Multigene phylogenetic analyses to establish new *Valsaria* species and taxonomic significance of spore ornamentation

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Abstract

During our studies on fungal diversity from plant substrates, a new species of *Valsaria* was isolated from dead branches of *Ostrya carpinifolia*. The taxon is morphologically similar to other taxa in Valsariaceae and is characterized by pseudostromata, apically free pseudo-paraphyses, bitunicate asci, and dark brown, 2-celled ascospores. However, it differs from extant species in number of guttules and ornamentation of spore. It is introduced herein as *Valsaria ostryae* sp. nov. Multigene phylogenies based on combined LSU, ITS and RPB2 DNA sequence data generated from maximum likelihood, maximum parsimony and MrBayes analyses indicate that *V. ostryae* is basal to *V. lopados-tomoides* and *V. rudis* and its establishment as a new species is strongly supported. No discordance was found between our morphological and phylogenetic species boundaries as postulated by other researchers and our molecular data strongly supports ornamentation of spore as useful for species delineation. *Valsaria* species do not appear to be host specific. Full morphological details are provided herein and phylogenetic relationships of *Valsaria* species are also discussed in light with host association.

Introduction

Valsariaceae was introduced by Jaklitsch et al. [1] and classified in the order Valsariales. Species of this family have a worldwide distribution occurring on sun-exposed, corticated logs, branches of coniferous and broadleaf trees and on bamboo [1] and exist as saprobes, plant pathogens or necrotrophs [2,3]. The family comprises three genera namely *Bambusaria*...
Jaklitsch et al., *Myrmaecium* Nitschke ex Fuckel and *Valsaria* Ces. & De Not [1,4]. The asexual morphs are known to be coelomycetous [4]. *Valsaria* was introduced by Cesati and De Notaris [5] with *Valsaria insitiva* (Tode) Ces. & De Not. as the type species. Ju et al. [2] examined numerous species to provide an update of its taxonomy. Multigene phylogenetic analyses have also been performed to clarify species taxonomy [1]. In this paper, we introduce one new species of *Valsaria*, which was found on dead terrestrial branches of *Ostrya carpinifolia* (Betulaceae). Combined analyses of LSU, ITS and RPB2 DNA sequence data using maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian inference analyses clearly indicate that this species belongs to the Valsariaceae with strong statistical support. Phylogeny also provides important clues on the taxonomic usefulness of morphological features that can be used to differentiate among *Valsaria* species and its allies. In this paper, the new species is described, illustrated and compared with similar taxa.

**Materials and methods**

**Isolates and morphology**

Specimens were collected from dead branches of *Ostrya carpinifolia* in the Province of Forlì-Cesena [FC], Santa Sofia, Camposonaldo, Italy and were pressed and dried individually between blotting papers and labelled. The geographic coordinates for the data collected is 43.952106, 11.873138 (Fig 1). Specimens were observed under a Motic SMZ 168 series dissecting stereo-microscope. Hand-cut sections of the fruiting structures were mounted in water for microscopic studies and photomicrography. Pure cultures were obtained from single spore isolation following the method designated in Chomnunti et al. [6]. Germinating ascospores were transferred aseptically to malt extract agar (MEA) plates and incubated at 16°C. Colony characters on media were observed and growth rates were measured after one week and at weekly intervals. No specific permissions were required for the location because permissions are standardized only for macro-fungi such as only 3 fruiting-bodies for each collection, the indication of the name of the species that it needs to collect and other things valid only for macrofungi. Also, the field studies did not involve endangered or protected species. The holotype specimen was deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand. Ex-type living cultures are deposited in the Culture Collection at Mae Fah Luang University (MFLUCC) and at the International Collection of Microorganisms from Plants (ICMP). Faces of Fungi and Index Fungorum numbers are provided as in Jayasiri et al. [7] and Index Fungorum [8]. New taxa were established based on recommendations outlined by Jeewon and Hyde [9].

![Fig 1. Geographical coordinates of sample collected.](https://doi.org/10.1371/journal.pone.0217982.g001)
DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on MEA media at 16 ± 2°C for 4 weeks. Fungal mycelium was extracted with Biospin Fungus Genomic DNA Extraction Kit-BSC14S1 (BioFlux, P.R. China). Amplification reactions were performed using known primer pairs, LR0R/LR5 for the 28S large subunit rDNA (LSU) [10], ITS5/ITS4 for 5.8S nrRNA gene with the two flanking internal transcribed spacers (ITS) [11] and fRPB2-5F/ fRPB2-7cR for RNA polymerase II second largest subunit (RPB2) [12]. The ultimate volume of the PCR reaction were 25 μl, containing 1 μl of DNA template (40 μg), 1 μl of each forward and reverse primer (0.2 μM) and 12.5 μl of 2xMaster Mix and 9.5 μl of ddH2O. The PCR condition was as follows: initial denaturation 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 48 s, elongation at 72°C for 1 min, and final extension at 72°C for 10 min for LSU, ITS and RPB2. PCR products were sequenced with the same primers mentioned above at Sangon Biotech, Shanghai, China.

Sequence alignment and phylogenetic analyses

Phylogenetic analyses were conducted on a combined dataset of LSU, ITS and RPB2 sequence data. Taxa used in the analyses were obtained through recent publications [1, 13]. Separate LSU, ITS and RPB2 DNA sequences were also subjected to BLAST search engine tool of NCBI for verification and selection of taxa for subsequent phylogenetic analyses. Maximum likelihood analysis was performed by using raxmlGUIv.0.9b2 [14]. The search strategy was set to rapid bootstrapping and the analysis carried out using the GTRGAMMAI model of nucleotide substitution. The number of replicates was inferred using the stopping criterion [15]. Maximum Likelihood bootstrap values equal or greater than 90% are given as the first set of numbers above the nodes (Fig 2). PAUPv4.0b10 was used to conduct the parsimony analysis to obtain the phylogenetic trees. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were setup to 500 and branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length [TL], Consistency Index [CI], Retention Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI]) were calculated for trees generated under different optimality criteria. Kishino-Hasegawa tests (KHT) [16] were performed in order to determine whether trees were significantly different. Maximum-parsimony bootstrap values equal or greater than 90% are given as the second set of numbers above the nodes (Fig 2). Bayesian Inference (BI) analysis was conducted with MrBayes v. 3.1.2 [17] to evaluate Posterior probabilities (BYPP) (Rannala and Yang 1996, Zhaxybayeva and Gogarten 2002) by Markov Chain Monte Carlo sampling (BMCMC). Two parallel runs were conducted, using the default settings, but with the following adjustments: Six simultaneous Markov chains were run for 5,000,000 generations and trees were sampled every 1000 generation. The distribution of log-likelihood scores was examined to determine stationary phase for each search and to decide if extra runs were required to achieve convergence, using the program Tracer 1.5 [18]. First 20% of generated trees were discarded and remaining 80% of trees were used to calculate posterior probabilities (PP) of the majority rule consensus tree. Phylograms were visualized with FigTree v1.4.0 program [19] and reorganized in Microsoft power point (2016) and Adobe Illustrator CS5 (Version 15.0.0, Adobe, San Jose, CA). The finalized alignment and tree is deposited in TreeBASE, submission ID: 22749 (http://purl.org/phylo/treebase/phylows/study/TB2:S22749).

Nomenclature

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic
publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to Index Fungorum from where they will be made available to the Global Names Index. The unique Index Fungorum number can be resolved and the associated information viewed through any standard web browser by appending the Index Fungorum number contained in this publication to the prefix www.indexfungorum.org/_. The online version of this work is archived and available from the following digital repositories: PubMed Central, LOCKSS.

Compliance with ethical standards

There is no conflict of interest (financial or non-financial) and all authors have agreed to submission of paper. The authors also declare that they have no conflict of interest and confirm that the field studies did not involve endangered or protected species.

Results

Phylogenetic analyses

The combined LSU, ITS, RPB2 dataset which comprises 38 strains of Valsariaceae species was analyzed to determine the placement of our new taxon and infer relationships at the

Fig 2. RAxML tree based on DNA sequence analyses of a combined dataset of LSU, ITS and RPB2 gene regions. Bootstrap support values for ML and MP equal to or greater than 90%, Bayesian posterior probabilities (PP) equal to or greater than 0.90 are defined as ML/MP/PP above or below the nodes. Taxonomic novelty is indicated in red and ex-type strains are in bold text. GenBank accession numbers are indicated at the end of the species name. The tree was rooted to Tubeufia chiangmaiensis MFLU 11–1149, Tubeufia javanica MFLU 13–0371.

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intragenic level. The phylogenetic trees obtained from maximum likelihood and parsimony analyses yielded trees with similar overall topology and in agreement with previous study based on maximum likelihood analysis [1]. The maximum parsimony dataset consists of 2414 characters with 1707 characters as constant information, 52 characters as variable characters are parsimony-uninformative, and 655 characters were counted as parsimony informative character. The most parsimonious tree had the following tree scores: TL = 1666, CI = 0.744, RI = 0.930, RC = 0.692, HI = 0.256 values. The best scoring RAxML tree is presented in Fig 2. For better phylogenetic inferences derived from our analyses, we divided the ingroup taxa in the phylogram (Fig 2) into eleven clades (A–K) which corresponds to different Valsaria species, including our new taxon. All V. robiniae strains clustered together in one clade (clade A) with high statistical support (100% ML/ 99% MP/ 1.00 PP). Clade B appearing in 100% ML/ 98% MP/ 1.00 PP of the bootstrap replicates comprises V. spartii while V. insitiva strains are basal (clade C) and all these taxa constitute a strongly supported monophyletic clade (100% ML/ 100% MP/ 1.00 PP). Valsaria neotropica (clade D) is basal to V. robiniae, V. spartii and V. insitiva, but its relationships to these species receive weak support while V. rudis (clade E) and V. lopadostomoides (clade F) cluster together in a monophyletic group but with no support. Our new taxon, V. ostryae (clade G) is basal to V. lopadostomoides and V. rudis. Individual LSU, ITS and RPB2 single gene trees were generated from individual datasets and phylogenies recovered were essentially topologically similar with no conflicting phylogenetic signals (data not shown). The new sequence data are deposited in GenBank (Table 1).

Taxonomy

Valsaria ostryae D. Pem, R. Jeewon, Camporesi & K.D. Hyde, sp. nov. Fig 3
[urn:lsid:indexfungorum.org:names:554758]
Facesoffungi number: FoF 04614
Etymology—Name reflects the host from which the fungus is isolated.
Holotype—MFLU 17–0837
Saprobic on dead branch of Ostrya carpinifolia. Sexual morph: Stromata pseudostromatic, erumpent from bark, scattered, sometimes aggregating in groups of 2–3; pustular, conical to sub-globose with flattened base, 500–800 μm high, 1000–1500 μm diam., enclosed on top and/or at sides by a black, 20–40 μm thick pseudoparenchymatous crust. Ectostroma often forming sub- or inversely stellate structures of 3–5 greyish, brown to black, tubercular segments; tissue beneath crusty pseudoparenchymatous; tissue between necks and at stromatal base prosenchymatous, grey, soft, mixed with bark cells. Ostiolar openings inconspicuous at the surface or indistinctly papillate. Ascomata arranged in valsoid configuration, monostichous, sub-globose to flask-shaped, 277–479 μm high, 284–603 μm diam., peridium 25–40 μm thick, of pale brown compressed cells of textura angularis. Paraphyses numerous, unbranched, tapering upwards, apically free, 2–3 μm wide. Asci 82–131 × 9–12 μm (x = 110 × 11 μm, n = 15), 8-spored, bitunicate, fissitunicate, cylindrical, apex containing an ocular chamber and a short cylindrical to barrel-shaped ring, 2–3 μm wide, 3–4 μm high (x = 2 × 3 μm, n = 10), staining in Congo Red. Ascospores 16–17 × 7–9 μm, (x = 16 × 7 μm, n = 20), ellipsoid, 2-celled, dark brown, with a dark, thick, central, slightly constricted septum; surface finely warted to reticulate. Asexual morph: Pycnidia 0.1–0.4 mm diam, globose, multiloculate, black, irregularly surrounded by white mycelium, wall pseudoparenchymatous, thick-walled, globose to clavate cells, giving rise to short, cylindrical to ellipsoid hyaline cells. Phialides 7–18 × 2–5 μm (x = 13 × 3 μm, n = 20) produced in more or less parallel, densely packed in palisades, narrow, lageniform, cylindrical or conical. Pycnoconidia 14–18 × 2–9 μm (x = 16 × 5 μm, n = 20) oblong, cylindrical to ellipsoid, sometimes subglobose, 1-celled, hyaline, smooth, lower end sometimes truncate (Fig 4).
Table 1. Culture collection code and GenBank accession numbers of fungal strains used in this study.

| Species                  | Isolate No. | Code  | Herbarium No. | Substrate/Host | Country          | LSU | ITS | Rpb2     |
|--------------------------|-------------|-------|---------------|----------------|------------------|-----|-----|----------|
| Bambusaria bambusae      | MFLUCC 12–0851 | DDQ0025 | MFLU 15–0050  | Thysrostachys siamensis | Thailand | K687812 | K687812 | K687890 |
| Bambusaria bambusae      | CBS 139763  | DDQ00254 | MFLU 15–0051 | Thysrostachys siamensis | Thailand | K687813 | K687813 | K687891 |
| Myrmaecium fulvopruinatum| CBS 139057  | VF     | WU 33433      | Fagus sylvatica  | Austria          | K687858 | K687858 | K687933 |
| Myrmaecium fulvopruinatum| VFi          | WU 33434 | Fraxinus excelsior, Alnus glutinosa | Austria | K687859 | K687859 | K687934 |
| Myrmaecium fulvopruinatum| VFB          | WU 33436 | Betula pendula | Austria | K687860 | K687860 | K687935 |
| Myrmaecium fulvopruinatum| CBS 139058  | VFi    | NY (M.E.B.B. 6905) (E) | Fagus grandifolia | U.S.A. | K687861 | K687861 | K687936 |
| Myrmaecium fulvopruinatum| CBS 139059  | VFQ    | VU 33438      | Quercus ceras | Austria          | K687863 | K687863 | K687938 |
| Myrmaecium rubricosum    | CBS 139067  | VRF    | WU 33447      | unidentifed bark | France          | K687881 | K687881 | K687955 |
| Myrmaecium rubricosum    | VRJ          | WU 33448 | Quercus robur | France | K687882 | K687882 | K687956 |
| Myrmaecium rubricosum    | VRJ1         | WU 33449 | Picea abies   | U.S.A. | K687883 | K687883 | K687957 |
| Myrmaecium rubricosum    | VRM          | WU 33449 | Picea abies   | Austria | K687884 | K687884 | K687957 |
| Myrmaecium rubricosum    | CBS 139068  | VRP    | WU 33450      | Quercus pubescens | Croatia | K687885 | K687885 | K687958 |
| Myrmaecium rubrum        | CBS 109505  | Quers sp. | Italy        | K6456324 | - | - | - |
| Tubeufia chiangmaiensis  | MFLUCC 11–0514 | - | MFLU 11–1149  | dead wood of an unidentified tree | Thailand | K301538 | K301538 | - |
| Tubeufia javanica        | MFLUCC 12–0545 | - | MFLU 13–0371,  | dead culm-sheath of bamboo | Thailand | K880036 | K880034 | - |
| Valsaria insitiva        | CBS 139056  | VA     | WU 33453      | Acer monspessulanum | Croatia | K687847 | - | K687922 |
| Valsaria insitiva        | VAC          | WU 33454 | Acer campestr | Austria | K687849 | K687849 | K687924 |
| Valsaria insitiva        | VAF          | WU 33455 | Ficus carica  | France | K687850 | K687850 | K687925 |
| Valsaria insitiva        | VCE          | WU 33456 | Cercis siliquastrum | Greece | K687854 | K687854 | K687945 |
| Valsaria insitiva        | CBS 127882  | VV     | WU 33462 (E)  | Vitis vinifera  | Croatia | K687866 | K687866 | K687959 |
| Valsaria insitiva        | CBS 139062  | VIQ    | WU 33470 (H)  | Quercus ilex | Greece | K687868 | K687868 | K687945 |
| Valsaria insitiva        | CBS 139064  | VJM    | WU 33471 (H)  | unidentified corticated twig | France | K687874 | K687874 | K687948 |
| Valsaria ostryae         | MFLUCC 11–0514 | - | MFLU 11–1149  | dead wood of an unidentified tree | Thailand | K301538 | K301538 | - |
| Valsaria ostryae         | MFLUCC 18–1123 | MFLU 17–0837 | Ostrya carpinifolia | Italy | M337873 | M337874 | M337875 |
| Valsaria robiniae        | CBS 121890  | VC     | WU 33472      | Hippocrepis emer | Slovenia | K687851 | K687851 | K687926 |
| Valsaria robiniae        | CBS 128015  | VCA    | WU 33474      | Caragana arborescens | Austria | K687853 | K687853 | K687928 |
| Valsaria robiniae        | CBS 125583  | VCo1   | WU 33475      | Colutea arborescens | Austria | K687855 | K687855 | K687930 |
| Valsaria robiniae        | V1R          | WU 33477 | Robinia pseudacacia | Italy | K687869 | K687869 | K687944 |
| Valsaria robiniae        | CBS 139063  | VIR1   | WU 33478      | Robinia pseudacacia | Italy | K687870 | K687870 | K687945 |
| Valsaria rubis           | VQC          | WU 33483 | Quercus ceras | Croatia | K687877 | K687877 | K687951 |
| Valsaria rubis           | CBS 139065  | VMQ    | WU 33484      | Quercus macrolepis | Greece | K687878 | K687878 | K687952 |
| Valsaria rubis           | CBS 139066  | VQP    | WU 33485 (E)  | Quercus pubescens | Austria | K687879 | K687879 | K687953 |
| Valsaria rubis           | V3           | WU 33486 | Quercus ceras | Italy | K687836 | K687836 | K687914 |
| Valsaria rubis           | V7           | WU 33487 | Quercus petraea | Austria | K687844 | K687844 | K687920 |
| Valsaria rubis           | V31          | WU 33488 | Quercus petraea | Austria | K687838 | K687838 | K687916 |
| Valsaria spartii         | CBS 139070  | V6     | WU 33505 (E)  | Spartium junceum | Italy | K687843 | K687843 | K687919 |
| Valsaria spartii         | V18          | WU 33515 | Ulex parviflorus | Spain | K687823 | K687823 | K687901 |
| Valsaria spartii         | V19          | WU 33516 | Teline linifolia | Spain | K687824 | K687824 | K687902 |
| Valsaria spartii         | V20          | WU 33517 | Cytisus baeticus | Spain | K687826 | K687826 | K687904 |
| Valsaria spartii         | V21          | WU 33518 | Retama monosperma | Spain | K687827 | K687827 | K687905 |

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Culture characteristics: Ascospores germinating on MEA within 24 h. Colonies growing on MEA, reaching 2 cm diam. in 1 week at 16˚C. Mycelium medium dense, circular, slightly raised, surface smooth, edge slightly crenate, thinly hairy, slightly irregular margin, above whitish to pale yellow, reverse dark brown in middle and yellowish at the edge.

Material examined: ITALY, Province of Forli-Cesena [FC], Santa Sofia, Campoisonaldo, on dead branches of *Ostrya carpinifolia* (Betulaceae), 18 March 2017, E. Camporesi, IT 3290 (MFLU 17–0837, holotype; HKAS 97468, isotype) ex-type living culture MFLUCC 18–1123, ICMP 22558.

Discussion

Species of *Valsaria* occur primarily on woody parts of ornamental plants but is also distributed on a wide range of hosts. Our new taxon was isolated from *Ostrya carpinifolia* which belongs to Eudicots and Betulaceae family [20]. Substrates colonised by members of *Valsaria* recorded until now are all dicotyledons of the Fabaceae, Fagaceae and Vitaceae. *Valsaria lopadostomoides* can be found on evergreen oaks, such as *Quercus ilex* (Fagaceae) while *V. rudis* occur on deciduous oaks, such as *Quercus cerris* and *Q. petrae* (Fagaceae). Woody plant hosts of *V. lopadostomoides* and *V. rudis* are closely related to each other as both are accommodated in the Fagaceae which is a monophyletic family close to Quercoideae [21]. *Valsaria neotropica* has been isolated from unidentified hosts in the Neotropics and seems to be distributed in the
Fig 4. *Valsaria ostryae*: Asexual morph (MFLU 17–0837). a-c Culture characteristics and mycelium. d, e hyphae with denticles and conidia (partly budding). f–j Phialides. j–o Conidia. Scale bars: c = 1000 μm, d = 20 μm, e = 10 μm, f–j = 10 μm, j–o = 10 μm. Notes—*Valsaria ostryae* resembles *V. insitiva* (type species), in having pseudostromatic stromata, pseudoparenchymatous ectostroma, bitunicate asci containing short cylindrical ring and ellipsoid, dark brown ascospores. However, *V. ostryae* differs from *V. insitiva* in having narrower pseudoparaphyses (2–3 μm vs. 1.5–5 μm), guttule number in each ascospore cell (0 vs. 2–3) and in ornamentation and constriction of the ascospores. The ascospores are
deeply constricted at septum and finely warted to reticulate in *V. ostryae* while those of *V. insitiva* are not or hardly constricted at the septum and finely tuberculate. Our multi-gene phylogeny suggests that *V. ostryae* is related to *V. lopadostomoides* and *V. rudis* but phylogenetically segregated from them with strong bootstrap support (100% ML, 100% MP, 1.00 PP). *Valsaria ostryae* has smaller stromata (500–800 μm high, 1000–1500 μm diam v.s 1200–1400 μm high, 1700–2500 μm diam), shorter asci (82–131 × 9–12 μm v.s (112–)115–128(–136)×(10.5–)11.0–14(–15.5) and have different ascospore ornamentation (finely warted to reticulate v.s distinctly warted, reticulate or spotted). Our new taxon is also morphologically different from *V. rudis* in having smaller stromata (1000–1500 μm diam v.s 1200–1700 μm high, 1200–2500 μm diam) and smaller ascocoma (277–479 μm high, 284–603 μm diam, v.s 3000–6000 μm high, 2000–5000 μm diam). Furthermore, a comparison of 600 nucleotides across the ITS (+5.8S) gene regions between *lopadostomoides* reveals 6.60% base pair differences respectively. Hence, *V. ostryae* is introduced as a new species in the genus *Valsaria*.

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...tropical terrestrial ecoregions of the Americas and the South American temperate zone. *Valsaria insitiva* was described mainly from non-fabaceous hosts and *Vitis* (Vitaceae) which is accommodated in its own order Vitales and closely related to Rhamnaceae in the order Rhamnales [22]. *Valsaria robiniiae* have been reported from *Amorpha, Caragana, Colutea, Hippocrepis* and *Robinia* species in Central Europe, submediterranean regions and Eastern USA while *V. spartii* occur on various species of woody Fabaceae in the Sub-Mediterranean. Host distribution of *V. robiniiae* and *V. spartii* seems to be related as most of the species are from Fabaceae which is the third largest family of angiosperms after Orchidaceae and Asteraceae [23]. Based on available literature, it is apparent that *Valsaria* has a wide host range and species are less likely to be host specific. As far as we are aware, no *Valsaria* species are known from *Ostrya carpinifolia*. We compare the new species with *V. insitiva*, the type species of *Valsaria* and they share similar characters. However, they differ in the number of guttules in each ascospore cell, as well as in the surface ornamentation of the ascospores (Fig 3). Our new taxon can also be compared to *V. spartii* and *V. robiniiae* and differs in ascospores that are not constricted. *Valsaria ostryae* also shares similar morphological characters to *V. rudis* and has 2–3 guttules in each ascospore cell, while *V. rudis* has no guttule in its ascospore cells. Previous morphological studies and phylogenetic investigations based on SSU, ITS-LSU RPB2 and TEF genes data have provided insights into the taxonomy and classification of *Valsaria* and its allies [1]. The most recent taxonomic work dealt with more than 100 collections of *Valsaria sensu lato*, including two new species, which has helped to resolve generic delimitation, circumscription and classification of *Valsaria* [1]. In this study, a new species with a unique morphological character is illustrated and its relationships with other species are investigated based on multigene phylogenetic analyses. Our molecular data analyzed herein generated phylogenies that are congruent with those of Jaklitsch et al. [1] and helps to resolve intergeneric relationships between *Bambusaria, Myrmaecium* and *Valsaria*. Three well supported monophyletic groups are recovered which support *Bambusaria, Myrmaecium* and *Valsaria* as phylogenetically distinct genera (Fig 2). Nevertheless, we observe that several morphological characters such as ostiole, apical ring, and pseudoparaphyses are interspersed in several clades which could be examples of homoplasy in morphological characters within the *Valsariaeae*. Our molecular phylogeny provides an overview of the usefulness of specific morphs for species and generic delineation.

One interesting finding herein is the significance of spore surface ornamentation in taxonomy. Ornamentation is a character commonly used to delineate species [24–28]. The taxonomy of fungi is based to a large extent on spore characteristics including spore size, shape, color, surface ornamentation and ontogeny [29–34]. Among asexual fungi, spore pigmentation and ornamentation have been crucial in the taxonomy of appendaged coelomycetes. Jee-won et al. [35, 36], Maharachchikumbura et al. [37] and Liu et al. [38] have demonstrated that versicolourous and nonversicolourous spore can be used to segregate species. Among Pleosporalean fungi, Punithalingam [39] used spore surface ornamentation as a diagnostic feature to differentiate *Coniothyrium* species. Among other sexual fungi, twenty-one species of
Eupenicillium were successfully segregated into two groups (with vs without distinct equatorial ridges) based on ornamentation of ascospores [40]. Spore septation and colour have also been used as characters for distinguishing genera and species of the family Lophiostomataceae by many mycologists such as Saccardo, who established 9 separate genera using these criteria [41]. Species of the genus Aspergillus Section Nigri, which are taxonomically confused and complex have also been identified and delineated based on spore ornamentation [42]. The latter has also been reported to be useful to demarcate sexual and asexual species at the familial and generic level [43–47]. Surface ornamentation and wall development have also been examined to segregate species of the Pachyphloides (Pezizaceae, Pezizales) as well as to differentiate between species, genera and families of the Basidiomycetes [48]. Villegas et al. [49] reported spore ornamentation as a useful character in differentiating various genera within Gomphales. Clitopilus reticulosporus was described as a new species (Entolomataceae, Agaricales) based on its unique spore ornamentation and the spore evolution theory has been well discussed by Morgado et al. [50]. Interestingly, ornamentation of ascospores as well as guttule number have also been significant to establish new genera and demarcate taxa within Helvellaceae (Pezizales, Ascomycota) such as Gyromitra subgenus Melaleucoides (warted, non apiculate, biguttulate ascospores), Gyromitra subgenus Caroliniana (coarsely reticulate ascospores with multiple spicules at the poles) and Helvella subgenus Cerebriformae (globose, echinate ascospores) [51].

A similar phylogenetic scenario is reported herein. The three clades corresponding to different genera can be easily distinguished based on spore ornamentation. Valsaria is characterized by fine warted to reticulate or finely tuberculate ascospores surface, whereas Bambusaria and Myrmaecium species possess densely reticulate and longitudinally ribbed ornamentation respectively. Even at the species level, ornamentation can be used to a certain extent to segregate species. For example, V. insitiva the type species, can easily be delineated from other Valsaria species herein, except V. neotropica, based on finely tuberculate spore ornamentation. Valsaria rudis and Valsaria lopadostomoides are similar in having ascospores surface 'warted to reticulate' but additionally the ascospores of V. lopadostomoides is described as ‘spotted’ [1]. In addition, M. fulvopruinatum, characterized by densely reticulate spores can be distinguished from M. rubrum and M. rubricosum, whose spore surface has labyrinth ornamentation. We can therefore rely mostly on such morphology to circumscribe species at this taxonomic level. However, relationships based on other morphs, such as stromatal characteristics, presence or absence of ostioles/pseudoparaphyses/apical ring, septum constriction and number of guttules show little congruence with findings from molecular data and are not phylogenetically significant for intergeneric segregation. Nevertheless some of these characters, especially the latter two, if used judiciously can be used to circumscribe species. For instance, V. lopadostomoides and M. fulvopruinatum can be distinguished from other Valsaria and Myrmaecium species on the basis of number of guttules. The number of guttules per spore has also been considered as a diagnostic feature to delineate taxa at the intraspecific level. For example, guttule character was found as important to separate species and varieties recognized in the genus Phoma [52] and helped to provide a key to differentiate among thirty taxa. Recently, Southworth et al. [53] demonstrated that number of guttules in each spore has been useful to delineate species of Balssania when other characters overlap. Vander [54] reported that the occurrence of large guttules was useful to distinguish species of Phyllosticta, especially when used in combination with other characters. Presence of guttule is especially useful when confirming diagnosis of herbarium material from fresh specimen and this has helped to validate Lophiostoma diminuens (Pers.) Fuckel [55]. The diagnostic microscopical characters supporting ranks of different taxa of the family Sarcoscyphaceae were mainly based upon differences in the guttule (lipid) number and pattern of living ascospores [41]. To differentiate genera within Gnomoniaceae, Monod [56] used characters such as presence or absence of small guttules. The latter has also
been considered as a diagnostic feature to separate \textit{Diaporthe} species from grapevine [57] as well as freshwater fungal species [58, 59]. Guttule characters can also be used to distinguish our new taxon, \textit{V. ostryae} from others. Phylogeny herein indicates \textit{V. ostryae} to be more closely related to \textit{V. lopadostomoides} and \textit{V. rudis} than any other species. Although these three species share several common morphological features (pseudostromatic stromata, apically free pseudoparaphyses, apical ring and dark brown ascospores), our new taxon is clearly distinct in number of guttules and surface ornamentation.

To further substantiate establishment of \textit{Valsaria ostryae} as a new species, the complete ITS and RPB2 gene regions were compared. Nucleotides differences reveal clear cut differences that warrant new species status. For example, \textit{V. ostryae} differs from \textit{V. lopadostomoides} by 5.31\% bp difference in the ITS gene regions. Considering both molecular and morphological data we regard \textit{V. ostryae} as a new taxon that warrants species rank. The genus \textit{Valsaria} is known to comprise taxa with minor morphological variations that result in taxonomic confusion. For example, the ascomata of \textit{Valsaria insitiva}, \textit{V. lopadostomoides} and \textit{V. neotropica} are monostichously arranged in valsoid configuration measuring 250–450 $\mu$m high, 180–400 $\mu$m diam, peridium 14–25 $\mu$m thick, cylindrical asci with a short cylindrical to barrel-shaped ring measuring in between 96–112 $\mu$m x 11–18.5 $\mu$m and 2-celled, ellipsoid dark brown ascospores with the size ranging from 12–19 $\mu$m x 6.5–11.7 $\mu$m. However, when analyzing the gene regions, they are all genetically different. ITS base pair difference between \textit{Valsaria insitiva} to \textit{V. lopadostomoides} is 2.4\%, \textit{V insitiva} to \textit{V. neotropica} is 11.8\%, and \textit{V. lopadostomoides} to \textit{V. neotropica} is 14.3\%. Likewise, other \textit{Valsaria} species such as \textit{V. robiniae}, \textit{V. rudis}, \textit{V. spartii} and our new taxon share similar morphological characters which are not really meaningful in species delineation.

This study provides delightful taxonomic insights across \textit{Valsaria} species. We anticipate that taxonomists will pay particular attention to these characters that can potentially aid in species identification of other genera. Our study also reveals that \textit{Valsaria} could be a speciose genus and there is a need for more collections targeting a wide variety of hosts which can help to answer pertinent questions with regards to fungal diversity issues.

**Author Contributions**

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