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Molecular Phylogeny of *Trametes* and Related Genera from Northern Namibia

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Abstract

Trametes Fr. is widely characterized as a polyporoid cosmopolitan genus which is presented in almost any type of forest environments. It is characterized by a combination of pileate basidiocarp, porous hymenophore, trimitic hyphal system and thin-walled basidiospores which do not react in Melzer's reagent. Dry polypores were collected from Northern Namibia and identified as *Trametes* species based on morphology. Molecular analysis of Internal Transcribed Spacer region 1 (ITS 1) and Internal Transcribed Spacer region 2 (ITS 2) of the collected material revealed inconsistency with morphological identification. The phylogenetic tree was reconstructed using the Neighbour Joining method and reliability for internal branches Assessment was done using the ML bootstrapping method with 500 ML bootstrap replicates applied to 44 unpublished sequences and sequences from GenBank database. Only specimens such as D1-D9, D11 and D13 and specimens F1, I2-I4 and K3-K6 were grouped in the trametoid clade together with *Trametes* species. Furthermore, the position of *Trametes trogii* (also known as *Coriolopsis trogii*) was confirmed to be outside the trametoid clade and more closely related to *Coriolopsis gallica*. The close relationships of *Pycnoporus* and *Trametes* were confirmed by grouping of *Pycnoporus sanguineus* in to trametoid clade. Alignment with GenBank sequences revealed identity to *Trametes* species with up to 99%. These results suggest that it is better to keep a single generic name of *Trametes* for the trametoid clade.

Key words: *Trametes* species, Phylogeny, Polyporoid, Namibia.

1. Introduction

Traditionally, fungal taxonomy was based mainly on morphological description of the fruit body, host specificity, and geographical distribution (Seo and Kirk, 2000; Olusegun, 2014). However, polyporoid fungi in the *Trametes* genus have a similar morphology and have proved to be challenging to identify based on this traditional technique (Ofodile *et al*., 2007). Ever since, mycologists have turned to molecular techniques to explain the taxonomic challenges in *Trametes* and related genera (Zakaria *et al*., 2009; Olusegun, 2014).

The *Trametes* classification has undergone extensive study and deliberation (Zhao *et al*., 1983). Although the Friesian (1835) description is widely accepted, there are ongoing studies to find a clear species delimitation for this Polyporoid group (Justo and Hibbett, 2011). *Trametes* Fr. is widely characterized as a polyporoid cosmopolitan genus which is presented in almost any type of forest environment. It is characterized by a combination of pileate basidiocarp, porous hymenophore, trimitic hyphal system and thin walled spores which do not react in Melzer's reagent (Tomšovsky *et al*., 2006; Carlson *et al*., 2014). *Trametes* Fr. was first named by Fries in 1835 because the hymenophore was considered a distinctive feature of the *Polyporus* genus and Fries wanted to accommodate *Coriaceous* species with a poroid hymenophore characterized by context continuously descending into the hymenophoral trama. At this stage, genera were created according to the hymenophore structure and were either grouped as lamellate, daedaleoid or regular (Trametoid) pores (Welti *et al*., 2012).

In 1886, Quèlet initially separated species by the shape of their pores but later considered other morphological features relevant to define new genera from the classical *Trametes*. The abhymenial surface of the tomentum was considered as a distinctive feature of the *Coriolus* group. Another description was suggested for a *Trametes* group consisting of all genera with di- or trimitic hyphal system with colorless, smooth and not amyloid basidiospores. This group included *Cerrena*, *Daedalea*, *Hexagonia*, *Pycnoporus*, *Coriolopsis*, *Datronia*, *Lenzites*, *Megasporoporus*, *Microporus*, *Trichaptum* and *Trametes* (Ko and Jung, 1999). Kavina and Pilát in 1936 also supported the view that hymenophoral morphology suggested by the Fries is devoid of generic systematic value. Therefore, species with lamellate, daedaleoid and poroid hymenophore were grouped into one, combining *Lenzites* and *Daedalea*. *Lenzites betulina* was combined with *Trametes sensu* Pilát (Welti *et al*., 2012).

Nobles (1958) further considered the significant role of wood rot type caused by the fungi as a distinguishing feature between the polypores. The white rot *Trametes* group was delineated from *Daedalea*, a brown rot fungus. In 1967, David argued that the heterocytic nuclear

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behavior with bipolar mating system separates *Funalia* and *Cerrena* from *Trametes* and *Coriolopsis* (Welti *et al*., 2012). In 1989, Corner observed many tropical species whose intermediate characters could change subject to basidiocarp conditions. The white flesh of *Trametes pubescens* would become brown due to age and mistakenly identified as *Coriolopsis polyzona* which has a brown context. As a result, Corner reported that pigment and rot type alone are not sufficient characters for species delimitation (Justo and Hibbett, 2011; Ko and Jung, 1999). Ryvarden's classification in 1991, corresponded with the widely accepted Friesian description of pileate basidiocarps, poroid hymenophore, trimitic hyphal system, smooth thin-walled basidiospores and white rot (Ko and Jung, 1999). Ryvarden also included all the genera synonymized by Corner in 1989, but excluded brown rot causing genera, such as *Daedalea* and *Fomitopsis* (Justo and Hibbett, 2011; Welti *et al*., 2012).

In 1995, Hibbet and Donoghue used the mitochondrial SSU rDNA to study the phylogeny of *Trametes* and related genera. It was concluded that trimitism in white rots was a common feature for all genera in the *Trametes* clade (Welti *et al*., 2012).

Ko (2000) used SSU mtDNA and ITS sequences to divide the Polyporaceae into two subgroups, where subgroup A contained: *Cryptoporus*, *Daedaleopsis*, *Datronia*, *Funalia*, '*Coriolopsis*' *gallica*, '*Tramella*' *trogii*, *Ganoderma*, *Lentinus*, *Microporus* and *Polyporus*. Subgroup B only had *Coriolopsis polyzona*, *Lenzites*, *Pycnoporus* and *Trametes*. Context pigmentation was also considered to be a distinctive feature for the identification of *Coriolopsis* Murril, which is now *Coriolopsis polyzona*. In 1881, *Pycnoporus* P. Karsten was created to distinguish trametoid specimens which had a brown or red (cinnabarin) colour (Ko and Jung, 1999).

These different concepts on the generic limits of *Trametes* have led to confusion and unresolved species delimitation in the genus (Carlson *et al*., 2014; Olusegun, 2014). It is not clear whether closely related genera, such as *Coriolopsis*, *Coriolus*, *Lenzites* and *Pycnoporus* in subgroup A, should be recognized as independent monophyletic genera or if they should be included in an enlarged *Trametes* genus (Welti *et al*., 2012).

Furthermore, during the past decade, researchers in Namibia developed an interest in studying the indigenous mushrooms of Namibia. Studies by Kadhila-Muandingi and Chimwamurombe (2012) and Ekandjo and Chimwamurombe (2012) focused mostly on medicinal mushrooms, specifically *Ganoderma* species. The problem remains that most Basidiomycetes in Namibia still need to be explored in order to document and preserve the Namibian mushrooms biota and biodiversity (Chang and Mshigeni, 2004).

Lastly, the incorrect taxonomy of many medicinal mushrooms jeopardizes the validity of current and future investigations of these mushrooms and their derivatives (Wasser, 2011; Zmitrovich *et al*., 2012). The use of general names like Turkey tail (*Trametes versicolor*) makes room for mistaken identity of specific species and type material (Wasser, 2011; Wasser, 2014). Therefore, there is a need for consistency in the identification of medicinal mushrooms, like *Trametes* species, to ensure that future investigations of their medicinal properties, composition and effectiveness are done on the right species. This study characterizes indigenous *Trametes* species from Northern Namibia in order to generate information on its genetic diversity. This study endeavors to confirm the identity of *Trametes* representatives from Northern Namibia using ITS region. The present work also attempts to reconstruct the phylogeny of indigenous Namibian *Trametes* using sequences from GenBank Database.

2. Materials and Methods

2.1. Material Studied

Dry mushrooms were collected from dead wood in three regions, namely Ohangwena, Omusati, and Oshana regions in Northern Namibia during late March and early April 2014. The samples were identified with pictures from Van der Westhuizen and Eicker (1994). The samples were recorded and kept in khaki paper bags labeled with name of village, region and host substrate type.

The collected mushrooms were dried in a cool shade for 6 hours and kept in a cool dry place. All visible sand and wood particles were removed before grinding the mushrooms to powder using sterilized mortar and pestles. For this study, samples C1-C4, C21, D1-D7, D9, D11, D13, I2-I4, J1-J4, J6-J9 and K3-K6 were collected from Ohalushu in Ohangwena region, while E1-E5 where collected from Okalumbi in Omusati region and M6 and M7 were collected from Omakango in Ohangwena region.

2.2. DNA Extraction

A Qiagen DNeasy® Plant Mini Kit (Hilden, Germany) and protocol was used to extract fungal genomic DNA from indigenous Namibian *Trametes* species and according to manufacturer's instruction. An adjustment was made to the protocol by reducing Buffer AE from 100 µL to 50 µL.

2.3. PCR and Sequencing

PCR amplification was performed in a 25 µl reaction consisting of 12.5 µL DreamTaq Green PCR Master Mix (2X), 10.5 µL nuclease free water, 1 µL ITS1-F primer (CTTGGTCATTTAGAGGAAGTA), 1 µL ITS2 primer (GCTGCGTTCTTCATCGATGC) and 1 µL DNA. PCR conditions were as follows: Pre-denaturation at 95°C for 4 mins, denaturation at 95°C for 30 s, annealing at 55°C for 1min and elongation at 72°C for 2 mins for 35 cycles. This was followed by a final extension of 72°C for 7 mins. PCR products were viewed using gel electrophoresis. Gel electrophoresis was performed in 0.5 % Tris-Borate EDTA (TBE) buffer. The DNA gel was prepared by dissolving 1 g agarose gel in 100 mL TBE buffer (1 %) and completely dissolving it by heating in the microwave. After slight cooling, 2.5 µL ethidium bromide was added to the gel before casting in a tray. Gel electrophoresis was run at 110 V for 60 minutes after which the gel was visualized under Ultra-Violet (UV) light. Sequencing of PCR product was performed in both directions using ITS1 and ITS2 primers at Inqaba Biotechnical Industries (Pty) Ltd in South Africa. 88 pairs of sequences of 200-250 base pairs were produced.

2.4. Alignment of Sequences

Sequences were analyzed and predicted by utilizing Chromas Lite201 version 2.1.1 (Queensland, Australia). A Local Alignment of the ITS1 and ITS2 sequences was performed in Bioedit to create contig sequences. A BLAST search was performed on the resulting 44 contig sequences using the NCBI GenBank database. The unpublished sequences as well as the sequences obtained from GenBank were aligned with Bioedit and Clustal W.

2.5. Phylogenetic Analysis

The Maximum Likelihood (ML) analysis was performed in MEGA version 6 (Tempe, USA). The phylogenetic tree was reconstructed using the Neighbour Joining method and reliability for internal branch assessment. It was done using the ML bootstrapping method with 500 ML bootstrap replicates.

3. Results

The mushrooms collected presented a morphology characteristic of *Trametes* species, although a high variation was observed in size and color of fruit bodies, size of pores, concentric zones and rigidity of mushroom upon breaking or tearing. Based on these morphological differences, at least four *Trametes* species were identified (Figure 1). Specimen A has a grey basidiocarp with 73 mm diameter with 1 to 2 mm wide pores underneath. Specimen B has a thin and dark brown basidiocarp 70 mm in diameter with black prickly 'hairs' on the top surface and 1mm sized pores on the hymenium. Specimen C was the smallest of the four types observed with 25 mm diameter basidiocarp and 1 mm sized pores. The basidiocarp was covered with distinct zones with different shades of grey, white and black. Specimen D basidiocarp was 45 to 75 mm in diameter with zones that have shades of tan, grey, black and white colour. The hymenium was covered with many small white pores ranging from 2-3 pores/millimeter. The mushrooms lacked a distinct pileus as they were attached directly to their host.

The ITS region of nuclear rDNA from 40 *Trametes* species from Northern Namibia was used for molecular identification. The sequences obtained were aligned with sequences from GenBank. Alignment with GenBank sequences revealed a variety of identities, with most sample species scoring 95-99 % similarity to the *Trametes* and *Coriolopsis* genera. The rest of samples showed similarity scores of 92-94 % to genera, such as *Hexagonia*, *Truncospora* and *Fomes*. For example, samples C1- C4 and C21 showed 99 % similarity to *Coriolopsis caperata* and D1-D7, D9, D11 and D13 are 99 % identical with *Trametes polyzona*. Samples E1, E3 and E4 showed 93 % similarity with *Truncospora macrospora*. Specimens F1, I2-I4, K3-K6 and M6 are 93-99 % identical with *Trametes* species, while E2, G6, J2, J6 and J7 resemble *Hexagonia* species with 92-94 % identity (Table 1).

The phylogenetic tree generated from the unpublished sequences and sequences from GenBank showed some variations. The phylogenetic tree has 8 major clades in total. Clade 1 contains specimens D1-D9, D11 and D13, and specimens F1, I2-I4 and K3-K6 as well as GenBank sequences *Trametes polyzona* (JN164979.1), *Coriolopsis polyzona* (FJ627248.1), *Trametes gibbosa* (FJ481048.1), *T. villosa* (KF573031.1), *T. hirsuta* (GU062274.1), *T. maxima* (JN164918.1), T. *cinnabarina* (AB735965.1), *Pycnoporus sanguineus* (AJ537499.1), *Trametes cubensis* (KJ654513.1), *T. orientalis* (AB735966.1), *T. elegans* (EU661879.1), *T. ljubarskii* (GU731579.1) and *T. marianna* (KC848334.1). Clade 2 contains specimens E1, E3 and E4 alongside *Truncospora macrospora* (JX941573.1). Clade 3 contains *Hexagonia tenuis* (KC414233.1), *Daedaleopsis* sp. (KF541330.1) and *Fomes fomentarius* (EF155494.1). Clade 4 contains specimens G6 and G9, while clade 5 contains specimens C1-C4, C21, M6, M7 and GenBank sequences *Coriolopsis caperata* (AB158316.1), *C. trogii* (KJ093492.1), *Funalia trogii* (EU273516.1), *Coriolopsis gallica* (JN165013.1), *Trametes suaveolens* (FJ478094.1) and *T. trogii* (HM989941.1). Clade 6 has *Hexagonia hirta* (KC867359.1), *C. aspera* (KP013018.1) and *H. apiaria* (KC867362.1). Clade 7 has specimens E2, E5, J1 and J3-J9, while Clade 8 only contains specimen J2. The specimens collected from Northern Namibia were distributed in 6 clades mainly alongside *Trametes* species *Truncospora* and more distantly *Coriolopsis* species.

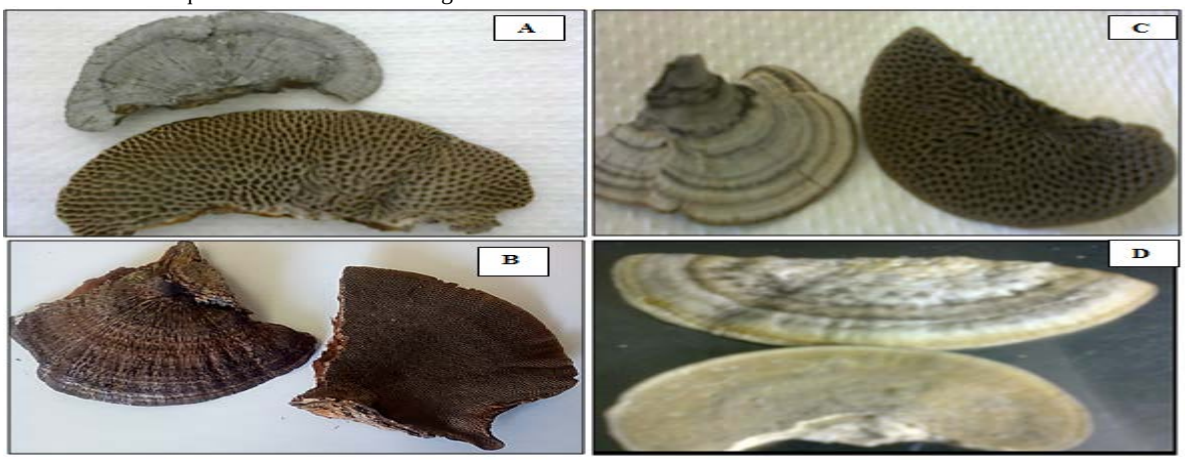

Figure 1. *Trametes* samples collected from Northern Namibia. The mushrooms collected had four distinct morphologies. Mushroom A had a grey basidiocarp with daedalean like pores. Mushroom B had black hair like structures with fine regular pores. Mushrooms C and D had distinct zones on the basidiocarp with different shades of tan, grey, white and black.

Table 1. Molecular identification of *Trametes* species from Northern Namibia based on ITS region

Sample	Organism name	Accession Number	Identity scores (%)
C1	*Coriolopsis caperata*	KF564288.1	99
C2	*Coriolopsis caperata*	KF564288.1	99
C3	*Coriolopsis caperata*	HQ323692.1	99
C21	*Coriolopsis caperata*	HQ323692.1	99
D1	*Trametes polyzona*	JN164979.1	99
D2	*Trametes polyzona*	JN164979.1	99
D3	*Trametes polyzona*	JN164979.1	99
D4	*Trametes polyzona*	KJ654516.1	99
D5	*Trametes polyzona*	JN164977.1	98
D6	*Trametes polyzona*	JN164980.1	99
D7	*Trametes polyzona*	JN164980.1	99
D9	*Trametes polyzona*	JN164978.1	99
D11	*Trametes polyzona*	KP013053.1	99
D13	*Trametes polyzona*	JX941573.1	99
I3	*Trametes marianna*	JQ806418.1	99
K3	*Trametes* sp	KP013021.1	99
M7	*Coriolopsis caperata*	AB158316.1	99
C4	*Coriolopsis caperata*	GQ372861.1	98
D5	*Trametes polyzona*	JN164977.1	98
M6	*Trametes trogii*	HM989941.1	98
I4	*Trametes ljubarski*	HM136871.1	97
K4	*Trametes ljubarski*	JQ806418.1	96
F1	*Trametes villosa*	KC414233.1	95
I2	*Trametes villosa*	KC848334.1	95
E2	*Hexagonia apiaria*	JX941573.1	94
J1	*Fomes* sp.	KC867359.1	94
J3	*Fomes* sp.	KF541332.1	94
J4	*Fomes* sp.	KC867362.1	94
J6	*Hexagonia apiaria*	KC867359.1	94
J8	*Fomes* sp.	KF541332.1	94
J9	*Fomes* sp.	KF541332.1	94
K6	*Trametes villosa*	JN164970.1	94
E1	*Truncospora macrospora*	KC867362.1	93
E3	*Truncospora macrospora*	JX941573.1	93
E4	*Truncospora macrospora*	HM136871.1	93
E5	*Fomes* sp.	KF573031.1	93
J2	*Hexagonia hirta*	HM136871.1	93
J7	*Hexagonia hirta*	KF541332.1	93
K5	*Trametes hirsuta*	JF439511.1	93
G6	*Hexagonia tenuis*	JN164995.1	92
G9	*Coriolopsis trogii*	JN164970.1	92

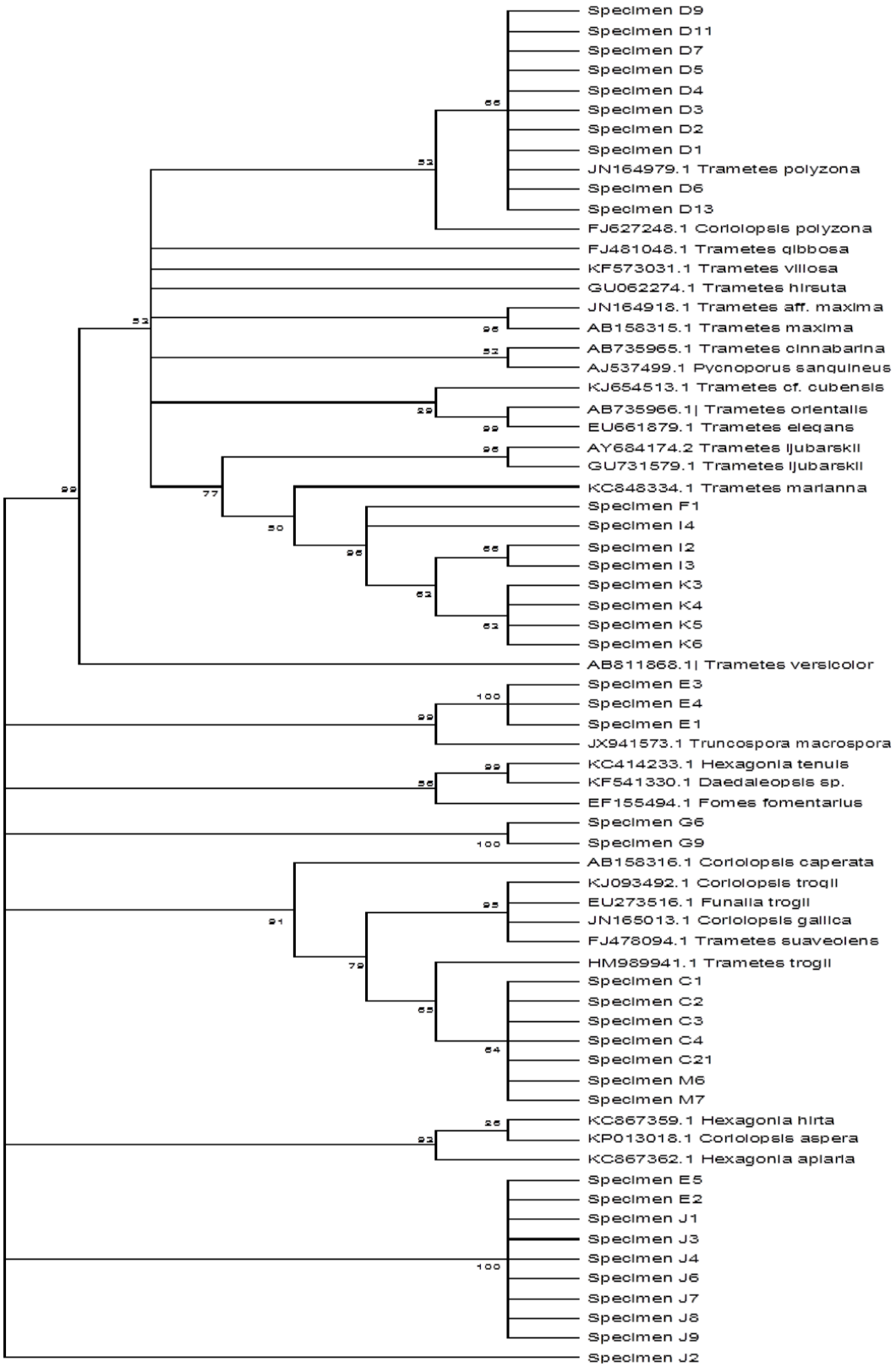

Figure 2. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbour-Joining method [1]. The optimal tree with the sum of branch length = 1.40518603 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [2]. The evolutionary distances were computed using the p-distance method [3] and are in the units of the number of base differences per site. The analysis involved 69 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 162 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [4].

4. Discussion

Dry *Trametes* specimens were collected from dead wood in three regions of Northern Namibia. The samples were identified positively with pictures from Van der Westhuizen and Eicker (1994). The fungi collected presented a morphology characteristic of *Trametes* species, although a high variation was observed in size and color of fruit bodies, size of pores, concentric zones and rigidity of the context upon breaking or tearing. Based on these morphological differences, at least four *Trametes* species were identified (Figure 1). Although these polypores were positively identified as *Trametes* species using morphologic features, molecular results show that not all the samples collected were indeed *Trametes* species. This proves that, although the traditional taxonomy of *Trametes* species was based on morphological features, it is not always reliable because these features are affected by nutrient status and growth conditions. Some species are so similar in their morphology that it is difficult to delineate them based on morphology alone (Gilbertson and Ryvarden 1987; Yang *et al*., 2010).

Maximum Likelihood and Neighbour Joining analysis was used to reconstruct a phylogenetic tree of *Trametes* species from Northern Namibia. The resulting phylogenetic tree showed 8 major clades in total. The placement of unpublished sequences into these different clades indicates that these sequences are not from the same ancestral origin as the sequences in GenBank (Olusegun, 2014). Sequences placed in the same clade share a common ancestor from whom they have inherited a set of unique characters (Baldauf, 2003).

It is interesting to observe that specimen D1-D9, D11 and D13 and specimens F1, I2-I4 and K3-K6 were all collected from Ohalushu village in Ohangwena region. However, specimens E2, E5, J1 and J3-J9 were also collected from Ohalushu village in Ohangwena region but they formed a separate clade. According to Olusegun (2014), these differences might occur because of geographical and environmental factors. Geographic factors are responsible for fungal diversity at a regional level in a radius of 1000-4000 km, while environmental factors may cause diversity at a local level within a radius of less than 1000 km.

In the phylogenetic tree obtained in the present study, specimens D1-D9, D11 and D13 and specimens F1, I2-I4 and K3-K6 were grouped in the trametoid clade together with *Trametes* species, such as *T. polyzona*, *T. gibbosa*, *T. villosa*, *T. hirsuta*, *T. maxima*, *T. cinnabarina*, *T. cubensis*, *T. orientalis*, *T. elegans* and *T. marianna*. These results are similar to those reported by Welti *et al*. (2012) and Carlson *et al*. (2014). Welti *et al*. (2012) reconstructed the phylogeny of the *Trametes* group using Bayesian analysis of ITS1-5.8S-ITS2 region and RPB2 protein coding gene to confirm the close relationship between the genera *Trametes*, *Coriolopsis* (*polyzona*) and *Pycnoporus*. One of the most recent and comprehensive works on *Trametes* is phylogeny using molecular data from the ribosomal Large Subunit (LSU) rRNA and ITS region as well as the RPB1, RPB2 and TEF1-alpha protein coding genes. Similar to the results obtained, the five-marker molecular analysis strongly supported a Trametoid clade which includes most *Trametes* species (*T. suaveolens*, *T. versicolor*, *T. maxima*, *T. cubensis*) and *Lenzites*, *Pycnoporus* and *Coriolopsis polyzona* species. Furthermore, the position of *T. trogii* (*Trametes trogii*) was confirmed to be outside the Trametoid clade and more closely related to *C. gallica* (Tomšovsky *et al*., 2006; Justo and Hibbett, 2011). The genus *Coriolopsis* is currently defined as polyphyletic with type species in the trametoid clade and two additional lineages in the core Polyporoid clade (Carlson *et al*., 2014). This explains why *Trametes trogii* was placed in a clade much further from other *Trametes* species but closer to *Coriolopsis gallica* and *C. trogii* (Figure 2). Except for the red color of *Pycnoporus* basidiocarp, it is morphologically similar to *Trametes*. Other biochemical characters between the two genera do not differ and molecular analysis of the ribosomal DNA groups the two genera in one clade (Tomšovsky *et al*., 2006), just as confirmed in the present study by grouping *Pycnoporus sanguineus* in the trametoid clade with *Trametes* species (Figure 2).

The authors support the decision to keep a single generic name of *Trametes* for the trametoid clade because, according to Justo and Hibbett (2011), which allows the preservation of the morphological concept of *Trametes*, the classification of additional species which may not yet be sampled or analysed and the classification of *Trametes* species using morphological features alone.

Any other decision to divide the trametoid clade is deemed extremely difficult or even impossible (Justo and Hibbett, 2011). Therefore, the question whether closely related genera, such as *Coriolopsis*, *Coriolus*, *Lenzites* and *Pycnoporus*, should be recognized as independent monophyletic genera, or whether they should be included in an enlarged *Trametes* genus (Welti *et al*., 2012) has been answered, albeit temporarily.

5. Conclusion

In conclusion, the present study is able to confirm for the first time, the identity of *Trametes* mushrooms from Northern Namibia using ITS region and to reconstruct the phylogeny of these indigenous *Trametes* using sequences from GenBank Database. From the results above, the authors identify specimens C1-C4, C21, D1-D7, D9, D11, D13, F1, I2-I4, K3-K6 as well as M6 and M7 to be *Trametes* species. Specimens E1, E3 and E4 are identified as *Pycnoporus* species.

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