr., two well-supported clades occurring near *G. gigas* were discovered that represent cryptic species, one previously described representing *G. ussuriensis* and the other an undescribed species. The goals of this study were to sample and sequence multiple representatives for these two taxa as well as *G. gigas* and to establish species boundaries, reconcile species relationships and biogeographical distributions, and assess the potential of ITS and LSU for resolving these cryptic species.

### Materials and methods

#### Specimens examined

Entire dried ascomata or small portions of the hymenophore of ascomata in 1.5 mL centrifuge tubes were sent to the first author either as loans or as gifts. Sequences generated during this study were obtained from DNA extracted directly from these dried ascomata, which were deposited at ILLS or are available at their home institution (BPI, CUP, F, ILLS, LE, MICH, MIN, NY, O, TAAM, TNS, and YSU). Fungarium acronyms follow Index Herbariorum (Thiers 2013, continuously updated).

Morphological descriptions are based on notes taken from fresh collections and associated photographs, dried fungarium specimens, and other sequenced material. Micromorphological examination followed Miller et al. (2020). The following were calculated for ascospore lengths and widths for each specimen: the range in minimum and maximum values, the mean length (*L*<sub>m</sub>), mean width (*W*<sub>m</sub>), length-width ratio (*Q*), and mean length-width ratio (*Q*<sub>m</sub>). The lengths of the ascospore apiculi are included in the measurements of ascospore length. Small fragments of the hymenial layer were rehydrated in 5% KOH, washed in distilled water, and sections were prepared at 25 μm thickness using a Physitemp BSF-3 freezing stage mounted on a Leica SM2000 sliding microtome. Images of micromorphological features were captured with an Olympus DP22 digital camera mounted on a BX52 compound microscope using Olympus Imaging Software Cell**®**D and processed using Adobe Photoshop 2021 (Adobe Systems Inc., Mountain View, California).

The following specimens were examined and annotated: *G. americanigigas* (CUP-A-024034, CUP-070734, MICH352014, MICH352015, MICH352016, MICH352017, MIN890906), *G. gigas* (ILLS00121401, NY01943083, O174609, OULU F25301, YSU-F-11757, YSU-F-11758), and *G. ussuriensis* (CUP-JA-000675, TAAM060483, YSU-F-08006). Voucher specimen number, locality, GenBank accession numbers, and source for all taxa included in the ITS and LSU analyses are shown in Table 1.

#### Molecular data

Methods for the extraction, PCR amplification, and sequencing of the internal transcribed spacer (ITS) region and the first 600 bp of the 5′ end of 28S nuclear ribosomal large subunit (LSU) followed Miller et al. (2020). Sequences were produced at the Roy J. Carver Biotechnology Center at the University of Illinois Urbana-Champaign, and consensus sequences were assembled with Sequencher 5.4 (Gene Codes Corp., Ann Arbor, Michigan, USA).

#### Phylogenetic analyses

ITS and LSU datasets were individually aligned using MUSCLE® as implemented in Sequecher 5.4. Since most taxa had missing data in the two datasets, portions of the 5′ and 3′ ends were excluded from all analyses. Both the ITS and LSU datasets possessed little sequence variation in their final alignments so removal of ambiguously aligned regions was unnecessary. The ITS and LSU alignments are included as FASTA-formatted files (Supplementary). Both ITS and LSU alignments were rooted with *G. montana* based on previous analyses (Miller et al. 2020). The Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985) was determined to be the best-fit model for both datasets by jModeltest (Darriba et al. 2012; Guindon and Gascuel 2003) based on the Akaika information criterion (AIC) (Posada and Buckley 2004). A maximum likelihood (ML) analysis with the HKY model and all parameters optimized was performed with 1000 bootstrap replicates using PhyML as implemented in Seaview 4.7 (Gouy et al. 2010). An additional ML analysis with a GTRCAT approximation and 1000 bootstrap replicates employing GAMMA model of rate heterogeneity and the rapid bootstrapping option (Stamatakis et al. 2008) was also performed using RAxML-HPC2 v.8.2.12 (Stamatakis 2014) in the CIPRES Science Gateway v.3.3 portal (Miller et al. 2010). Clades with bootstrap values (BV) ≥ 70% were considered significant and supported (Hillis and Bull 1993). Bayesian analyses were performed under the above model using MrBayes v 3.2.7 (Huelsenbeck and Ronquist 2001, 2005) on the CIPRES 3.3 portal. The Bayesian analyses were run for 1,000,000 generations which was when the average standard deviation of split frequencies was below 0.01 with trees saved every 1000 generations and burn-in set at 25%. Bayesian posterior probabilities (BPP) were determined from
Table 1  Specimens used in this study including type designation, voucher specimen number, locality, ITS and LSU GenBank accession numbers, and source of sequences. Newly generated sequences are in boldface

| Species               | Voucher specimen no. | Locality                  | ITS GenBank no. | LSU GenBank no. | Source                  |
|-----------------------|----------------------|---------------------------|-----------------|-----------------|-------------------------|
| *Gyromitra americanigigas* |                      |                           |                 |                 |                         |
|                       | MICH 352017          | Canada: New Brunswick     | ON527892        | ON532828        | This study              |
|                       | MICH 24092           | Canada: Nova Scotia       | ON527893        | ON532829        | This study              |
|                       | HOLOTYPE MICH 352014 | USA: Michigan             | ON527894        | ON532830        | This study              |
|                       | MICH 352015          | USA: Michigan             | ON527895        | ON532831        | This study              |
|                       | MICH 352016          | USA: Michigan             | ON527896        | ON532832        | This study              |
|                       | NY 01797009          | USA: Michigan             | ON527897        |                  | This study              |
|                       | MIN890906            | USA: Minnesota            | MT373909        |                  | Healy unpublished       |
|                       | BPI 895482           | USA: New Hampshire        | ON527898        |                  | This study              |
|                       | CUP-A-024034         | USA: New York             | ON527899        |                  | This study              |
|                       | CUP-070734           | USA: New York             | ON527900        | Deposited as ITS-LSU | This study              |
|                       | F-C0228434F          | USA: Wisconsin            | ON527901        |                  | This study              |
| *Gyromitra gigas*     |                      |                           |                 |                 |                         |
|                       | NY 01943083          | Austria                   | ON527902        |                  | This study              |
|                       | EPITYPE TUR-A 208088 | Czech Republic            | MH938663        | MH938309        | Carbone et al. 2018     |
|                       | TAAM190163           | Estonia                   | MW076963        | MW076976        | Miller et al. 2020      |
|                       | TAAM040189           | Estonia                   | ON527903        | ON532833        | This study              |
|                       | TAAM060481           | Estonia                   | ON527904        |                  | This study              |
|                       | TAAM072130           | Estonia                   | ON527905        |                  | This study              |
|                       | TAAM076736           | Estonia                   | ON527906        |                  | This study              |
|                       | TAAM040364           | Estonia                   |                  | ON532834        | This study              |
|                       | TU117077             | Estonia                   | UDB020355       | UDB020355       | Tedersoo et al. Global soil samples |
|                       | G4178                | Estonia                   | UDB0485039      |                  | Tedersoo et al. Global soil samples |
|                       | G4382                | Estonia                   | UDB0435061      |                  | Tedersoo et al. Global soil samples |
|                       | G4390                | Estonia                   | UDB0466718      |                  | Tedersoo et al. Global soil samples |
|                       | G4510                | Estonia                   | UDB0392834      |                  | Tedersoo et al. Global soil samples |
|                       | G4626                | Estonia                   | UDB0327095      |                  | Tedersoo et al. Global soil samples |
|                       | G4627                | Estonia                   | UDB0337494      |                  | Tedersoo et al. Global soil samples |
|                       | G4768                | Estonia                   | UDB0483315      |                  | Tedersoo et al. Global soil samples |
|                       | G4813                | Estonia                   | UDB0192402      |                  | Tedersoo et al. Global soil samples |
|                       | OULU-F 23717; ILLS00121402 | Finland               | MW076967        | MW076977        | Miller et al. 2020      |
|                       | OULU-F 23577; ILLS00121403 | Finland               |                  | MW076978        | Miller et al. 2020      |
|                       | OULU-F 25304; ILLS00121404 | Finland               |                  | MW076979        | Miller et al. 2020      |
|                       | OULU-F 25301; ILLS00121405 | Finland               | MW076968        | MW076980        | Miller et al. 2020      |
|                       | LY NV 2007.04.20     | France                    | MH938665        | MH938311        | Carbone et al. 2018     |
| Species | Voucher specimen no. | Locality | ITS GenBank no. | LSU GenBank no. | Source |
|---------|---------------------|----------|----------------|----------------|--------|
| HMAS254604; ILLS00121400 | France | MG846996 | MG847005 | Wang and Zhuang 2019 |
| ILLS00121401 | France | MW076969 | MW076969 | Miller et al. 2020 |
| ILLS00121407 | France | MW076970 | MW076970 | Miller et al. 2020 |
| ILLS00121408 | France | MW076971 | MW076971 | Miller et al. 2020 |
| ILLS00121409 | France | MW076972 | –––––– | Miller et al. 2020 |
| ILLS00121410 | France | MW076973 | –––––– | Miller et al. 2020 |
| ILLS00121411 | France | MW076974 | MW076974 | Miller et al. 2020 |
| LK95 04 08 | Hungary | MH938664 | MH938310 | Carbone et al. 2018 |
| TUR-A 208089 | Italy | MH938666 | –––––– | Carbone et al. 2018 |
| TUR-A 208091 | Italy | MH938667 | MH938312 | Carbone et al. 2018 |
| TUR-A 208092 | Italy | MH938668 | MH938313 | Carbone et al. 2018 |
| TUR-A 208093 | Italy | MH938669 | MH938314 | Carbone et al. 2018 |
| 14754 | Italy | JF908781 | –––––– | Osmundson et al. 2013 |
| O174609 | Norway | MW076964 | KX008328 | Miller et al. 2020 |
| O174628 | Norway | MW076965 | KX008329 | Miller et al. 2020 |
| H.546 | Turkey | KX420694 | –––––– | Gungor et al. unpublished |
| H.559 | Turkey | KX420695 | –––––– | Gungor et al. unpublished |
| H.815 | Turkey | KX420696 | –––––– | Gungor et al. unpublished |

**Gyromitra montana**

| DAOM 706056; ILLS00121424 | Canada: Newfoundland | MW077459 | MW077445 | Miller et al. 2020 |

**ISOTYPE**

| BPI 566707 | USA: Wyoming | MW077452 | MW077442 | Miller et al. 2020 |

**Gyromitra ussuriensis**

| HMAS89008 | China: Jilin | MG846995 | MG847004 | Wang and Zhuang 2019 |
| TNS-F-15631 | Japan: Hokkaido | ON527916 | –––––– | This study |
| TNS-F-31137 | Japan: Ibaraki | ON527917 | ON532837 | This study |
| CUP-JA-00675 | Japan: Ishikari | ON527918 | –––––– | This study |
| TNS-F-17980 | Japan: Tochigi | ON527919 | –––––– | This study |
| TNS-F-66010 | Japan: Tochigi | ON527920 | ON532838 | This study |
| TNS-F-66526 | Japan: Tochigi | ON527921 | –––––– | This study |
| YSU-F-08006; ILLS00121415 | Russia: Khanty-Mansiysky Autonomous Okrug | MW076975 | MW076981 | Miller et al. 2020 |

**NEOTYPE**

| TAAM060483 | Russia: Primorsky Kray | ON527922 | –––––– | This study |
| LE 304601 | Russia: Siberia, Krasnoyarsky Krai | ON527923 | –––––– | This study |
| LE 304603 | Russia: Siberia, Krasnoyarsky Krai | ON527924 | –––––– | This study |
| LE 323486 | Russia: Siberia, Krasnoyarsky Krai | ON527925 | –––––– | This study |
| KH:KA19-0027 | South Korea | MZ567190 | MZ573189 | Cho et al. 2021 |
a consensus tree using PAUP* 4.0b10 (Swofford 2002), and clades with BPP \( \geq 95\% \) were considered significant and strongly supported (Alfaro et al. 2003; Larget and Simon 1999).

**Sequence similarity comparisons**

Comparisons between ITS and LSU sequences and between the ITS1 and ITS2 regions were made in PAUP v.4.0a (build 166) (Swofford 2002) with distance set to uncorrected “p.” Mean and range were calculated for infraspecific and interspecific variation. The ITS1 and ITS2 regions were delimited using the ITSx program in PlutoF (Abarenkov et al. 2010).

**Results**

**Phylogenetic analyses**

PCR amplification and Sanger sequencing of ITS and LSU were largely successful from DNA extracted from most specimens, even those over 60 years old (Table 1). The final ITS alignment of 75 sequences consisted of 738 nucleotides after the removal of nucleotides on the 5’ and 3’ ends due to missing characters in most taxa. No ambiguously aligned characters occurred in the ITS alignment. The ITS contained 49 parsimony-informative characters with gaps treated as missing characters: 34 in the ITS1 region and 15 in the ITS2 region.

The final LSU alignment of 40 sequences consisted of 864 nucleotides after the removal of nucleotides on the 5’ and 3’ ends due to missing characters in most taxa. No ambiguous regions were present in the LSU dataset. The LSU contained only 3 parsimony-informative characters and lacked sufficient phylogenetic signal to differentiate among these putative taxa (data not shown) so phylogenetic relationships are based only on the ITS dataset.

Analyses of the ITS dataset generated identical most-likely trees in both the PhyML and RAxML analyses, except BV were higher in the RAxML tree (Fig. 1). Three distinct, well-supported monophyletic clades were formed that corresponded to three closely related, but separate species. *Gyromitra gigas* formed a highly supported clade with 90% BV and significant BPP. The clade containing members of *G. ussuriensis* was supported with 70% BV and significant BPP. The new species, *G. americanigigas*, is well-supported with 96% BV, but without significant BPP. *Gyromitra gigas* and *G. ussuriensis* occurred as sister taxa in a clade supported by significant BPP.

**Distribution**

Each of these three species inhabits a specific geographic region with some overlap in central Russia between *G. gigas* and *G. ussuriensis* (Fig. 2). All known species in the *G. gigas* species complex are shown on the map for clarity. *Gyromitra americanigigas* occurs throughout northeastern USA and southeastern Canada. Its range overlaps with *G. korfii* in Michigan and New York and with *G. montana* in Michigan and Canada. *Gyromitra gigas* occupies a large range extending from western Europe to central Russia and overlaps with *G. ticiniana* in France, Italy, and Turkey and with *G. ussuriensis* in central Russia. *Gyromitra ussuriensis* occurs mostly in eastern China, Japan, eastern Russia, and South Korea. *Gyromitra khanspurensis* is known only from its type locality in Pakistan, whereas *G. pseudogigas* has only been collected in Sichuan Province of China.

**Taxonomy**

*Gyromitra americanigigas* Dirks, A.N. Mill. & Methven, sp. nov. Fig. 3

Mycobank: MB844178

*Type:* USA, Michigan, Washtenaw County, Stinchfield Woods, 42.41 N, 83.92 W, on soil in a *Pinus* sp. plantation, 5 May 2020, A.C. Dirks (ACD0256), Holotype (MICH352014), Isotype (ILLS00114755), GenBank ON527894 (ITS), GenBank ON532830 (LSU).

*Etymology:* Named for the combination of “americana” and “gigas” to describe this new species from North America that is closely related to the European *G. gigas*.

*Description:* Ascomata consisting of an apical hymenophore and stipe, 5–12 cm high. Hymenophore 2–6 cm high, 5–9 cm diam., convoluted and folded; hymenium pruinose, brownish orange to brown; margin free; sterile surface smooth, tan. Stipe 3–6 cm long, 2–5 cm diam., off-white, longitudinally wrinkled and pitted, hollow. Excipulum one-layered, of *textura intricata*, hyaline. Paraphyses cylindric, apices clavate, inflated up to 7.5–10 μm diam., thin-walled, septate, unbranched, golden-brown in

| Species | Voucher specimen no. | Locality | ITS GenBank no. | LSU GenBank no. | Source |
|---------|----------------------|----------|-----------------|-----------------|--------|
| KH:KA21-0152 | South Korea | MZ567197 | MZ573196 | Cho et al. 2021 |
| KH:KA19-0153 | South Korea | MZ567198 | MZ573197 | Cho et al. 2021 |
KOH. Asci 275–325 × 17.5–20 μm, cylindric, operculate, thin-walled, hyaline, eight-spored. Ascospores uniseriate, 28–34 × 10–12 μm (Lm 30.3 μm, Wm = 11.0 μm, Q = 2.3–3.0, Qm = 2.8), ellipsoid to fusoid, at times inequilateral; surface finely wrinkled; apiculi knob-like, 2–3 μm long; perispore up to 1 μm thick, cyanophilic; content triguttulate, with one large central oil drop and two smaller polar oil drops, hyaline. Ascospores white in mass.

Ecology and distribution: Solitary to scattered on soil and wood in temperate coniferous and deciduous-coniferous forests in May. Known from New Brunswick and Nova Scotia in Canada and from Michigan, Minnesota, New York, and Wisconsin in USA.

Notes: *Gyromitra americanigigas* is macro- and micromorphologically similar to *G. gigas*, *G. korfii*, and *G. montana*. *Gyromitra gigas*, which is restricted to Europe and Asia, has slightly broader ascospores at 12–13(14) μm (Carbone et al. 2018). *Gyromitra korfii*, which also occurs in eastern North America, has longer ascospores ((29.2)31.5–37(37.3) × (9.7)10.4–10.9(12) μm; Raitviir 1970) and more prominent apiculi. *Gyromitra montana*, which occurs in western North America and Canada, has ascospores that measure (21.4)24.3–35.8(37.5) × (9)10.7–15.8 μm with shorter apiculi (0.1–1 μm) (McKnight 1971).

Additional specimens examined: CANADA, New Brunswick, Albert, 45.88 N, 64.82 W, on soil in mixed forest of balsam fir, birch, aspen, ash, and maple, 4 May 2021, T. Gilchist (ACD0418, MICH352017) (immature); USA, Michigan, Washtenaw County, Stinchfield Woods, 42.41 N, 83.92 W, on and at base of pine in conifer plantation, 5 May 2020, A.C. Dirks (ACD0257, MICH352015); on soil and dead conifer wood in conifer plantation, 5 May 2020, A.C. Dirks (ACD0258, MICH352016); Minnesota, Saint Louis County, Duluth, Lester State Park, off Seven Bridges Road, on buried wood underneath dead spruce in mixed spruce, aspen, birch forest, 29 May 2003, B.T. Dentinger (BD179, MIN890906); New York, Tompkins County, Dryden, Observation Hill, McLean Moor, 42.65 N, 76.49 W, in mossy ground, 26 May 1917, V. Dunlap (CUP-A-024034); Tioga County, Spencer, 42.21 N, 76.49 W, 3 May 2019, A. Schmalfuss (CUP-070734) (immature).

**Fig. 1** RAxML phylogram inferred from ML and Bayesian analyses of 75 ITS sequences from type and voucher specimens of *Gyromitra americanigigas*, *G. gigas*, and *G. ussuriensis*. *Gyromitra montana* is used as an outgroup. Specimen numbers are given followed by country and state/province. Type specimens for each species are given in parentheses. RAxML bootstrap support values above 70% are shown at the nodes, and Bayesian posterior probability scores above 0.95 are shown as thickened branches.

**Fig. 2** Distribution map for all eight species in the *G. gigas* species complex. Type specimens for each species are shown as stars, and sequenced voucher specimens are shown as circles. Colors for each species are shown in the legend.
Holotype (from Vassiljeva 1950): USSR: Extremus Oriens, distr. Voroschilov in valle fl. Suputinka, ad truncos putridos Betulae et Pini korajensis, V 1946, leg. auctor; distr. Schkotovo, Maiche-Daubiche plato, ad truncos putridos Betulae costatae, VI 1947, leg. B.P. Kolesnikov et V.A. Rosenberg.

Description: Ascomata consisting of an apical hymenophore and stipe, 9–16 cm high. Hymenophore 4–10 cm diam., inflated, plicate-lobate, deformed; hymenium brown to ochraceous-brown; margin free. Stipe 4–10 cm long × 2–6 cm diam., white, longitudinally wrinkled and pitted, hollow. Excipulum one-layered, of textura intricata, hyaline. Paraphyses cylindrical, apically clavate, inflated up to 7–10 μm, thin-walled, septate, unbranched, yellow-brown in KOH. Asci 250–325 × 17.5–20 μm, operculate, thin-walled, cylindrical, hyaline, eight-spored. Ascospores uniseriate, (27)28–
32(33) × 12–13 μm ($L_m = 29.5$ μm; $W_m = 12.5$ μm; $Q = 2.2$–2.6; $Q_m = 2.4$), ellipsoid to fusoid; surface roughened; apiculi knob-like, up to 2.5 μm long; perispore up to 1 μm thick, cyanophilic; content triguttulate, with one large central oil drop and two smaller polar oil drops, hyaline.

Ecology and distribution: Solitary to scattered on soil or decayed wood of Betula, Picea, Pinus koraiensis, and Populus tremula in temperate coniferous and deciduous-coniferous forests in May and June. Known from eastern China, Japan, South Korea, and central and eastern Russia.

Notes: Gyromitra ussuriensis is similar to G. gigas, and several researchers (Raitviir 1970; Van Vooren and Moreau 2009; Carbone et al. 2018) have placed G. ussuriensis in synonymy with G. gigas. However, molecular data supports recognition of G. ussuriensis as a distinct species. Vassiljeva (1950) described the ascospores as “33–40 × 11–13 μm, golden brown apices (bar = 50 μm) (DIC), e asci with immature ascospores (bar = 50 μm) (DIC), f ascus apex (bar = 20 μm) (DIC), g-i ascospores (DIC), i ascospore stained with lactophenol in cotton blue (bar = 10 μm)”
broadly fusiform; surface rugose-tuberculate, hyaline to pale brown.” She also stated that *G. ussuriensis* is distinguished from *G. gigas* by the “smaller hymenophore that has a free margin, long stipe, and growth on wood.” The epitype of *G. gigas* selected by Carbone et al. (2018) is similar macromorphologically and micromorphologically to *G. ussuriensis*. Carbone et al. (2018) described the ascospores of the epitype of *G. gigas* as “(25)27–32(34.5) × (11.5)12–13(14) μm on free spores [the most frequent 27–30 × 12–13 μm], Q = (2.1)2.2–2.5(2.75).”

The holotype specimen at VLA is missing and presumably lost, and no original material or illustration exists (Eugenia Bulakh, pers. comm.). Therefore, a specimen collected by Vassiljeva from the same locality as the holotype is designated as neotype here (TAAM060483). Carbone et al. (2018) described the ascospores of the epitype of *G. gigas* as

\[ (25)27–32(34.5) \times (11.5)12–13(14) \text{ μm}, Q = (2.1)2.2–2.5(2.75). \]

Sequence similarity comparisons

The ITS region was compared to LSU to investigate the infraspecific and interspecific variability of these two common molecular markers. Infraspecific ITS sequence variation on average was zero in *G. americanigigas* and *G. gigas* and 0.1% in *G. ussuriensis* (Table 2). Infraspecific LSU sequence variation on average was zero in all three species. Interspecific ITS sequence variation on average ranged from 0.7% between *G. americanigigas* and *G. gigas* to 1.2% between *G. americanigigas* and *G. ussuriensis*. This is compared to 5.6–6.5% sequence variation between these three species and *G. montana*. Interspecific LSU sequence variation on average ranged from zero between *G. americanigigas* and *G. ussuriensis* to 0.1% between the other two combinations. Sequence variation was equally low ranging from 0.2 to 0.4% between the three target species and *G. montana*. Whereas the ITS displayed a significant barcode gap and can be used to recognize these three species, the LSU had no barcode gap with infra- and interspecific variation nearly identical.

The ITS1 and ITS2 regions were compared to determine whether either one of these two regions could be used as a barcode marker for molecular identification of these taxa as is the case for most environmental sampling studies (Table 3). Infraspecific sequence variation in the ITS1 ranged between 0 and 0.3% and averaged 0–0.1%. Infraspecific sequence variation in the ITS2 ranged between 0 and 0.7% and averaged 0–0.1%. The ITS1 region contained more than twice the number of parsimony-informative characters compared to the ITS2

|         | *G. americanigigas* | *G. gigas* | *G. ussuriensis* | *G. montana* |
|---------|---------------------|------------|------------------|--------------|
|         | ITS = 0             | 0.7        | 1.2              | 5.6          |
|         | (0 – 0.3)           | (0 – 1.0)  | (0.9 – 1.7)      | (3.4 – 6.4)  |
| LSU     | 0.1                 | 0.1        | 0.1              | 0.2          |
|         | (0 – 0.3)           | (0 – 0.3)  | (0 – 0.5)        | (0 – 0.3)    |
|         | ITS = 0             | 1.0        | 6.5              | 0.2          |
|         | (0 – 0.3)           | (0 – 1.4)  | (3.3 – 9.1)      | (0 – 0.3)    |
| LSU     | 0                   | 0.1        | 6.1              | 0.4          |
|         | (0 – 0)             | (0 – 0.7)  | (3.5 – 6.6)      | (0.2 – 0.7)  |
|         | ITS = 0             | 0.2        | 0.2              | 0.2          |
|         | (0 – 0)             | (0 – 0.1)  | (0 – 0.1)        | (0 – 0.1)    |
| LSU     | 0.2                 | 0.4        | 0.4              | 0.4          |
|         | (0 – 0.3)           | (0 – 0.7)  | (0 – 1.4)        | (0 – 0.3)    |
region (34 vs. 15), and thus, it was expected that, in general, the ITS1 would vary twice as much as the ITS2. Interspecific variability was three times higher in the ITS1 versus the ITS2 region in comparisons of *G. montana* to the three target species, a result consistent with our previous findings (Miller et al. 2020). Interspecific ITS1 sequence variation on average ranged from 0.9% between *G. americaniigigas* and *G. gigas* (and 0.9% between *G. americaniigigas* and *G. ussuriensis*) to 1.2% between *G. gigas* and *G. ussuriensis*. Interspecific ITS2 sequence variation on average ranged from 0.5% between *G. americaniigigas* and *G. gigas* to 0.7% between *G. americaniigigas* and *G. ussuriensis*. Whereas infraspecific variation averaged less than 0.1%, interspecific variation averaged 0.6–1.3% for all three species comparisons. The ITS1 and ITS2 regions both displayed similar sequence variability among these three species, justifying the use of either the ITS1 or ITS2 region or both as a molecular barcode marker for species identification.

**Species concepts**

Species within the *G. gigas* monophyletic group are characterized by stipitate ascomata, hymenophores that are saddle-shaped to irregularly lobed or cerebriform and wrinkled, and yellow-brown to brown to reddish brown, ribbed to sulcate, white to yellow-brown stipe, ellipsoid to fusiform ascospores that are roughened to finely reticulate and uniguttulate or triguttulate with inconspicuous to distinctive single apiculi that are up to 4 μm long. Although we attempted to apply a polyphasic approach to species delimitation, our morphological examination revealed no significant characters to distinguish *G. americaniigigas*, *G. gigas*, and *G. ussuriensis*. However, there appears to be some geographical signal to the speciation that has occurred in this group (Fig. 2). Attempts to culture species in the *G. gigas* complex failed, and we are unaware of anyone else successfully doing so (Healy et al. 2022), preventing the use of intercompatibility tests to facilitate the delimitation of species boundaries under the biological species concept. The LSU cannot be used as a barcode marker since the infra- and interspecific variation among sequences of these three species was nearly identical. However, the ITS does contain an adequate barcode gap and can be used for species identification of these three species using a phylogenetic species concept.

**Table 3**  Intraspecific and interspecific sequence variation of the ITS1 and ITS2 regions for *G. americaniigigas*, *G. gigas*, *G. ussuriensis*, and the outgroup, *G. montana*. Mean and range (in parentheses) of percent differences based on uncorrected “p” sequence differences are shown for ITS1 along the upper diagonal and for ITS2 along the lower diagonal.

|                | *G. americaniigigas* | *G. gigas* | *G. ussuriensis* | *G. montana* |
|----------------|----------------------|------------|------------------|--------------|
| **ITS1**       | 0.9 (0.9 – 1.3)      | 0.9 (0.9 – 1.1) | 0.9 (0.9 – 1.1) | 9.5 (8.9 – 10.3) |
| **ITS2**       | 0.6 (0.5 – 1.1)      | 1.2 (1.2 – 1.9) | 0.1 (0.0 – 0.5) | 10.3 (9.8 – 10.7) |
| **ITS1**       | 0.7 (0.0 – 1.4)      | 0.7 (0.0 – 1.4) | 0.7 (0.0 – 1.4) | 10.1 (9.5 – 11.9) |
| **ITS2**       | 1.3 (0.8 – 1.7)      | 1.3 (0.8 – 1.7) | 1.3 (0.8 – 1.7) | 10.1 (9.5 – 11.9) |
| **ITS1**       | 3.3 (3.2 – 3.5)      | 3.2 (3.2 – 3.5) | 3.2 (3.2 – 3.5) | 3.1 (2.9 – 3.6) |
| **ITS2**       | 3.3 (3.2 – 3.5)      | 3.2 (3.2 – 3.5) | 3.2 (3.2 – 3.5) | 3.1 (2.9 – 3.6) |
| **ITS1**       | 3.3 (3.2 – 3.5)      | 3.2 (3.2 – 3.5) | 3.2 (3.2 – 3.5) | 3.1 (2.9 – 3.6) |

**Discussion**

Although species concepts in the *Gyromitra gigas* species complex were believed to be resolved (Miller et al. 2020), some secrets still remained due to cryptic speciation. Three well-supported clades representing *G. gigas*, a new species (*G. americaniigigas*), and a rediscovered species (*G. ussuriensis*) were revealed in our phylogenetic analyses (Fig. 1). Unfortunately, these three species do not possess any significant differences by which to delimit them morphologically. Although Vassiljeva (1950) originally described the ascospores of *G. ussuriensis* as being longer than in *G. gigas*, we could not confirm her results. She also distinguished it from *G. gigas* as having a smaller hymenophore with a free margin, long stipe, and growth on wood, but these characters are also
shared by G. gigas (Carbone et al. 2018). However, these three species do occupy somewhat distinct geographical areas with G. americanigigas confined to Canada and USA, G. gigas occurring mostly in Europe, and G. ussuriensis found in Asia (Fig. 2). Some overlap between G. gigas and G. ussuriensis does occur in central Russia, and G. gigas and G. ticiniana are both found in France, Italy, and Turkey. There is also overlap in the distributions of G. americanigigas, G. korfii, and G. montana, with all three species found in Michigan, USA. Sequencing of all or part of the ITS region is highly recommended for species identification in this group.

The ITS1 and ITS2 regions both display a small, but adequate barcode gap for identifying these three species either through nBLAST similarity searches or phylogenetic analyses. The infraspecific variation averaged less than 0.1%, whereas the interspecific variation averaged 0.6–1.3% for all three species comparisons. Ideally, species hypotheses should be based on ITS interspecific variation higher than 0.5–1.5%, but as more fungal species complexes are studied and more cryptic speciation is discovered, these lower percentages for species differentiation using the ITS fungal barcode may be the norm (Lücking et al. 2020). Future studies of freshly collected specimens should sequence one or more protein-coding genes, or ideally whole genomes, and use genetic discontinuity models to test our species hypotheses (Matute and Sepúlveda 2019), which would most likely corroborate our ITS data derived from mostly older fungarium specimens.

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Author contribution ANM was responsible for the study conception and design. ANM, AD, and EP generated molecular sequence data. AD, EP, and NF provided voucher specimens. Molecular analyses were performed by ANM. Morphological analyses were performed by ASM. The first draft of the manuscript was written by ANM, ASM edited the manuscript, and all authors commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

Data availability All data and materials have been deposited in publicly accessible holdings.

Declarations

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