Mycosphere 8(10): 1975–1983 (2017) www.mycosphere.org ISSN 2077 7019
Article
Doi 10.5943/mycosphere/8/10/17
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What is the best: A four marker phylogenetic study of the dark-spored myxomycete *Fuligo septica*

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Hoppe T 2017 – What is the best: A four marker phylogenetic study of the dark spored myxomycete *Fuligo septica* Mycosphere 8(10), 1975–1983, Doi 10.5943/mycosphere/8/10/17

Abstract

Myxomycetes are mono-cell eukaryotic protists with a sexual and asexual life cycle. A morphological system was established within the last 200 years and most of the species are easily distinguishable. During the last decades, however, a number of gene sequences have been made available for many myxomycete species with different gene markers. Forty-three specimens of the myxomycete species *Fuligo septica*, including its three varieties, were used for a comparison of phylogenetic relations with four different gene markers namely: (i) elongation factor-1alpha (EF-1alpha), (ii) small ribosomal subunit (18S rRNA), (iii) ribosomal internal transcribed spacer (ITS2) and (iv) mitochondrial small ribosomal subunit (mtSSU). Phylogenetic trees calculated for the four different gene markers all showed similar results. Haplotype networking for the gene markers showed differences between the networks of the different varieties for the 18S rRNA and mtSSU, but not for EF-1alpha and ITS2.

Key words – 18S rRNA – EF-1alpha – ITS2 – haplotype analyses – morphological analysis – mtSSU – population study

Introduction

Myxomycetes, also called slime molds, have a worldwide distribution. These organisms are characterized by a multinucleated one-cell plasmodia and a resistant fruiting body. The life cycle can be divided into a haploid and a diploid part (Stephenson 2011). In both parts, a multiplication of the cell is possible. Within a short time, a large population of the same best adapted genotype can expand. This possibility allows these organisms to establish rapidly in a variety of habitats (El Hage et al. 2000). Consequently, many of the species described have a worldwide distribution. Myxomycetes are heterothallic or/and non-heterothallic (Clark & Haskins 2010) depending on the available environmental conditions. A good example would be the species *Fuligo septica* that has a typical representative of the asexual and sexual life cycles (Hoppe & Kutschera 2010). A haploid spore produces an amoeba. They divide mitotically and have predatory roles in soils (Hoppe & Kutschera 2015). A fusion event of two haploid myxamoeba (apomixes) generates diploid plasmodia. The negative phototactic plasmodia feed on organic materials and change the behavior for fruit body (aethalia) development into a positive phototactic one. First, the diploid nuclei are enclosed in one spore. Here they are converted by means of reduction division in a haploid state.

There are five orders of myxomycetes, which have been widely been studied as a large group. From population studies of morphological and molecular-genetic characters became clear that this

Submitted 16 October 2017, Accepted 21 December 2017, Published 30 December 2017
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organismic group is heterogenic (Nandipati et al. 2012, Winsett & Stephenson 2011). Therefore, we used the common species *Fuligo septica*, with its known varieties, to examine its population structure. The genus *Fuligo* is placed in the order Physarales. It is characterized by very large yellow, rarely white plasmodia (phaneroplasmodia). An important characteristic of *Fuligo septica* are the brown spores of less than 8-10 µm in diameter (Table 1, Poulain et al. 2011).

Table 1 – Identification guide of the genus *Fuligo*.

1	Cortex spongiose		2
1*	Cortex smooth, rough		7
2	Spore 6-10 µm		3
2*	Spore 10-22 µm		4
3.1	Cortex yellow and calcareous nodes white	***F. septica* var. *septica***	
3.2	Cortex and calcareous nodes white	***F. septica* var. *candida***	
3.3	Cortex and calcareous nodes yellow, older collections show color only at regions between cortex to substrat	***F. septica* var. *flava***	
3.4	Cortex and calcareous nodes purple	***F. septica* var. *rosea***	
3.5	Cortex white to purpel or red, calcareous nodes purpel, smaller spores	***F. septica* var. *rufa***	
4	Spore 10-13 µm		5
4*	Spore 14-22 µm	***F. megaspora***	
5	Cortex yellow, Spore oval	***F. licentii***	
5*	Spore nodular		6
6	Aethalium more than 10 mm, calcareus nodes white	***F. intermedia***	
6*	Aethalium mostly max. 10 mm, calcareus nodes yellow	***F. muscorum***	
7	Aethalium white, calcareus nodes white, spore oval, 10-12 x 14-17 µm, spore ornamentation net-like	***F. cinerea***	
7*	Aethalium coloured, spore ornamentation without network-structure		8
8	Aethalium yellow, calcareus nodes white, spore oval, smooth with few humps, 7 x 8-10 µm	***F. luteonitens***	
8*	Spore nodular with small humps		9
9	Aethalia yellow		10
9*	Aethalia red-brown, spore 7-10 µm,	***F. leviderma***	
10	Spore mass black, spore equally-dyed	***F. aurea***	
10*	Spore mass black-brown, spore lopsided brightened, spores smaller than 10 µm	***F. laevis***	

At present *Fuligo* has five varieties and the cortex and the calcareous nodes are important features to distinguish them. The species may have a yellow, white or pink aethaloid fruiting bodies. *Fuligo septica* var. *candida* (Fig. 1A) quickly loses the pigmentation after the fruit body matures. The pigment and the lime of the cortex and the pseudocolumella is clearly recognizable. The aethalia of *F. septica* var. *flava* (Fig. 1B) is permanently yellow. The cortex is early dwindling. The pseudocolumella is yellow to colorless. The aethalia of *F. septica* var. *rufa* (Fig. 1C) has a pink-white to pink-brown aethalia. The pseudo-capillitium is white to pink. The spores of these subspecies are significantly smaller than the other varieties. The investigated strains were described as heterothallic and non-heterothallic (Clark & Haskins 2010). In this study with investigate three varieties of *Fuligo* with molecular data to establish if the varieties are supported.

Due to the wide distribution of this species and the various reproductive strategies, geographic genotypes are hard to determine. A non-heterothallic reproduction allows (in combination with the clonal reproduction of the haploid amoebae) a rapid manifestation of characteristics (genetic expression results in morphological diversity).

Figure 1 – The investigated varieties: A *Fuligo septica* var. *candida* with a typical white cortex, B *F. septica* var. *flava* with the yellow pigmentation and C a fresh aethalia of *F. septica* var. *rufa* with a red pigmentation.

Material and Methods

Fruiting bodies were sampled in the field or obtained from materials of M. Schnittler (University of Greifswald, Germany), Y. Novozhilov (Russian Academy of Sciences, Russia) and W. Nowotny (Riedau, Austria). Species and variety determinations were carried out using current literatures (Neubert et al. 1995, Poulain et al. 2011).

Morphological characters: Fruiting bodies were measured by light microscope. Spores were air-dried and prepared for scanning electron microscopy (Hoppe & Kutschera 2010). SEM pictures were taken with a magnification of 2000×. The size of spores and nodes structures were analyzed by the software's AxioVison SE64 (Carl Zeiss Microscopy, Germany). The number of ornaments was measured within a range of 2.4 µm. The measurement was automated using ImageJ (NIH, Maryland, USA).

Genomic characters: DNA was isolated from 43 aethalia of *Fuligo septica*. Spores were transferred into an Eppendorf cup and homogenized by using a Ribolyzer (Hybaid, USA). Genomic DNA was extracted with Invisorb Spin DNA Extraction Kit (Stratec Biomedical AG, Germany) according to the manufacturing protocol. PCRs were done for four different gene markers (18S rRNA, EF-1alpha, ITS2 and mitochondrial DNA - mtSSU). Primers used for this study came from different publications or in some instances new primers were designed (Table 2).

Table 2 Used primers for the four investigated gene markers.

Gene Marker	Name	Sequence (5' → 3')	Reference
18S rRNA	S3c_F	CTGAATCTGCGWACGGCTCCGC	Hoppe & Schnittler 2015
	S31_R	AATCTCTCAGGCCCACTCTCCAGG	Hoppe & Kutschera 2016
EF-1alpha	MYX f3	CGGAAGCTTTCAAYAARATGGA	Baldauf & Doolittle 1997
	EF-F4D	CGTCAAGAAGATCGGWTWCAAYCCCGAGAA G	in this study
	EF-L3f	GAAGWCCGCYAACCTCSCATGGTACAAGGGA	in this study
	EF-SF1r	ACACATGGGCTTGGAAGG	Hoppe & Kutschera 2010
ITS2	phf1b	AAAACTCACCAGGTCCRGAT	Kamono & Fukui 2006
	phf2	GTCATGCCCTTAGATGTT	Kamono & Fukui 2006
	phr2b	TACAAAGGGCAGGGACGCAT	Kamono & Fukui 2006
mtDNA	mtCore 1F	TAGTGTTATTCGTGATGACT	Hoppe 2013
	mtCore 2F	CTCGAATTAAACCACAT	Hoppe 2013
	mtCore_4R	GTTTCKCGCGTTARCTTYAAACC	Hoppe 2013

The PCR-products were sequenced by Beckman Coulter Genomics (GB) and checked by Chromas 2.6 (Technelysium Pty Ltd, Australia, Table 3).

Table 3 Specimens used in this study.

Specimen	Ref. Num.	Coordinates	18S rRNA	ITS2	EF-1alpha	mtSSU
F. septica	LE254971	N51°46'10.9/ E87°41'3.0	KT778649	KU057879	KT958794	KT778618
F. septica var. *candida*	MYX1415	N54°02'13/ E13°25'44	KT778652	KU057886	KT958797	KT778625
F. septica var. *candida*	MYX1418	N54°07'37/ E13°18'16	KT778653	KU057887	KT958798	KT778626
F. septica var. *candida*	MYX48	N51°13'/ E8°54'	KT778656	KU057880	FJ546670	KT778619
F. septica var. *candida*	MYX490	N50°55'32.9/ E7°56'33.5	KT778657	KU057881	KT958801	KT778620
F. septica var. *candida*	MYX815	N50°50'36.2/ E8°02'22.2	KT778659	KU057883	KT958803	KT778622
F. septica var. *flava*	MYX1452	N48°34'15.6/ E8°14'20.2	KT778655	KU057889	KT958800	KT778628
F. septica var. *flava*	MYX1043	N50°56'15.9/ E8°02'28.1	KT778660	KU057890	KT958804	KT778629
F. septica var. *flava*	MYX1044	N50°56'15.9/ E8°02'28.1	KT778661	KU057891	KT958805	KT778630
F. septica var. *flava*	MYX1172	N50°50'42.4/ E8°02'27.0	KT778662	KU057892	KT958806	KT778631
F. septica var. *flava*	MYX1226	N50°50'36.9/ E8°02'25.4	KM977866	KU057893	KT958807	KT778632
F. septica var. *flava*	MYX1234	N50°50'41.4/ E8°02'27.7	KT778663	KU057894	KT958808	KT778633
F. septica var. *flava*	MYX1235	N50°50'43.8/ E8°02'23.0	KT778650	KU057884	KT958795	KT778623
F. septica var. *flava*	MYX1240	N50°50'42.3/ E8°02'26.1	KM977867	KU057895	KT958809	KT778634
F. septica var. *flava*	MYX1249	N50°55'22.7/ E7°56'36.1	KP323378	KU057896	KT958810	KT778635
F. septica var. *flava*	MYX1254	N50°50'36.2/ E8°02'23.5	KT778664	KU057897	KT958811	KT778636
F. septica var. *flava*	MYX1409	N54°07'47/ E13°20'51	KT778665	KU057898	KT958812	KT778637
F. septica var. *flava*	MYX1410	N54°07'28/ E13°18'12	KT778666	KU057899	KT958813	KT778638
F. septica var. *flava*	MYX1413	N54°07'27/ E13°18'11	KT778667	KU057900	KT958814	KT778639
F. septica var. *flava*	MYX1414	N54°07'19/ E13°18'16	KT778651	KU057885	KT958796	KT778624
F. septica var. *flava*	MYX1419	N54°02'45/ E13°25'32	KT778654	KU057888	KT958799	KT778627
F. septica var. *flava*	MYX1480	N49°06'01.2/ E13°15'03.7	KM977869	KU057901	KT958815	KT778640
F. septica var. *flava*	MYX1519	N49°05'56.1/ E13°13'59.6	KM977870	KU057902	KT958816	KT778641
F. septica var. *flava*	MYX412	N51°19'30.7/ E10°49'41.9	KT778668	KU057903	KT958817	JX125025
F. septica var. *flava*	MYX458	N50°50'/ E8°02'	KM977871	KU057904	KT958818	JX125026
F. septica var. *flava*	MYX459	N50°50'/ E8°02'	KM977872	KU057905	KT958819	JX125027
F. septica var. *flava*	MYX462	N50°50'/ E8°02'	KT778669	KU057906	KT958820	JX125028
F. septica var. *flava*	MYX466	N50°50'/ E8°02'	KT778670	KU057907	KT958821	JX125029
F. septica var. *flava*	MYX467	N50°50'/ E8°02'	KT778671	KU057908	KT958822	JX125030
F. septica var. *flava*	MYX469	N50°50'/ E8°02'	KT778672	KU057909	KT958823	JX125031
F. septica var. *flava*	MYX471	N50°50'/ E8°02'	KT778673	KU057910	KT958824	JX125032
F. septica var. *flava*	MYX482	N50°50'/ E8°02'	KT778674	KU057911	KT958825	JX125033
F. septica var. *flava*	MYX501	N50°50'35.3/ E8°02'24.1	KT778675	KU057912	KT958826	JX125035
F. septica var. *flava*	MYX502	N50°50'35.5/ E8°02'24.1	KM977873	KU057913	KT958827	JX125036
F. septica var. *flava*	MYX514	N51°00'15.4/ E8°05'46.2	KT778658	KU057882	KT958802	KT778621
F. septica var. *flava*	MYX525	N50°56'20.3/ E8°02'59.6	KT778676	KU057914	KT958828	JX125038
F. septica var. *flava*	MYX551	N51°06'56.8/ E8°57'23.9	KT778677	KU057915	KT958829	KT778642
F. septica var. *flava*	MYX602	N50°55'22.4/ E7°56'35.7	KT778678	KU057916	KT958830	KT778643
F. septica var. *flava*	MYX683	N50°55'20.3/ E7°56'45.8	KT778679	KU057917	KT958831	KT778644
F. septica var. *flava*	MYX847	N50°50'36.7/ E8°02'24.8	KT778680	KU057918	KT958832	KT778645
F. septica var. *flava*	MYX865	N50°50'40.9/ E8°02'48.3	KT778681	KU057919	KT958833	KT778646
F. septica var. *rufa*	MYX1742	N52°55'15.2/ E14°01'37.4	KT778682	KU057920	KT958834	KT778647
F. septica var. *rufa*	MYX372	N48°18'7.36/ E13°38'3.13	KM977874	KU057921	JF317291	KT778648

Alignments were made with MEGA 6 analysis software (Tamura et al. 2013). Relations between haplotypes for every marker were constructed with TCS 1.21 (Clement et al. 2000). A neighbor joining tree of a joined alignment was calculated by TOPALI v2.5 (Biomathematics & Statistics Scotland, GB).

Results

Phylogenetic trees were constructed for every genetic marker with Neighbor-Joining method and phylogenetic networks for the specimens were estimated (Figs 2–4).

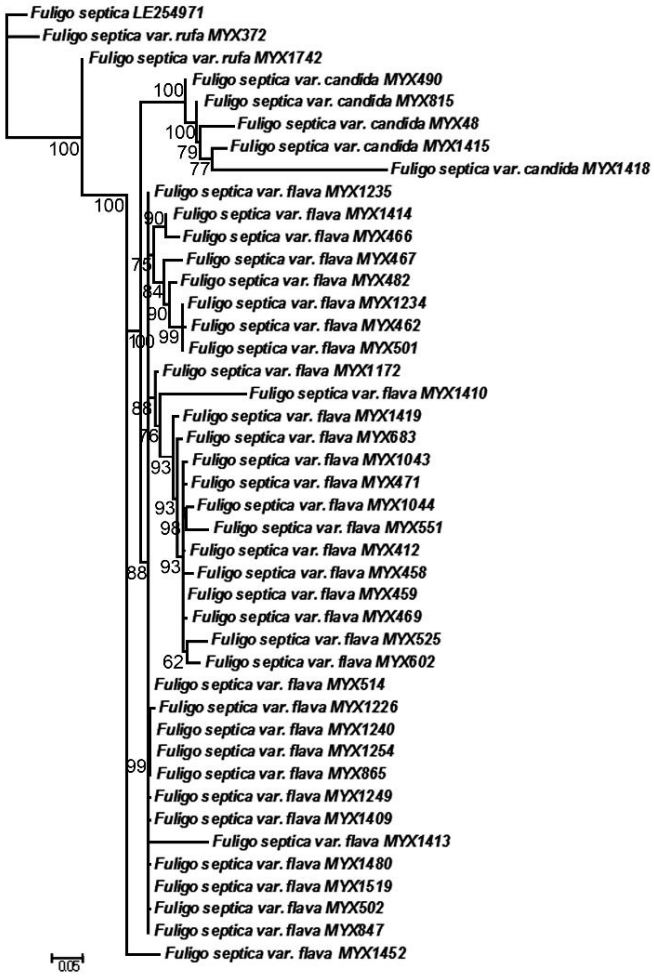

Figure 2 – Phylogenetic tree of the joined alignment from four gene markers (charset=1038bp, 1.000 runs, 1.000.000 generations, sample freq. 10, burnin 50%).

The analysis of the individual markers showed similarities to each other and to the morphologic system. The investigated specimens of the three varieties clustered together in similar arrangements. With the combination of the four genetic markers, a combined alignment was constructed and a concatenated tree was calculated (Fig. 2). However, bootstrap values and radiation events are not identical. In all four phylogenies (Fig. 3-4), two major clades within the *F. septica* var. *flava* are formed. The numerically small groups of *F. septica* var. *candida* and *F. septica* var. *rufa* clustered together and the phylogenetic tress of the markers 18S rRNA (Fig. 3A) and mtSSU (Fig. 4B) showed radiation events.

An analysis of the haplotype network for 18S rDNA (Fig. 5A) show three haplotypes of each variety, which are not attached to each other. There are seven haplotypes for *F. septica* var. *flava*, three haplotypes for *F. septica* var. *candida* and three haplotypes for *F. septica* var. *rufa* calculated. The analyzing of the sequence from EF-1alpha (Fig. 5B) distinguished 22 haplotypes (14 *F. septica* var. *flava*, six *F. septica* var. *candida* and two *F. septica* var. *rufa*). The four haplotypes of *F. septica* var. *flava* and *F. septica* var. *candida* showed no connection to each other. The analysis of the fragment of the ITS2 region (Fig. 5C) shows relations between *F. septica* var. *flava* (eight haplotypes) and *F. septica* var. *candida* (four haplotypes). Here, MYX1415 shares the haplotype with representatives of the variety *flava*. Two haplotypes showed no relation to a different haplotype. The variety *rufa* has two haplotypes. The analysis of the haplotypes for the mtSSU has shown 21 haplotypes (Fig. 5D). Two haplotypes (MYX1418 and MYX1044) have no connection to another haplotype. All varieties show only connections to haplotypes of the same variety.

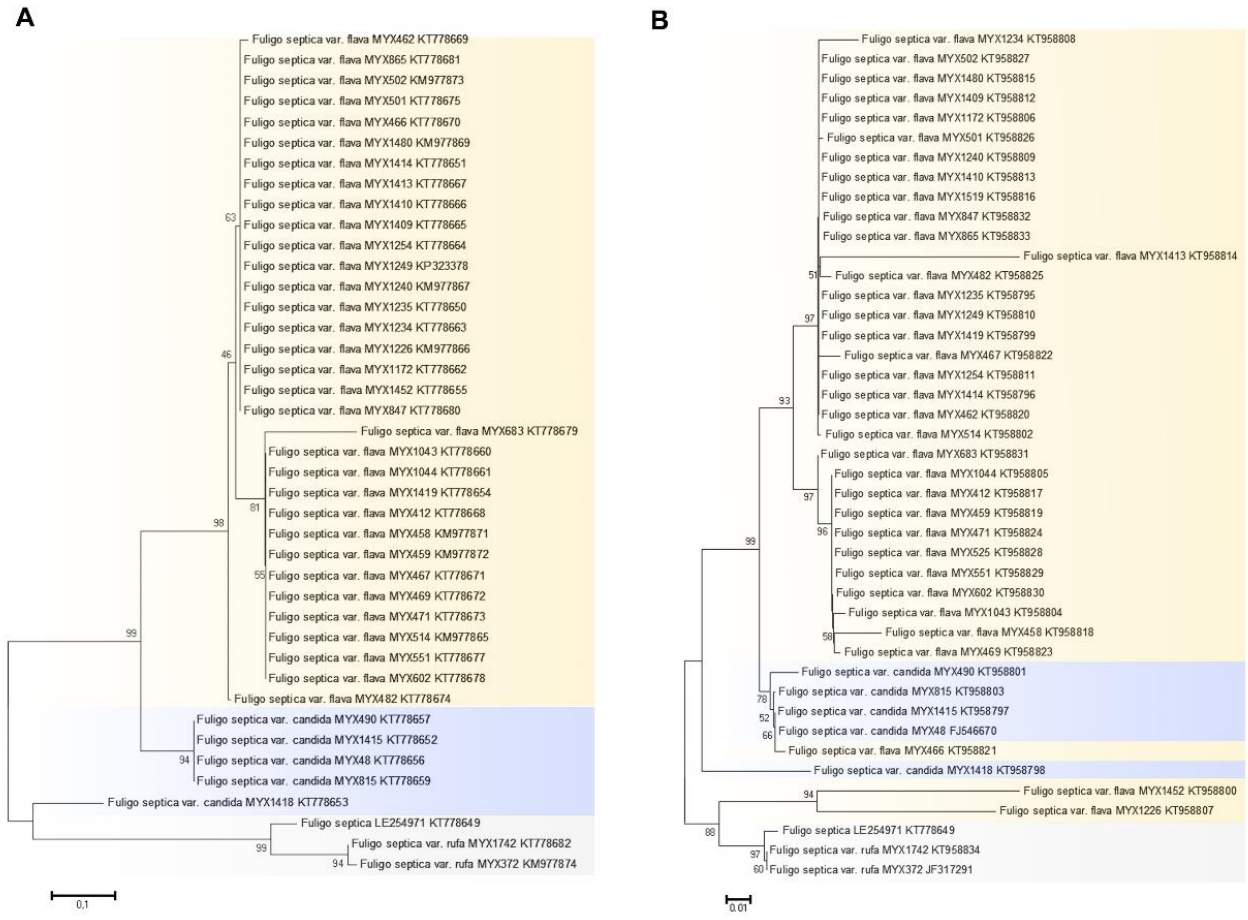

Figure 3 – Phylogenetic trees calculated with Neighbor Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. A) phylogenetic tree based on 251 positions of partial 18S rRNA sequence; B) phylogenetic tree based on 548 positions of partial EF-1alpha sequence.

The loss of pigmentation after a short storage of time was made clearly visible for all candida collections. This variety showed a significant limy cortex (single and double). In *F. septica* var. *flava*, the yellow pigment is stable for a longer time and can also be seen in older collections (sometimes only in the basal region of the cortex next to the hypothallus). The cortex of *F. septica* var. *rufa* is slightly reddish (not as much as in *F. septica* var. *rosea*). The cortex has at least two layers, wherein the outer carries the reddish pigment. For the studied representatives this outer cortex broke quickly after collecting from the inside layer. The varieties *F. septica* var. *candida* and *F. septica* var. *rufa* have a higher content of lime than *F. septica* var. *flava*. The spore diameter was measured and values between 6.7 and 9 µm. Here all varieties were similar to each other: *F. septica* var. *flava* 7.58 µm (n=1988), *F. septica* var. *candida* 7.53 µm (n=380) and *F. septica* var. *rufa* 7.26µm (n=270). The number of warts or spines on the spores was slightly smaller at *F. septica* var. *candida* (0.24 µm) than *F. septica* var. *flava* (0.29 µm). The number of spikes per area was for *F. septica* var. *candida* statistically higher (23.3 spines/ circle) than in *F. septica* var. *flava* (18.8 spines/ circle) and *F. septica* var. *rufa* (21.0 spines/ circle). *F. septica* var. *candida* showed less variations within the different individuals. The analysis of other characteristics such as cortex structure, number of nodes or fruiting body size showed no differences.

Discussion

Fuligo septica is a myxomycete species with conspicuous fruiting bodies. The systematic position from three varieties could be investigated by four different gene markers. All three varieties differed (except MYX1415 in ITS2, MYX1452 and MYX1226 in EF-1alpha). The 18S

rRNA and mtSSU trees corroborate with the morphological system. The calculated concatenated tree of all four markers distinguishes these morphological varieties. The radiation events are different in each of the four calculated trees, as well as the haplotype networking analyses. After the comparison of the data for these four different markers, the markers 18S rRNA and mtSSU seems to be the most appropriate tools for the analysis of populations. In the haplotype networking analysis, with any of the markers used here, every of the individuals were placed in a relationship. This could be due to the too small range of possible genotypes. Within all analysis, the variety *F. septica* var. *rufa* was clearly separated from the other varieties (also in the phylogenetic trees). The 18S rRNA is relatively highly conserved, with an intron (Johansen et al. 1996, Fiore Donno et al. 2008 2013, Nandipat et al. 2012). This intron is evolving faster than the flanking elements.

ITS2 and the partial 18S rRNA are two adjacent fragments, but greatly differ from each other. The ITS2 is conserved by more than 60% and therefore can be used for species determination (Kamono & Fukui 2006). This resulted in good discriminating ability of phylogenetically closely related individuals. However, the haploid-analysis shows no systematic relationship between the studied varieties. Only *F. septica* var. *rufa* is disconnected.

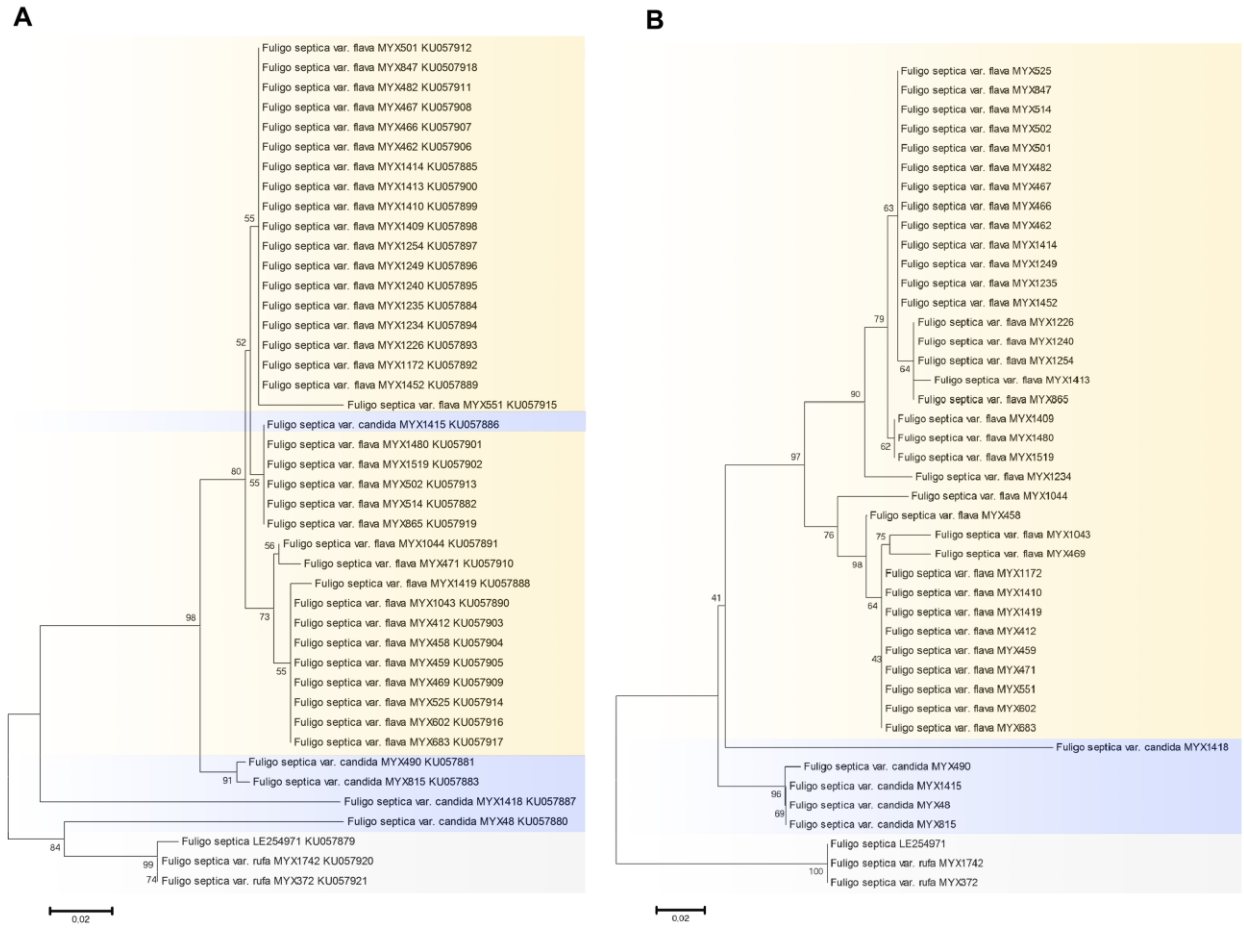

Figure 4 – Phylogenetic trees calculated with Neighbor Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. A) phylogenetic tree based on 145 positions of a partial ITS2 sequence and B) phylogenetic tree based on 145 positions of a partial mtSSU sequence (blue – *F. septica* var. *candida*, yellow – *F. septica* var. *flava*, grey – *F. septica* var. *rufa*).

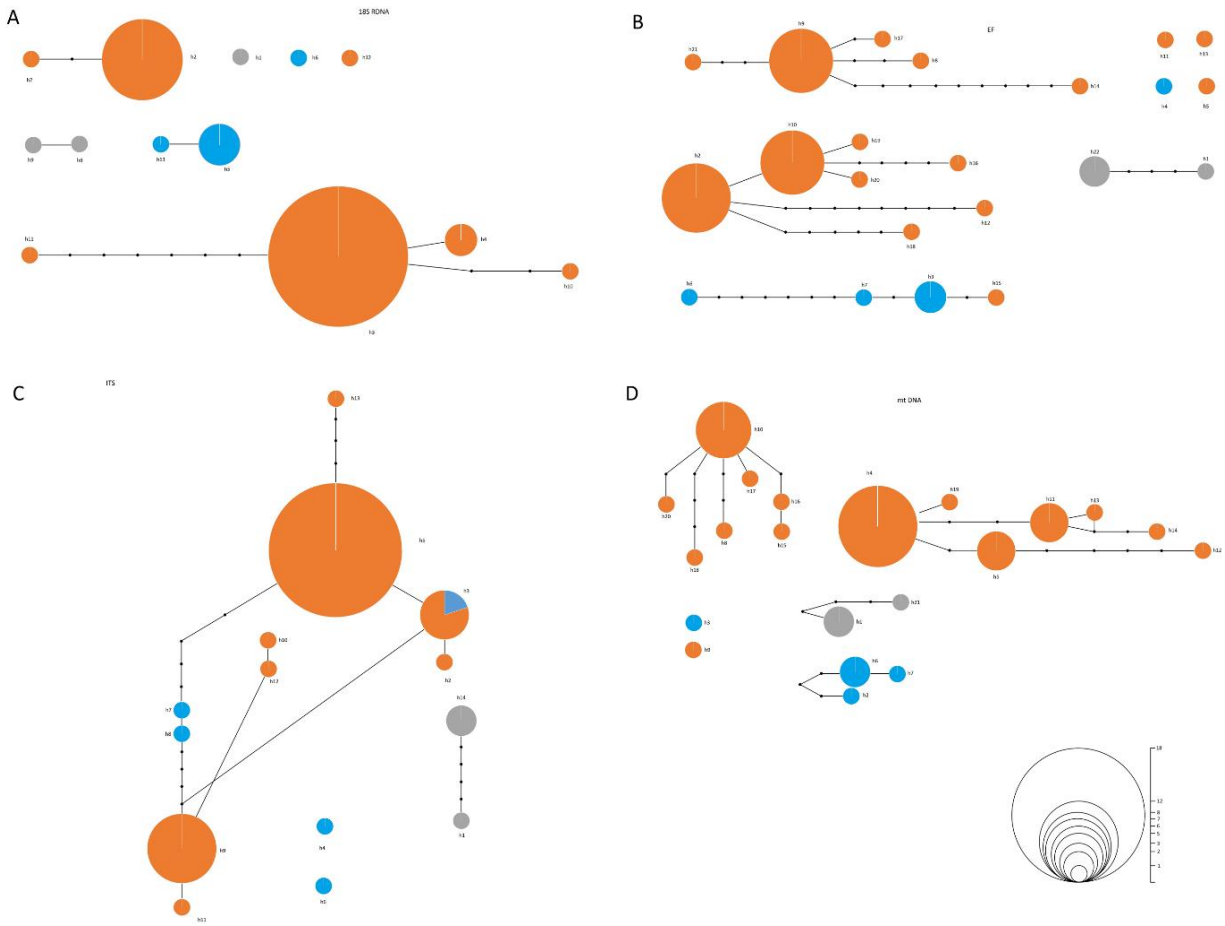

Figure 5 – The haplotype network of 43 *Fuligo septica* specimens. A) 18S rRNA, B) EF-1alpha, C) ITS2 and D) mtSSU (blue – *F. septica* var. *candida*, yellow – *F. septica* var. *flava*, grey – *F. septica* var. *rufa*).

Morphological characteristics, such as colour of peridia, size of the fruiting body, length of the stem or lime are often varying characteristics (Clark et al. 2004). In the text books, the colour of the fruiting-bodies and the nodes are major determination characters. The colour differences of the cortex could be used in most fresh collections as major feature, but appears to be a variable character (pigment loss of older collection material, influence by habitat conditions). The colouring of the lime nodes and the analysis of the spore surface is much more reliable. The features of the spore surface are seemingly less plastic. Also environmental influences have little effect on the change of these characteristics (Walker & Stephenson 2016).

The reduction of a complex organismic system to the basis of one gene or another aspect appears to be sufficient for certain hypotheses. More characters however, should be involved for a comparative study, in order to understand as precisely as possible how different types or individuals are related to each other.

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