universität wien

DISSERTATION

Titel der Dissertation

Molecular Phylogeny and Taxonomy of the *Physarum notabile* Species Complex (Myxomycetes)

Verfasser

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angestrebter akademischer Grad

Doctor of Philosophy (PhD)

Wien, 2014

Studienkennzahl lt. Studienblatt:	A 094 490
Dissertationsgebiet lt. Studienblatt:	Molekulare Biologie
Betreut von:	Univ.-Prof. Dr. Arndt von Haeseler

Abstract

Im Gegensatz zu Tieren und Pflanzen sind die Modelle der Verbreitung von mikroskopischen Organismen eher schlecht erforscht. Der Grund dafür sind ihre geringe Größe und bestimmte Merkmale des Lebenszyklus. Zur selben Zeit trägt die geringe Größe zusammen mit der hohen ökologischen Toleranz zur weltweiten Verbreitung der Mikroorganismen bei. In letzter Zeit wurden die Modelle des Kosmopolitismus (Fenchel, 2005; Fenchel, Finlay, 2004; Finlay, Fenchel, 2004) sowie des „Moderate Endemism“ (Foissner, 2006) im Bezug auf Mikroorganismen öfters diskutiert (Fontaneto, 2011; Martiny et al., 2006).

Um diese Fragen genauer zu studieren, sind Modell-Mikroorganismen benötigt, die leicht im Feld sowohl auch im Labor identifiziert werden. Eine solcher Gruppen sind die Myxomyceten (Eumycetozoa). Die Gruppe beinhaltet ca. 1000 Spezies in 5 Ordnungen (Martin, Alexopoulos, 1969; Olive, 1975). Als aktive Bakteriophagen sind Myxomyceten wichtige Regulatoren der bakteriellen Biota in Böden. Die Erforschung der Phylogenie der Myxomyceten trägt dazu bei, die evolutionäre Geschichte der Protisten besser zu verstehen (Adl et al., 2012).

Bis vor kurzem basierte das taxonomische System der Myxomyceten fast ausschließlich auf den morphologischen Eigenschaften der Fruchtkörper (Sporokarpen) (Martin, Alexopoulos, 1969; Schnittler et al., 2012) und das morphologische Spezies-Konzept wurde überwiegend benutzt. Erst im letzten Jahrzehnt wurden die Ergebnisse von molekularen Studien an Myxomyceten veröffentlicht (Fiore-Donno et al., 2008). Dennoch ist das taxonomische System der Myxomyceten sehr unstabil (Schnittler et al., 2012).

Die Morphospezies aus der Gattung *Physarum* mit grauen Sporokarpen gehören zu einem Spezies-Komplex mit sehr verschwommenen Grenzen (Hagelstein, 1944). Zuvor wurde ein Morphotyp aus der Gattung *Physarum* beschreibt, welches in Steppen- und Wüstenregionen auf allen Substrata dominierte (Novozhilov et al., 2006; Novozhilov, Schnittler, 2008). Dieser wurde anhand von Ähnlichkeiten in der Morphologie der Fruchtkörper vorläufig der Spezies *Physarum notabile* T. Macbr. zugeordnet. Diese Spezies wurde von uns als Modellobjekt für eine phylogeographische Studie auserwählt, da wir im Besitzt einer großen Sammlung von Isolaten dieser Spezies aus aller Welt sind, einschließlich Proben von einer 3500 km langen Transekte, die durch Zentralasien führt.

Die folgende Arbeit präsentiert die Ergebnisse einer Studie des *Ph. notabile* Spezies-Komplexes, die molekulare und morphologische Methoden einbezieht. Wir benutzen eine Kombination von zwei genetischen Markern – der 18S SSU rDNA und des tef1 Genes – um die phylogenetischen Zusammenhänge in dieser verwickelten Gruppe von Organismen zu erforschen.

Auf Grund von morphologischen und phylogenetischen Analysen beschreiben wir die aride

Form von *Ph. notabile* als eine neue Spezies, *Ph. pseudonotabile*. Wir untersuchen die Verwandtschaft zwischen *Ph. notabile* und *Ph. leucophaeum* und zeigen, dass diese Taxa sich sowohl in Morphologie als auch in ökologischen Vorzügen ähnlich sind und synonym sein könnten. *Ph. album* wurde anhand von markanter Morphologie und molekularen Merkmalen als die am weitesten vom Komplex entfernte Spezies ermittelt. Dabei ist das Spezies-Komplex, wie unsere Forschungen zeigen, polyphyletisch; die morphologische Ähnlichkeit dessen Vertreter könnte konvergenten Ursprungs sein. Die phylogeographische Analyse deutet auf eine schwache Korrelation zwischen geographischen und genetischen Distanzen bei den Isolaten von *Ph. pseudonotabile*; diese Daten unterstützen das Modell des „Moderate Endemism“ im Bezug auf Myxomyceten.

In contrast to animals and plants, the distribution patterns of microscopic organisms are studied rather poorly. This is mostly due to their small size and certain features of their life cycle. At the same time, the small size, together with high ecological tolerance, contributes to their world-wide distribution. The model of cosmopolitism in regards to microorganisms (Fenchel, 2005; Fenchel, Finlay, 2004; Finlay, Fenchel, 2004) as well as the model of moderate endemism (Foissner, 2006) has been discussed widely recently (Fontaneto, 2011; Martiny et al., 2006) in the context of the rule "Everything is everywhere, but the environment selects" (EiE hypothesis).

To study these questions, model microorganisms are required that can be easy identified in both field and laboratory conditions. One of such groups are the Myxomycetes, or plasmodial slime molds (Myxomycetes = Eumycetozoa). This group consists of appr. 1000 species combined to five orders (Martin, Alexopoulos, 1969; Olive, 1975). Being active bactreriophages, the Myxomycetes are important regulators of bacterial biota in soil (Feest, Madelin, 1985). The research of Myxomycete phylogeny is an essential part of studying the evolutionary history of protists (Adl et al., 2012).

Until recently, the traditional systematics of Myxomycetes has been based on the morphological features of their fruiting bodies (sporocarps) and spores (Martin, Alexopoulos, 1969; Schnittler et al., 2012) and the morphological species concept prevailed in taxonomical studies of this group. Only in the last decade, results of molecular studies in Myxomycetes have been published (Fiore-Donno et al., 2008 and papers thereafter). Still, the taxonomical system of Myxomycetes is very unstable (Schnittler et al., 2012).

The morphospecies of the genus *Physarum* with gray sporocarps belong to a species complex with highly blurred borders (Hagelstein, 1944). Previously, a morphotype belonging to the genus *Physarum* was described that dominated in desert and steppe communities on all substrate complexes (Novozhilov et al., 2006; Novozhilov, Schnittler, 2008). This form was tentatively assigned to *Ph. notabile* T. Macbr based on similarities in sporocarp morphology. We selected this species as a model object for a phylogeographic study since we had a large collection of isolates from various regions of the world, including samples from a 3500 km long transect going through Central Asia.

The present work shows the results of a study of the *Physarum notabile* species complex involving molecular and morphological methods. We use a combination of two genetic markers – the 18S SSU rDNA gene and the nuclear tef1 gene – to elucidate phylogenetic relationships in this entangled group of organisms.

Based on morphologic and phylogenetic results we describe the arid form of *Ph. notabile* as a new species, *Ph. pseudonotabile*. We investigate the relationship of *Ph. notabile* and *Ph. leucophaeum*, showing that these taxa share both similar morphology and ecological preferences and thus could be synonymous. *Ph. album* is shown to be the most outstanding species from the complex,

having both a prominent morphology and molecular features. The species complex on its own is shown to be polyphyletic; thus, the morphological similarity of its representatives might be of convergent origin. The phylogeographic analysis displays the presence of a weak correlation between geographical and genetic distances for the isolates of *Ph. pseudonotabile*, thus supporting the model of moderate endemism.

Parts of this thesis have been published in the following articles:

1. Y. K. Novozhilov, M. Schnittler, D. A. Erastova, M. V. Okun, O. N. Schepin, E. Heinrich (2012) Diversity of nivicolous myxomycetes of the Teberda State Biosphere Reserve (Northwestern Caucasus, Russia). *Fungal Diversity*. (DOI: 10.1007/s13225-012-0199-0)
2. D. A. Erastova, M. V. Okun, Y. K. Novozhilov, M. Schnittler (2012) Phylogenetic position of the enigmatic myxomycete genus *Kelleromyxa* revealed by SSU rDNA sequences. *Mycological Progress*. (DOI: 10.1007/s11557-013-0892-8)
3. Y. K. Novozhilov, M. V. Okun, D. A. Erastova, O. N. Shchepin, I. V. Zemlyanskaya, E. Garcia-Carvajal, M. Schnittler (2013) Description, culture and phylogenetic position of a new xerotolerant species of *Physarum*. *Mycologia*, 105(6), 1535-1546. (DOI: 10.3852/12-284)

Acknowledgements

I would like to thank my supervisor, Professor Arndt von Haeseler for his agreement to support me in my project during the PhD study, his constant guidance and valuable advises. His aspiration for finding unusual, fresh ways in science has inspired me over this time.

I also would like to thank my teachers and collaborators, Prof. Yuri K. Novozhilov and Prof. Martin Schnittler for introducing me into the mysterious world of Myxomycetes and never letting me down in hard times.

Thanks to all my colleagues at CIBIV I spent the time of my study in a great atmosphere of friendship. Special thanks to Dr. Minh Bui and Dr. Heiko Schmidt for the help in the many phylogenetic questions that I had in this time. Thanks to Milica Krunic, Olga Chernomor, Steven Mueller, Celine Prakash and Konstantina Kyriakouli for the amazing game evenings and other activities that let me feel like being a part of a great collective.

I would like to thank my reviewers and examiners for a critical review of my thesis.

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Introduction

In contrast to animals and plants, the distribution patterns of microscopic organisms are studied rather poorly. This is mostly due to their small size and certain features of their life cycle. The small size of protists, microscopic fungi and other microorganisms as well as their high ecological tolerance contribute to their global dispersal and limit the possibility of allopatric speciation. In the last decade, the model of cosmopolitism in regard to microorganisms has been discussed widely (Fenchel, 2005; Fenchel, Finlay, 2004; Fenchel, 2004), with the main idea stated as "everything is everywhere, but the environment selects" (EiE hypothesis). One of the arguments supporting this hypothesis is the low level of genetic differentiation between distantly located populations of one morphospecies. On the contrary, several authors declare the hypothesis of moderate endemism, stating that there are indeed patterns of genotypic diversity present in populations across large habitat areas (Foissner, 2006; Telford et al., 2006; Vyverman et al., 2007).

In order to study the problem of the distribution of microscopic organisms, species are required that can easily be seen and identified in both nature and laboratory. Such are the Myxomycetes, or plasmodial slime molds (Eumycetozoa). This group of organisms consists of appr. 1,000 species in five orders: Echinosteliales, Trichiales, Stemonitales, Physarales and Liceales (Martin, Alexopoulos, 1969; Olive, 1975; Lado, 2005; 2013). Myxomycetes are active predators feeding mostly on bacteria, thus contributing to the composition of bacterial biota in soils (Feest, Madelin, 1985). Moreover, several Myxomycete species like *Physarum polycephalum* or *Didymium iridis* are used as model objects in genetical and cytological researches. Overall, studying the Myxomycetes is vital for the general research of phylogeny and evolutionary history of protists (Adl et al., 2012).

A distinctive feature of Myxomycetes is the combination of different life forms in their life cycle: microscopic amoeba, zoospores, macroscopic plasmodia, and fruiting bodies. The reproductive system of Myxomycetes is very flexible. Their morphospecies often consist of a mixture of asexual clones and heterothallic lineages. Thus, shedding light on the influence of geographical and ecological barriers on the processes of genetic divergence in Myxomycetes is of great importance.

Until recently, the taxonomy of Myxomycetes was based exclusively on morphological features of fruiting bodies (sporocarps) and spores (Martin, Alexopoulos, 1969; Schnittler et al., 2012), and the morphological species concept prevailed in this group. However, this concept has been criticized more and more in recent researches. Firstly, developmental aberrations caused by fluctuations of environmental factors can lead to modifications of morphological traits and thus to erroneous descriptions of new species (Schnittler, Mitchell, 2000). Secondly, studies in

Myxomycetes' reproductive systems have shown, that several reproductively isolated biological species are described as a morphospecies (Clark, Haskins, 2010).

Only in the last decade the first molecular studies of Myxomycetes were published (Fiore-Donno, 2008 and papers thereafter). According to the latest researches, all Myxomycetes are monophyletic (Fiore-Donno et al., 2010). Despite that, the taxonomic system of Myxomycetes is rather unstable, and most of the traditional families and genera are not monophyletic (Schnittler et al., 2012).

The morphospecies from the genus *Physarum* belong to a group of species with rather blurred taxonomic borders (Hagelstein, 1944). In previous studies carried out in arid regions of Eurasia, a morphospecies was discovered that belong to the genus *Physarum*. This species was dominant in all studied plant communities on all substrate types. It was tentatively assigned to *Ph. notabile* as the closest in terms of morphology (Novozhilov et al. 2006, 2010).

This morphospecies was selected as the model object in our study, since a huge collection (over 400 specimen) was assembled and identified that covers a transect going through 3500 km of Central Asia, covering the territories of the Caspian Lowland (Russia), Western, Central and Eastern Kazakhstan, South-Western China and Western Mongolia. Analyzing the genetic diversity of the *Ph. notabile* morphocomplex would shed light on the problem of distribution strategies of Myxomycetes. If there were a genetic structure present among the populations of this species, this would agree with the hypothesis of "moderate endemism" (Foissner, 2006). On the contrary, the absence of such structure would point towards the EiE hypothesis (Finlay, Fenchel, 2004).

This work presents the results of a research carried out on the *Ph. notabile* species complex using both morphological and phylogenetic methods. Most of contemporary researches in the field of systematics and taxonomy are based on such an approach, that allows to analyze the taxonomical system of the studied organisms from the point of view of evolutionary relationships. This approach shows greater reliability than the ones relying only on morphological features alone (Schnittler, Mitchell, 2000), and allows to create a natural system based on the phylogenetic relationships between taxa and their evolutionary history. The usage of two independent marker genes – the 18S Small Subunit ribosomal DNA (or SSU rDNA) and the nuclear translation elongation factor alpha gene (tef1) increases the reliability of the research, since the results of the two markers can be compared.

The computational methods for phylogenetic researches are in constant progress: both the performance of computational algorithms and the power of computers constantly improve. At the same time, there are plenty of phylogenetic methods available, each of those having their strong and weak sides. The results obtained and the overall scientific value depend heavily on the chosen

methodology. In order to account for this, the present study was performed in tight collaboration with phylogenetic software developers, allowing the tree reconstruction algorithms to be used in the most efficient and adequate way.

The main aim of this work was to study the genetic diversity and the phylogenetic structure of the *Ph. notabile* morphocomplex. In order to achieve this, the following goals were set:

1) Determine the taxonomic status of the arid morphotype of *Ph. notabile*;
2) Reveal the phylogenetic relationships in the *Ph. notabile* species complex;
3) Search for a correlation between genetic distances and geographical localities in the isolates of the arid morphotype of *Ph. notabile*;
4) Perform a selection of the most efficient methods for the phylogenetic analysis of the studied specimens.

The results of the following work were presented at the following conferences: XVI Congress of European Mycologists (Halkidiki, Greece, 2011), International Conference of young scientists "Lomonosov" (Moscow, Russia, 2011, 2012), International Conference on Molecular Phylogenetics "MolPhy-3" (Moscow, Russia, 2012), International Conference on Biosystematics "BIOSyst 2013" (Vienna, Austria, 2013).

Chapter 1. Microorganism distribution theories

The model of cosmopolitism is widely spread in the scientific community and is best formulated by the phrase "Everything is everywhere, but the environments selects" (Beijerinck, 1913; Becking, 1934). In other words, microorganisms can be found everywhere on earth if there are appropriate habitat conditions. In modern science, this theory is reflected in the works of Fenchel and Finlay (2004). One of the main arguments speaking for the theory of cosmopolitic microorganisms is the fact that, due to their huge numbers, the microbes will distribute in a highly efficient way and occupy all possible niches (Finlay, 2002). Importantly, the initial cosmopolitic theory, similar to many of its contemporary analogs, deals with taxonomic units that were separated based on morphological traits, such as morphospecies (Finlay, 1996). Indeed, it seems that certain protist morphospecies are spread ubiquitously. There are many examples of such distribution among protists; these include not only species where the amount of morphological traits is limited like lobose amoebae or certain ciliates, but also species with well-defined features like shelled amoebae (Wylezich et al., 2007; Kristiansen, 2008; Smith et al., 2008, Chapter 7). At the same time, there are multiple cases of moderate endemism in microorganisms, especially for isolates in poorly studied regions, and more rarely – from well-studied habitats with a suitable environment. Such cases were reported for testate amoebae (Mitchell, Meisterfeld, 2005; Smith, Wilkinson, 2007), unicellular green algae (Coesel, Krienitz, 2009), diatoms (Vanormelingen et al., 2008), and other protist groups. However, it cannot be excluded, that a more extensive research will show these taxa to have a wider habitat area.

Many aspects of the cosmopolitism theory still have no explanation. For example, we cannot fully describe the diversity of many microorganisms, quantitatively characterize their ecological niches and geographic ranges, and determine their distribution potential. Moreover, our understanding of how microorganisms disperse globally is far from being full. Can speciation events occur without the presence of geographical barriers between populations? If microorganisms are not restricted to a certain geographic area, how can they retain their species status? Well-defined taxonomic criteria are needed in order to map their distribution. Many species regarded as cosmopolitic are in fact complexes combined of several geographically restricted species. In order to solve such cases, combined approaches are required, making use of both traditional morphological as well as molecular methods. The latter is increasingly used as an efficient tool to study both already described morphotypes and determining species new to science (Hills, Dixon, 1991; Nei, 1996).

With the development of novel methods in molecular biology, new questions and possibilities arose in the field of the biogeography of microorganisms. The use of primers with different levels of taxonomic specificity combined with the latest algorithms for phylogenetic reconstruction allow for

an easier research of specific group of protists. The results of such studies show that the levels of genetic diversity are much higher than those of plants and animals (Berney et al., 2004; Cavaier-Smith, 2004) and also higher than expected from their morphological diversity (Chao et al., 2006). Moreover, many, if not all morphospecies consist of a mixture of lineages differing in ecological, physiological, ultra-structural and/or other traits (von der Heyden, Cavalier-Smith, 2005; Stoeck et al., 2006). This phenomenon is a consequence of a long-term morphological conservation and convergent evolution of protists; a direct consequence of this is the difficulty or even impossibility of using the morphospecies concept to study evolutionary relationships among microorganisms.

The 18S Small Subunit ribosomal DNA (18S SSU) gene sequence is often used to measure the genetic diversity in microorganisms (Wuyts et al., 2000). This gene acts as a good phylogenetic marker with high resolution. The sequences of this gene can be found in high numbers in various databases; this allows one to use it as a marker in phylogenetic analysis. The 18S SSU gene is a multi-copy gene and is thus easy to amplify even when the DNA is extracted from a small amount of cells. It has a sufficient length and variability, allowing using it in an unusual (for a single gene) range of phylogenetic studies on different taxonomical levels (Sunnucks, 2000).

At the same time, it remains unclear which level of phylogenetic resolution is needed to delimit species (or OTU – operational taxonomic units) in protists. It is clear that this level will be different in various groups, since the genetic marker and the evolution of traits are not connected directly (Kimura, 1984). Thus, this question is to be answered separately for each group using a combined approach including molecular, morphological and ecological methods to delimit biologically meaningful OTU's. There are already prerequisites for such an approach: according to latest results (Pither, 2007; Nolte et al., 2010), only ecologically dominant taxa can be widely spread, since only they can achieve a high population density, and, as a consequence, high dispersal efficiency. It was shown that increasing the level of phylogenetic resolution leads to an increase in the amount of endemic OTU (Fontaneto et al., 2008). This tendency is, generally speaking, applicable to all organisms, but in the case of protists, it is hard to interpret because of the lacking knowledge about the relationships between molecular phylogeny and taxonomy. As it can be seen from Figure 1, higher-level taxa have a wider distribution range. On the other hand, an individual is defined by a certain spot in space. Thus, the areal decreases with the taxonomic level. However, the usage of the morphospecies concept is complicated in many protistean taxa. The evaluation of habitat borders of a morphospecies depends heavily on the limits of this species. The idea of a large habitat range of many morphospecies is often based on a large size of their taxa.

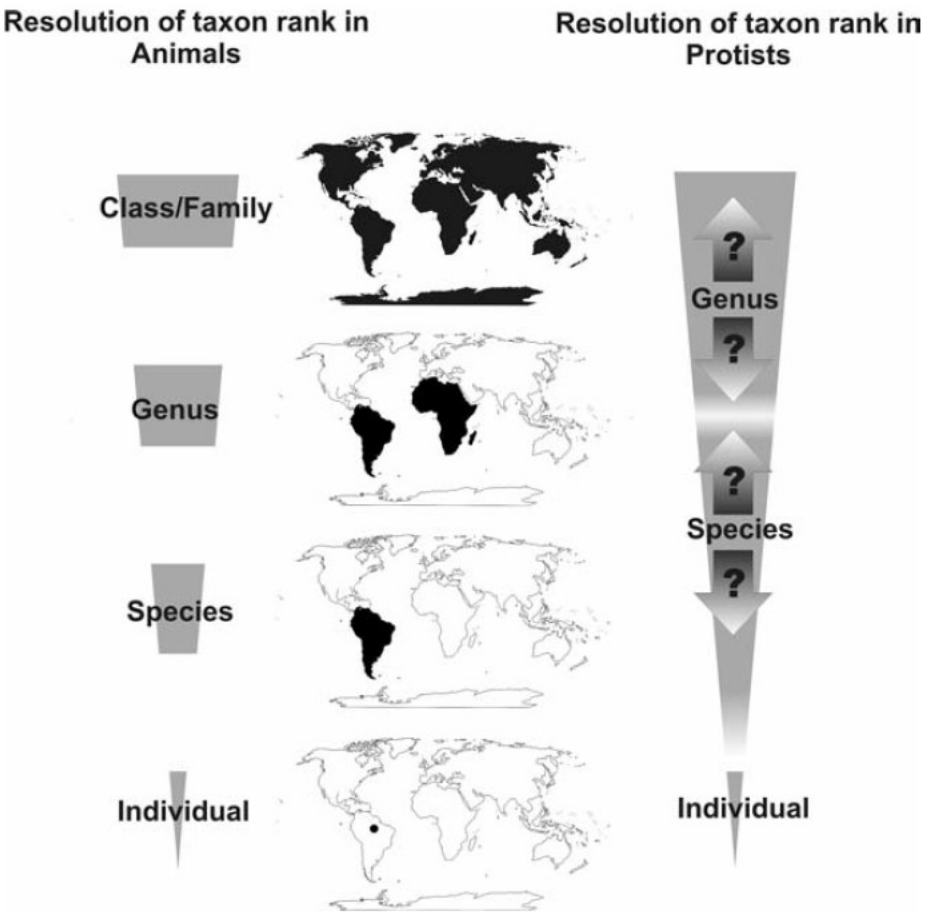


Figure 1. Schematic representation of the relationships between taxonomic ranks and biogeography in animals and protists (Bass, Boenigk, 2011).

An approach based on searching for endemic species appears to be not efficient when it comes to proving different microorganism distribution theories. It seems that a more appropriate approach to elucidate the taxonomic structure has to include an analysis of morphological variability, genetical diversity and ecological niche preferences.

Chapter 2. Systematics and geography of Myxomycetes

2.1. The systematic position of Myxomycetes

The Myxomycetes comprise of appr. 1,000 species from five orders: Echinosteliales, Liceales, Physarales, Stemonitales and Trichiales (Lado, 2005; 2013). According to latest researches, they are the largest group of ameboid soil organisms (Stephenson et al., 2011; Urich et al., 2008) and play an important role in the regulation of abundance and composition of bacterial soil microflora (Feest, 1985; Olive, 1975), thus keeping a balance between the bacterial and fungal processes of organic matter destruction (Madelin, 1984).

The class Myxomycetes is part of the phylum Amoebozoa, which, together with the phylum Opistokonta, forms the monophyletic superkingdom Unikonta (Cavalier-Smith, 2010; Adl et al., 2012). Molecular data shows that Myxomycetes are a considerably old group of organisms. The maximal age of fossil sporocarps is estimated to be about 50 mio. years, some of the fossilized spore samples are dated as from Oligocene and Pleistocene (Graham, 1971). However, paleontological evidence of plasmodial slime molds is quite rare (Domke, 1952; Waggoner, Poinar, 1992; Dorfelt et al., 2003) and cannot be used for phylogenetic analysis. The life cycle of Myxomycetes (Figure 2) includes microscopic mixamoebae and zoospores as well as macroscopic multinuclear plasmodia and spore-bearing fruiting bodies (sporocarps). Sporocarps have a considerable amount of morphological traits and display a huge variability among Myxomycetes (Figure 3), and they give a good basis for global distribution studies (Stephenson et al., 2008). However, the identification of Myxomycete species is based almost exclusively on the morphological features of their fruiting bodies. Plasmodia do not exhibit a sufficient number of morphological traits for a precise identification. Only in the last two decades studies on Myxomycete phylogeny were published that make use of methods of molecular systematics (Fiore-Donno et al., 2008). According to present-day data, the Myxomycetes are monophyletic, and together with the Dictiostelids they form the “Macromycetozoa” group (Fiore-Donno et al., 2010). Still, the taxonomic system of Myxomycetes is very unstable, and many of the traditional families and genera are not monophyletic (Fiore-Donno et al., 2012; Schnittler et al., 2012).

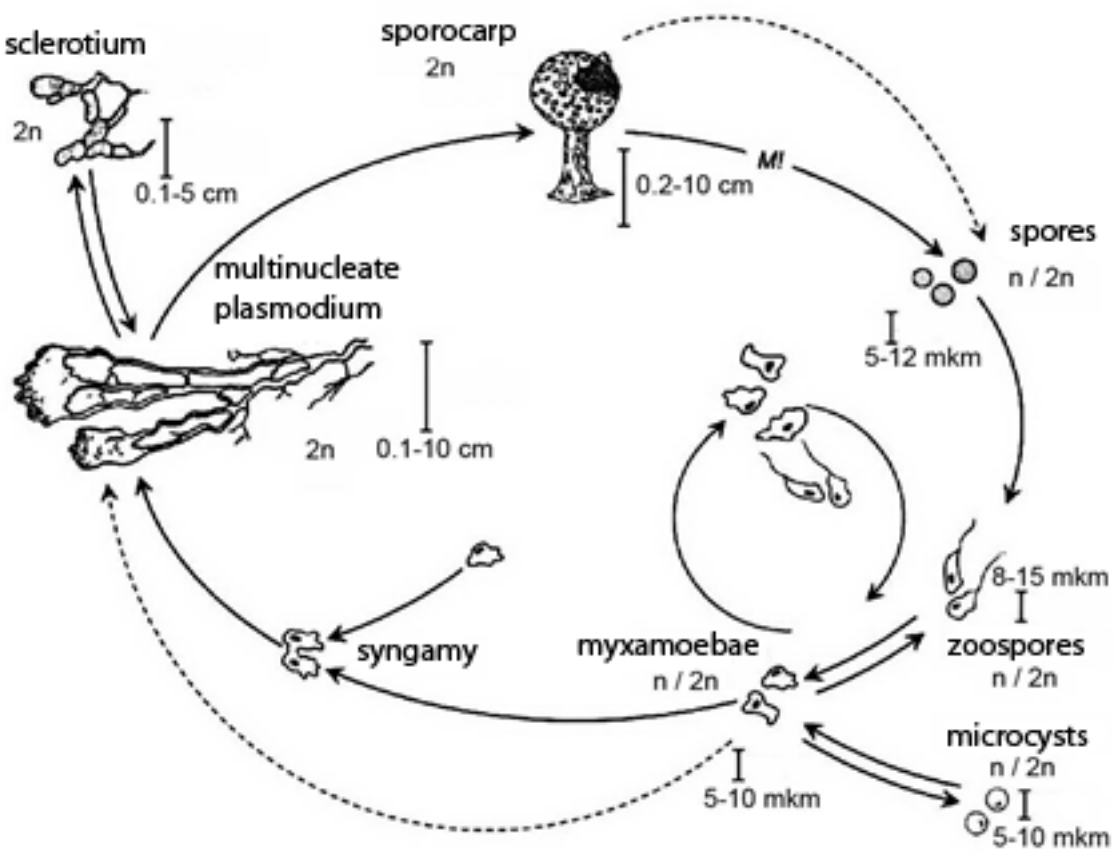


Figure 2. A Myxomycete life cycle with interchanging haploid (n – spore, amoeba and zoospore) and diploid (2n – plasmodium) stages (modified from Schnittler et al., 2012).

Figure 3. The diversity of Myxomycete fruiting bodies. **A** – *Physarum roseum*; **B** – *Trichia varia*; **C** – *Stemonitis virginiensis*; **D** – *Diderma montanum*; **E** – *Hemitrichia serpula*; **F** – *Arcyria helvetica*; **G** – *Lycogala epidendrum*; **H** – *Metatrichia vesparum*. Scale bar = 1 mm.

The present-day taxonomy of Myxomycetes is based on the morphological species concept (Mallet, 2007), which has been criticized for two major reasons. Firstly, developmental aberrations

caused by fluctuations of environmental factors can lead to modifications of morphological traits and thus to erroneous descriptions of new species (Schnittler, Mitchell, 2000). Secondly, studies in Myxomycete reproductive systems have shown that several reproductively isolated biological species can exist inside a morphospecies (Clark, Haskins, 2010). Whereas the first problem leads to over-splitting of true biological entities, the second lumps them together.

2.2. Myxomycetes: reproduction and species concepts.

According to one of the definitions, a species is a genetically delimited group of populations, those individuals are characterized by common intrinsic features and are able to freely mate and produce viable offspring (Mayr, 1982; Grant, 1981). The vast majority of studies in the field of biodiversity, ecology and biogeography make use of this species concept. Still, there is no uniform species concept that could be applied to all groups of organisms (Mallet, 2007). This means that, depending on which concept is applied, the number of species known may differ in magnitudes. At the same time, ecology and biogeography consider the species as the main taxonomic rank. In ecology, it represents a group of individuals, the divergence among which can be ignored for the sake of e.g. studying the relationships among different species. In biodiversity studies, species are important as entities those numbers has to be counted on a certain area. It would be very convenient to have a uniform species concept that satisfies all these needs, but such one is not present. Instead, we deal with several species definitions that are based on different methodological approaches.

One of the main features of the biological species concept is the reproductive isolation, which assures “the safety of the gene pool” (Mayr, 1982). When asexual, parthenogenetic or self-fertilizing species are studied, this concept is difficult to apply, and species borders are blurred. It is known that many of the widely distributed myxomycete species are actually complexes of geographically restricted apomictic clones who switched to asexual reproduction (Clark, 2000; Clark, Stephenson, 2000; ElHaage et al., 2000; Irawan et al., 2000). Some of these forms are found in special habitats like withering inflorescences of tropical herbs. These clones are adapted to a certain habitat, and their sporocarps show distinctive features (e.g. color and size) that differ from sporocarps found in more typical environments (Schnittler, Stephenson, 2002). About 50% of all Myxomycetes are known from holotype habitats or from less than five localities. It seems probable that many of these “species” are just morphologically distinct lineages of one species, adapted to specific habitats (Schnittler, Mitchell, 2000).

At the same time, the question on one unified species concept in biology remains unanswered. For over 200 years, since the first description of a Myxomycete by Persoon (1801), the descriptions

were made based on the morphological features of the fruiting bodies (sporocarps). Still, despite the fact that these sporocarps have a decent number of morphological features compared to similar structures in other microorganisms (Schnittler et al., 2012), their number is still less than the one present in “true” macroscopic organisms like animals and higher plants.

Due to the reasons described above a conceptual shift occurred in the systematics of Myxomycetes, making the phylogenetic species concept the preferred one. According to it, the monophyly of a certain group is the main criterion. Gene trees are used as basis for species description (Fiore-Donno et al., 2012). At the same time, it is known that the ability of many lineages to stop recombination processes and leading to cloning can hamper phylogenetic and systematic studies (Grebelny, 2008).

Indeed, mating experiments have shown Myxomycete reproductive systems to be very flexible (Collins, 1981; Clark et al., 2004) (Table 1). If a variety of strains of a certain morphospecies is tested, some will reproduce sexually and behave heterothallic (will form plasmodia only if amoebae from two different spores mated). Multiallelic mating gene systems with one or two loci have been already discovered in Myxomycetes. Other strains will from plasmodia from one spore, thus behaving non-heterothallic (Collins, 1981). This can be achieved either by true homothallism (sexual reproduction without a mating gene system) or by asexual reproduction. Compatibility experiments cannot reveal the difference since crossing between amoebae originating from one spore cannot be avoided. However, quantitative studies of nuclear DNA content during the life cycle show that such asexual strains remain diploid during the whole life cycle, bypassing meiosis and cytogamy (Therrien, Yemma, 1974) (Figure 4).

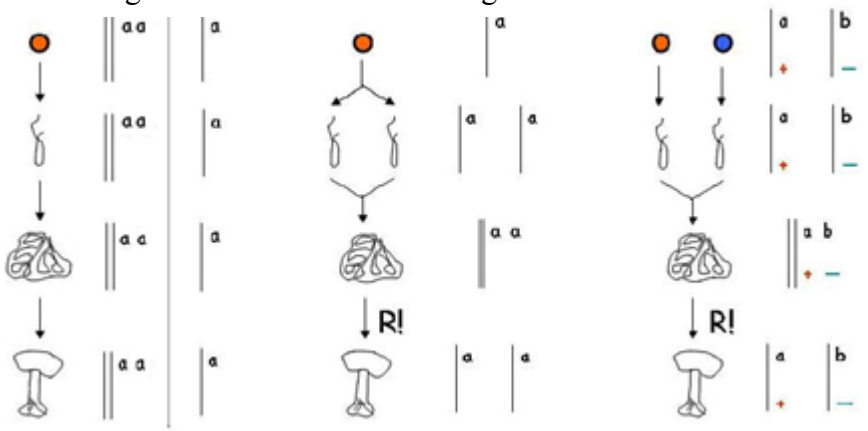
mating system	Apomictic	Homothallic (no mating system)	Heterothallic (with mating system)
Spores	All stages n	All stages 2n	
Myxamoebae			
Plasmodia			
Sporophores			
Obtain plasmodia from monospore cultures	Yes	Yes	No
Expected changes in DNA content during amoeba-plasmodia transition	No	Yes	Yes
Expected genetic diversity	Very low (clones)	Low	High

Table 1. Schematic representation of possible Myxomycete reproduction systems and their contribution to the expected genetic diversity for model morphospecies, as well as analysis options. A and b denote possible nucleotide mismatches in DNA sequences, "+" and "-" - various mating systems.

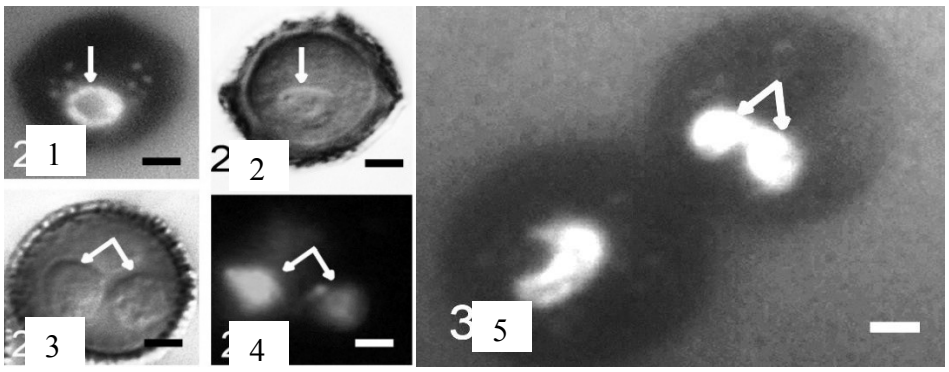


Figure 4. Myxomycete spores can contain several nuclei (marked with arrows), which can be confirmed by both standard light microscopy (2, 3) and by DAPI staining (1, 4, 5) (Novozhilov et al., 2013). Scale bar = 1 μm.

A recent study of wild populations of two bryophylous (moss-inhabiting) species from the genus *Lamproderma*, showed that the sequences of three markers (partial 18S, ITS1-2 and tef1) were identical among the representatives of one clonal population (Fiore-Donno et al., 2011). This is best explained by the fact that asexual reproduction dominates in this group, which leads to independently evolving clones that differ slightly in morphological features. The existence of mating types assumes the presence of separate biological species based on incompatible mating types. This was shown in an impressive way on the model object Didymium (Clark, 1995; Clark, Landolt, 1993; ElHaage et al., 2000). For example, among the 39 studied Myxomycete species, 21 had non-heterothallic mating systems, 6 were heterothallic and 12 species included strains behaving in both ways (Clark et al., 2004). However, the phylogenetic relationships between sexual and asexual forms have not been studied. It still unclear if asexual isolates represent discrete ancestor lineages that diverged millions years ago and are actually separate asexual species, or the sexual lineages dominate, whereas the asexual lines are a result of a recent mutation. It is also still unclear, in which environmental conditions the asexual lineages have evolutionary advantage over the sexual ones.

2.3. Genus *Physarum*: systematics and morphology

As mentioned, the most appropriate species for biogeographic research are the ones that can be easily identified in the field and cultivated in the laboratory. Among the Myxomycetes, such are representatives from the genus *Physarum* Pers., 1794. Species of this genus are often used as model objects in various experimental works (such as the tropical *Ph. polycephalum*) (Imoto et al., 201). *Physarum* is placed in the order Physarales T. Macbr, which is the largest among the Myxomycetes, including two families: *Physaraceae* Chevall. and *Didymiaceae* Rostaf. *ex* Cooke. Characteristic

features of the order are dark-colored spores, lime in all elements of the sporocarps and the ability to form all types of fruiting bodies (sporocarps, ethalia and pseudoethalia). The assimilative stage is represented by a phaneroplasmodium.

The genus *Physarum* was described by Persoon (Persoon, 1794) based on *Ph. aureum* Pers. (= *Ph. viride* (Bull.) Pers.). Most of the species from this genus have a well-defined capillitium divided into hyaline tubules and lime nodes (Figure 5).

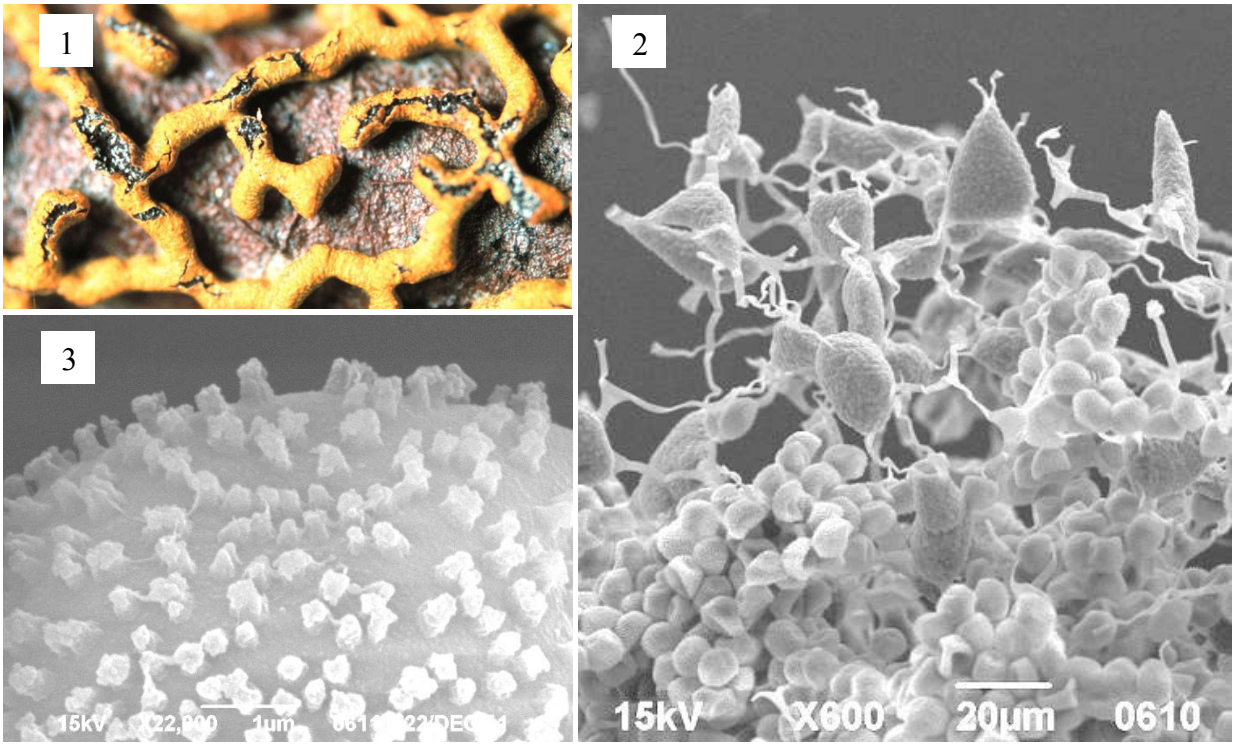


Figure 5. Morphology of the fruiting body and its elements in representatives of the genus *Physarum*. 1 – fruiting bodies of *Ph. aureum*; 2 – *Ph. notabile*, capillitium with lime nodes and spores; 3 – *Ph. notabile*, spore surface with warts.

The genus *Physarum* includes over 130 species (www.nomen.eumycetozoa.com); approximately one fifth of those was described in the last two decades. Main features used to identify the species of this genus are sporocarp stalk (presence/absence, lime presence, color), form of the capillitium (radial or isodiametric), and the form and color of the lime nodes in the peridium, although it has been shown that the last trait can vary (Aldrich, 1982). The spores all have a similar structure: about 10 µm in diameter, mostly ornamented with spikes or warts. The shape of the sporocarp and the degree of stalk development can vary even within one morphospecies, depending on the environmental conditions during development. The genus *Physarum* is not monophyletic (Nandipati et al., 2012), and the border between the genera *Physarum* and *Badhamia* is mostly artificial, since

some of the *Physarum* species tend to display a capillitium consisting of a continuous network of tubules – a feature characteristic for *Badhamia* species.

The genus *Physarum* contains several morphospecies, such as *Ph. leucophaeum* Fr., *Ph. album* (Bull.) Chevall. and *Ph. notabile* T. Macbr. that form a so-called "taxonomic continuum" (Hagelstein, 1944) because of sharing similar morphological and ecological traits. Because of this, defining taxonomic borders and identifying systematical position of the mentioned taxa is often difficult. During a study of Myxomycetes in arid regions of Kazakhstan, isolates of the genus *Physarum* with small greyish sporocarps were obtained using moist chamber techniques (Schnittler, Novozhilov, 2000b; Schnittler, 2001). Similar morphotypes were discovered in Colorado (Novozhilov et al., 2003a). In investigations of Myxomycete biodiversity it has been discovered that these morphotypes can often be found in arid regions of the lower Volga basin (Novozhilov et al., 2003, 2006), in the Altay mountains (Novozhilov et al., 2009, 1010), in western Mongolia (Novozhilov, Schnittler, 2008), Kazakhstan (Schnittler, 2001; Schnittler, Novozhilov, 2000a) and western China (Schnittler et al., 2013). All these forms did not match any of the known descriptions of *Physarum* species and presented a huge level of variability in lime distribution over the peridium as well as in shape and proportion of the sporotheca and stalk. In all mentioned papers, they were tentatively assigned to the species *Ph. notabile* as the closest in terms of morphology.

2.4. The *Ph. notabile* species complex

According to the literature (Hagelstein, 1944; Martin, Alexopoulos, 1969), *Ph. notabile* belongs to a group of *Physarum* species with greyish sporocarps and consists of a variety of morphological forms (Figure 6). This complex includes several species like *Ph. album* (Figure 6, 3) and *Ph. leucophaeum* (Figure 6, 1). All these species display a considerable variability of several key features like the amount and distribution of lime in the peridium, shape, color and ornamentation of spores. These species are often found on rotten wood in moderate deciduous and boreal coniferous forests. Normally, the spores of *Ph. notabile* (Figure 6, 6) are not as dark as those of *Ph. compressum*, but the color of the spores cannot be seen as a reliable criterion because of its variability. Some colonies can have both stalked and sessile sporocarps as well as all possible types of plasmodiocarps. *Ph. notabile* is connected with *Ph. compressum* by sessile sporangia and the presence of plasmodiocarps, but can be distinguished from the latter by pointed lime nodes. Flatten sessile forms of *Ph. notabile* may sometimes represent *Badhamia affinis*. The sporocarps resemble those of *Badhamia panicea* with a similar capillitium and sometimes a false columella (Hagelstein, 1944). Another representative of this group is *Ph. pusillum* (Berk. & M.A. Curtis) G. Lister. It can be

identified by a rather reddish color of the stalk, although this trait may depend on environmental conditions.

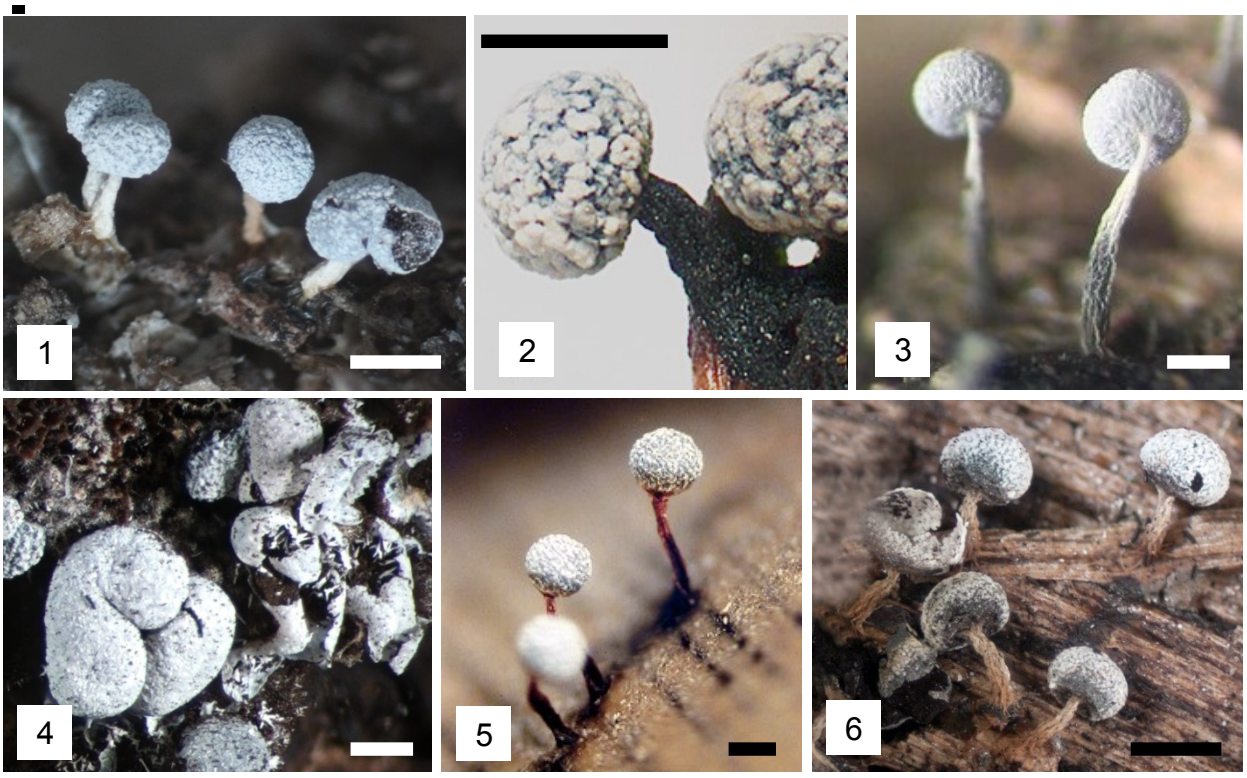


Figure 6. Sporocarps of various representatives of the *Ph. notabile* morphocomplex. 1 – *Ph. leucophaeum*; 2 – *Ph. notabile* (arid morhotype); 3 – *Ph. album*; 4 – *Ph. nivale*; 5 – *Ph. pusillum*; 6 – *Ph. notabile*. Scale bar = 500 µm.

However, the relative "weight" of many morphological traits used in species identification is still unclear; a molecular analysis allows us to re-estimate the importance of such features in taxonomic studies. An example of such a research is presented in Figure 7. In the described work, clusters of samples of the genus *Lamproderma* obtained in phylogenetic analyses display different patterns of spore features (diameter and ornamentation density). Thus, these features can be considered important for the microsystematics of *Lamproderma*.

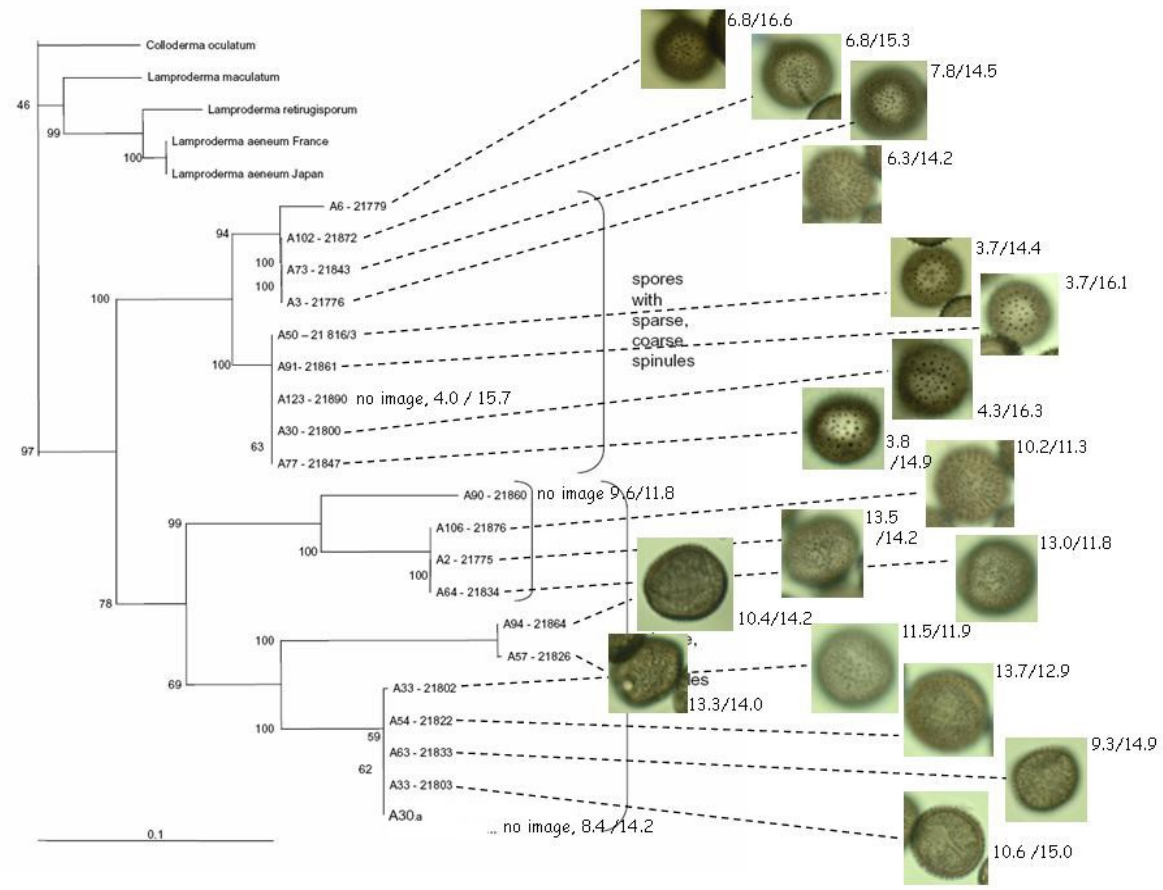


Figure 7. Phylogenetic tree based on an MP (Maximum Parsimony) analysis of 20 partial 18S SSU sequences of *Lamproderma puncticulatum* agg., (Schnittler et al., 2010). *Colloderma oculatum* as well as 4 other *Lamproderma* species were used as outgroups. The wart density/spore diameter are shown for each picture. Bootstrap support (100 replicates) is shown for each node. Scale bar = 0.1 nucleotide substitutions per site.

Chapter 3. Molecular Phylogenetics

3.1. The methodology of molecular phylogenetics

Molecular phylogenetics makes use of comparing various types of sequence data (DNA, RNA or proteins). Closer relationships between organisms are usually characterized by a higher similarity in the sequences of studied macromolecules, and the ones less related to each other differ more (Suarez-Diaz, Anaya-Munoz, 2008). In the easiest case of DNA sequence analysis, the number of differing positions between two sequences is counted. The degree of kinship is then expressed in percent of mismatches on the analyzed DNA fragment. The obtained distance matrix is subjected to a cluster analysis, and the result is displayed in form of a phylogenetic tree, or dendrogram, that displays the hypothetical evolutionary pathway of the studied organisms (Suarez-Diaz, Anaya-Munoz, 2008). An outgroup (a group of organisms that belong to a distinct taxonomical clade) is sometimes used to root the tree. The usage of additional statistical methods such as the bootstrap allows to estimate the reliability of the clusters in the obtained tree.

The simple method of tree reconstruction based on a DNA sequence similarity matrix described above belongs to the group of so-called distance methods. This group also contains methods such as UPGMA (Unweighted Pair Group Method with Arithmetic mean), and NJ (Neighbor Joining method) (Table 2). Such methods mostly do not require time-consuming calculations and therefore are often used on large data sets, where the usage of more sophisticated methods is impossible due to high calculation needs. However, these methods mostly make use of distance measures that are prone to underestimating the true phylogenetic distance between taxa, because they do not take into account the possibility of multiple mutations occurring at one site (e.g. back-mutations).

More reliable results on mid-sized data sets are obtained using computational methods that rely on single characters (nucleotides or amino acids) in the analyzed sequence. Such methods are ML (Maximum Likelihood), MP (Maximum Parsimony) and BA (Bayesan Inference).

The MP method is based on the “Okkam's Razor” principle, postulating that of all possible solutions for a problem, the easiest will be the best. MP searches for the tree with the least number of substitutions that leads to the sequence alignment (Fitch, 1971). At the same time, this method has several limitations. Only sites matching the informativity criterion are used in the MP analysis. For this, a column of the alignment has to contain at least two different states of characters, and of those at least two have to be present at least twice. Moreover, the MP algorithms do not make use of an evolutionary model, so different rates of substitution or back-mutations cannot be taken into account.

Analyzed data	Method	Evaluation criterion
Alignment	MP	Parsimony
	ML, BA	Evolutionary model
Distances	UPGMA, NJ	

Table 2. Main methods of phylogenetic analysis, their estimation criteria and types of analyzed data.

In the ML tree reconstruction method, the likelihood function is used to describe the goodness of fit of the phylogenetic tree and the alignment data (Felsenstein, 2004). Algorithms making use of the ML method try to maximize the likelihood during tree reconstruction, thus making the analyzed data described most probably by the tree inferred and the evolutionary model. This procedure has high computational costs, thus, an exhaustive search – an analysis of all possible tree topologies for a given alignment – is impossible in most cases: for 10 taxa there are more than 2 mio. possible trees. Therefore ML algorithms use a heuristic approach, meaning that, beginning from a certain starting tree (usually obtained by NJ or MP methods) they "move" through the space of possible tree topologies, where each spot has its value of the likelihood function. This "movement" is actually a series of random tree perturbations. Three perturbation methods are normally applied: Nearest Neighbor Interchange (NNI) exchanges the connectivity of four subtrees within the main tree; Tree Pruning and Regrafting (TPR) selects and removes a subtree from the main tree and reinserts it elsewhere on the main tree to create a new node; finally, Tree Bisection and Reconnection (TBR) detaches a subtree from the main tree at an interior node and then attempts all possible connections between branches of the two trees thus created. Thus, the tree rearrangement process is virtually infinite, so these algorithms include a stopping rule that finishes the analysis based on a certain condition. Usually this happens when a local maximum of the likelihood value is reached, and moving in any direction for a certain amount of time does not improve this value. Nevertheless, no algorithm can guarantee that the tree found will represent the true evolutionary relationship of the studied taxa.

In contrast to parsimony methods, the ML approach makes use of an explicit evolutionary model – a set of parameters that define the frequency of nucleotide or amino acid substitutions and other related processes. These parameters are optimized for the given data set and allow to consider such evolutionary events like multiple mutations on a single site. The evolutionary model plays a key role during the ML tree search since the likelihood of every topology depends on it. Thus, the correct selection of the model is a vital step of every phylogenetic analysis. For nucleotide data the General Time Reversible model (GTR; Tavare, 1986) is often used, which allows for all possible transitions and transversions with independent frequencies. However, an unjustified usage of a model with a high number of independent parameters can lead to an overfitting effect, where the usage of simpler models would lead to better results because no excessive fluctuation of model parameters would occur

(Ruano-Rubio, Fares, 2007). An optimal approach would be selecting the most appropriate model based on the given alignment. This can be done, for example, with the ModelTest software (Posada, Crandall, 1998). A similar approach was also used in this study.

Another ML-based heuristic approach to search the tree space is Bayesian phylogenetic reconstruction (BI). In contrast to the ML methods described above in BI one first needs to specify prior beliefs in the model parameter values before the analysis. The algorithm then generates a Monte-Carlo-Marcov chain, where each state represents a tree topology that deviates from the previous state by certain characteristics. The likelihood function is used in this case as a measurement of tree fitness, and substitution parameters are defined by an evolutionary model (Felsenstein, 2004). Similar to the ML search, this algorithm is not stopping: usually, several chains are run in parallel and the results are compared from time to time. The degree of convergence between those runs is used as the measurement of search success; if two or more chains run with high convergence for a certain amount of generations, they must have achieved the same local maximum of the likelihood function. After this, a certain amount of starting trees is discarded ("burn-in"), and the rest is summarized to a consensus tree. Several ways exist to determine the amount of trees to be discarded; in the fixed approach, for example, a given percentage of trees is discarded (e.g. 25%). This is probably not the best solution since it is independent of the alignment or the topologies obtained. A rather optimal approach includes discarding all the trees before a certain level of run convergence was achieved. This approach is also suggested by many Bayesian software developers.

In most cases, the result of a phylogenetic analysis needs to be statistically proven. The are several ways to do this; the most common are the bootstrap methods (Efron, 1979) and the posterior probability values. Whereas the latter are drawn from split frequencies of the Bayesian consensus tree, the first requires a separate calculation to be performed. During bootstrapping, character columns are randomly selected from the initial alignment, and the tree reconstruction is repeated based on the data set obtained this way. This procedure is performed several times, and the results from the iterations are summarized in support values. There are two similar approaches used in tree reconstruction: in the first case, the final dendrogram is a consensus tree of all obtained bootstrap trees, and the branch support values (in percent) represent the frequency of a given branch being found in all bootstrap trees. In the second approach, support values from bootstrap trees are extrapolated on the main tree that is based on the original data set (and was often obtained with a more sophisticated approach). According to most software manuals, the bootstrap support value of 75% or more means this branch can be seen as reliable. In case of Bayesian inference, the posterior probability of a branch is calculated from the occurrence of this branch in all the found trees. Branches with support values of 95% or more are considered reliable. There also several variations of the

bootstrap method, such as the rapid bootstrap implemented in the RAxML software and the ultra-fast bootstrap used in IQ-Tree software.

3.2. Theoretical approaches in genosystematics

One of the advantages of molecular phylogenetic methods is that the DNA or RNA sequences studied directly reflect the evolutionary pathway. At the same time, this is the "weak spot" of this approach: molecular phylogeny works with an assumption that the genetic distance between DNA sequences of two species points to the time both diverged from a common ancestor, and the genetical genealogies actually represent the organismal ones, with the gene tree representing the species tree. However, this is not always the case; a schematic example of this is given on Figure 8. A duplication event in the ancestor of species A and B is not followed by an immediate speciation event.

Besides the difficulties in the interconnection of genetical and organismal genealogies, there are two theoretical approaches to the interpretation of inferred phylogenies. Scientists performing research is the areas of molecular phylogeny have to stick to one of two possible tendencies in the theory of molecular evolution: cladistics or evolutionary taxonomy.

According to the cladistic theory (also called phylogenetic systematics), derived from W. Hennig, organisms form clusters according to certain features that distinguish them from their last common ancestor – so-called apomorphies. The usage of traits that are as well present in the ancestor is not allowed in species descriptions, because it can lead to the creation of paraphyletic clades, i.e. a clade that does not include all the descendants of one ancestor.

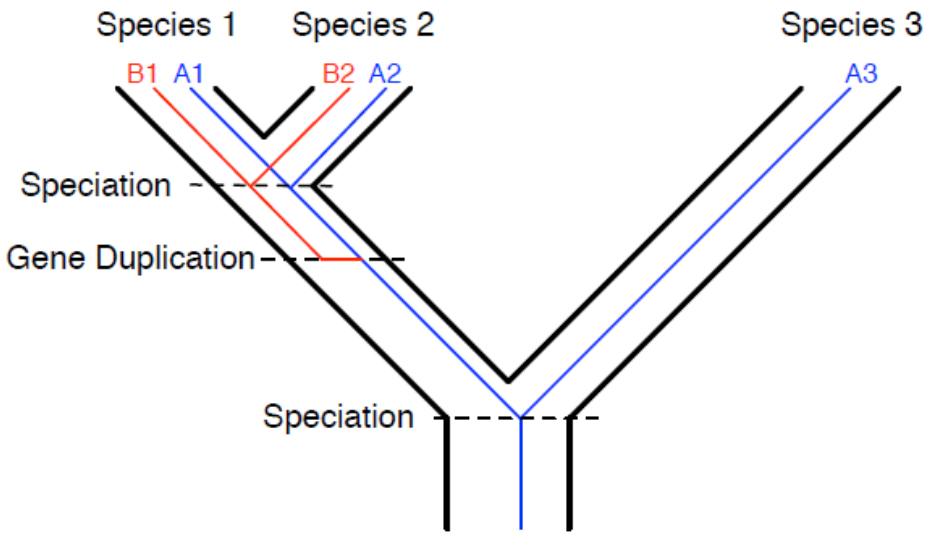


Figure 8. Schematic representation of evolutionary relationships between species and their genes. The black tree shows the phylogeny of species 1, 2, and 3. The blue and red lines represent the phylogeny of the genes A and B. (Koestler 2012)

On the other side, evolutionary taxonomy, being supported by W. Mayr, G. Simpson and others, accepts paraphyletic clades as a sub-type of monophyletic ones, whereas the classical cladistic approach rejects them as not appropriate for describing evolutionary relationships. One of the contemporary supporters of evolutionary taxonomy is Thomas Cavalier-Smith, who specializes in macrosystematics. Many taxonomic groups in Myxomycetes are paraphyletic, so the ideas of evolutionary taxonomists have to be taken into account while working on Myxomycete phylogenetics.

The theoretical background for molecular systematics was created in the 1960s by E. Zuckerkandl, E. Margoliash, L. Pauling and F. Fitch. The first time principles of molecular systematics were applied in research was in the work of Ch. Sibley (on birds), Dessor (herpetology), and Goodman (primates). The first methods used were protein electrophoresis and DNA-DNA hybridization.

An important prerequisite for performing a phylogenetic analysis is the availability of additional data on sequences of the studied organism group. The involvement of additional data increases the range of the study and its scientific value. In 1983, the 5,8S rDNA sequence of *Physarum polycephalum* was obtained (Otsuka et al., 1983). In 1995, a study began where different rDNA regions of Myxomycetes were amplified (Rusk et al., 1995). In 1997, first reviews were published based on researches performed, that addressed the origin and evolution of Myxomycetes in the light of molecular studies (Baldauf, Doolittle, 1997). At the present time, over 1170 nucleotide sequences of rDNA from over 700 species of Myxomycetes are deposited in the NCBI GenBank database (www.ncbi.nlm.nih.gov/genbank). These data allow us to significantly increase the amount of specimen studied and raise the reliability of molecular studies in taxonomical areas.

3.3. Genetical markers in Phylogenetics

An important part of a phylogenetic analysis is the selection of an appropriate region of the DNA to analyze. Depending on the scope of the analysis, more or less variable sequences must be used. Macrosystematic researches are mostly based on sequences of conserved genes, such as the tubulin synthesis genes (TUB) (Tourancheau et el., 1998). Due to selection pressure, mutations occur less often in such genes, and they are thus more conservative. On the contrary, investigations on the species level do not require conserved genes to be involved. The ribosomal DNA (rDNA) is widely used in such studies. It consists of multiple copies located in the nucleolus. Each copy contains the 18S, 5.8S and 28S genes separated by internal transcribed spacers (ITS) (Figure 9). The latter are subjected to splicing and are therefore more variable. An average size of 600-700 bp makes them easy

to amplify and sequence in one run. However, while the ITS regions are widely used in fungal research, in case of Myxomycetes, the partial 18S region of the rDNA is mostly used (Small Subunit, SSU) (Fiore-Donno et al., 2005, 2008, 2011, 2012; Aguilar et al., 2013). This part of the rDNA is less variable, and is used to study Myxomycete phylogenies from the species to the family level (Fiore-Donno et al., 2008; Erastova et al., 2013).

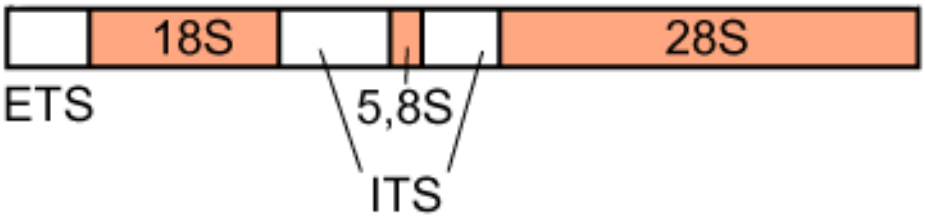


Figure 9. Schematic representation of one copy of the rDNA in eukaryotes. ETS – External Transcribed Spacer; ITS – Internal Transcribed Spacer; 18S, 2.5S, 28S – rDNA genes encoding respective rRNA molecules, that together constitute the ribosome.

Moreover, the NCBI GenBank database contains a huge amount of partial 18S SSU sequences of Myxomycete origin, allowing to add third-party data to our analyses. Besides that, the full 18S SSU gene can be sequenced with a length of appr. 1500-2000 bp. The length can vary because of 6 possible introns with a total length of around 1000 bp. We have shown that increasing the length of the analyzed 18S SSU fragment increases the support values of the obtained phylogenetic trees (see 5.7. Phylogenetic analysis of the *Ph. notabile* species complex).

One of the drawbacks of molecular phylogenetic methods is the fact that the obtained trees reflect the evolutionary pathway of the studied gene, not the taxon in question. At the same time, those pathways do not need to be the same: the speciation process as such does not need to be connected with changes in the rDNA. Not all changes in nucleotide sequences need to be followed by speciation events. Adaptations to certain ecological or physiological environments can occur with a speed that differs from the evolutionary rate of a single marker gene. Such cases have been shown for Dinoflagellates (Logares et al., 2007), where two species clearly differing in morphological traits had identical ITS 1-2 sequences. Similar results were obtained by Barth et al. (2006) in research on species of the genus *Paramecium*. Vice versa, cases of disproportionally fast evolution in protistean marker genes are known, e.g., in Foraminifera (Pawlowski, Berney, 2003). Such ambiguities can be avoided by using several unlinked marker genes. In addition, increasing the size of the studied DNA fragment raises the informativity of the analysis. Because of this, together with the main phylogenetic

marker (partial 18S SSU sequence) also the full sequence of the 18S SSU gene was used in this study.

Besides the 18S marker gene, the cytochrome-oxidase I gene (COI) is often used in phylogenetic researches (Ward et al., 2009). However, its usage with protists is hampered by low numbers of sequences available in public sequence databases. Instead, we used the nuclear tef1 gene to verify the topologies based on the SSU sequence. If both analyses produce same results, it can be concluded with high probability that the true evolutionary pattern was obtained.

The tef1 gene is more conservative than the SSU, encoding the translation elongation factor eF1a. Even single-nucleotide mutations that lead to a frame shift (insertions and deletions) would lead to malfunctions in protein synthesis and would therefore be lethal. The sequences of this marker gene in Myxomycetes are used on the genus, family and order levels (Baldauf, Doolittle, 1997; Fiore-Donno et al., 2005, 2009, 2011). The NCBI GenBank databases contains 137 sequences of the tef1 gene from about 100 Myxomycete species, which, as it was in the case of the 18S gene, widens the possibilities of a phylogenetic analysis considerably. In addition, since our collection of Myxomycete samples included specimens of the studied morphotype from different regions of the world (see Supplementary Table S1), we had the possibility to evaluate the relationship between the genealogy and geography of this species. This question expands the work into the field of Phylogeography.

3.4. Phylogeography: history of development, main aspects

The term “Phylogeography” is used to designate a new scientific field that combines approaches from populational biology, genomics, systematics and biogeography. Phylogeography can be considered as a part of a larger discipline – biogeography. In contrast to ecogeography, which studies the factors of genetical selection that influence the geographical distribution of clusters, phylogeography mostly deals with historic aspects of the taxon distribution over the earth (Avise, 2000). The analysis and interpretation of data on lineage distribution patterns is only possible when involving information from several disciplines: molecular genetics, genosystematics, populational genetics, ecology and historic geography. It is obvious that historical events that represent a complex of geographical and/or ecological factors define not only the present-day biodiversity of a community, but also the spatial distribution of a species. Thus, phylogeography can be seen as an integrative discipline. J. Avise et al. (1987) suggested the term “intraspecific phylogeograhy” to describe this field of study.

The development of phylogeography is tightly connected with rapid development of different molecular methods used in mitochondrial DNA analysis. Initially, birds, mammals (including humans) were studied in such researches. The study of the mentioned DNA type is justified in these

organisms, since the mitochondria are inherited maternally. This allows to follow the historical development of the studied genealogy. In 1998, a special issue of "Molecular Ecology" was published and in 2000 the book "Phylogeography" was published in the Harvard University (Avise, 2000).

According to J. Avise, Phylogeography is an area of study that deals with processes and principles behind the geographical distribution of genealogies, especially on the intra-specific level. Clusters that are obtained on intra-specific phylogenetic trees often demonstrate a clear geographic pattern. Many different types of features (morphological, behavioral, molecular and others) can be used for phylogeographic (broadly speaking) reconstructions; however, standard phylogeography deals with patterns of spatial distributions of alleles, those phylogenetic relationships are known or can be assessed. Phylogeography analyses gene lineages of different loci in mitochondrial and nuclear DNA. According to Avise, because of several evolutionary benefits the mitochondrial genome fits the given task better than the nuclear one, allowing one to access the "family archive of species" (Avise et al., 1989). However, since the establishment of Phylogeography as a science several works appeared where nuclear genes are involved in a phylogeographic context (Aquadro et al., 1991; Bernardi et al., 1993; Palumni, Baker, 1994; Avise, 1998). The relationships between the gene genealogy and geography were called "phylogeographic pattern". Data on intra-specific DNA diversity are studied from two different points of view: a) genealogical relationships between DNA molecules and b) geographical distribution of phylogenetic groups. These two elements combined constitute intra-specific phylogeography.

Phylogeography created a shift in the genealogical thinking on the intra-specific level that was based on a huge amount of data obtained worldwide. Reaching deep into the history of genealogies with the help of molecular markers now becomes an essential part of species concepts. The phylogeographic approach underlines historical microevolutionary aspects and connects population genetics and phylogenetics. Molecular phylogeography changes the understanding of the species as a simple network of organisms sharing a common gene pool (according to Dobzhansky) or as groups of mating populations (according to Mayr). This is of great importance for the research of taxonomic systems in microorganisms, since the above mentioned species concepts often cannot be applied in their case. Now a new definition can be set for the species, and this has to be done by a joint collaboration of molecular biologists and specialists in intra-specific biodiversity.

In order to establish phylogeographic approaches in the research of microscopic organisms, new empirical data are needed on comparative phylogeography in regional biota. These are of great importance nowadays because of the wide spread of molecular methods and the prevalence of the phylogenetic species concept in systematics. This often leads to an "over-splitting" of already described taxa and to creation of new ones, something called "taxonomic inflation" (Isaac et al.,

2004). This situation can be observed nowadays in regards to microorganisms. It is known that our understanding of the number of species in microorganisms is far from being complete. In this light, studying closely related species that are divided by barriers (spatial, ecological, reproductive) is of great importance.

It has been shown that desert storms carry tons of dust over Africa and Asia every year. They create a peculiar atmospheric bridge for various groups of protists (Kellog, Griffin, 2006; Gorbushina et al., 2007). Dust transport from the Gobi desert to western US states is possible (Griffin 2007). We have collected a variety of isolates of model morphospecies collected in desert regions of Russia, Kazakhstan, Mongolia, China, USA and Oman. This allows us to consider the possibility of trans-continental transfer of myxomycete spores and microcysts together with dust particles. The analysis of myxomycete distribution in deserts is valuable to understand the evolution of ecosystems and population structures in heterogenous desert habitats. So far such research including molecular, ecological and geographical data was carried out only for some groups of microorganisms (Martiny et al., 2006).

Chapter 4. Materials and Methods

4.1. Specimens used in this study

199 samples of sporocarps from different individuals were selected for this study including the representatives of the genus *Physarum* from the Herbarium of the Komarov Botanical Institute RAS (LE), as well as samples from the private collections of Prof. M. Schnittler (University of Greifswald, Germany, samples marked with "sc"), prof. S. Stephenson (Arkansas University, USA), E-G. Carvajal (Madrid, Spain), and from the myxomycete collection of the Botanical Institute of Lithuania (BILAS, Vilnus, Lithuania) (Table 3).

Analysis of partial 18S SSU gene sequence	173
Analysis of full 18S SSU gene sequence	9
Analysis of the partial tef1 gene sequence	18
Morphological analysis	83
Specimens studied in total	199

Table 3. Number of samples used in this study.
Note: for a detailed list on specimens studied by each method, see Supplementary Table S1.

104 samples belong to the morphospecies *Physarum notabile*. These samples were collected in the arid regions of the Caspian lowland, Eastern, Central and Western Kazakhstan, as well as adjacent territories of Mongolia and China – the Khovd aimag and Xinjiang provinces, respectively. DNA was also extracted from the sporocarps of the arid morphotype of *Ph. notabile* found in Patagonia (Argentina), Arizona (USA) and Oman (Novozhilov et al., 2013) (Figure 10). Besides this, representatives of the "classic" form of *Ph. notabile* from deciduous and coniferous forests in Northern America, Germany and the Novosibirsk region in Russia were studied. A list of all studied isolates and their geographical localities are presented in Supplementary Table S1.

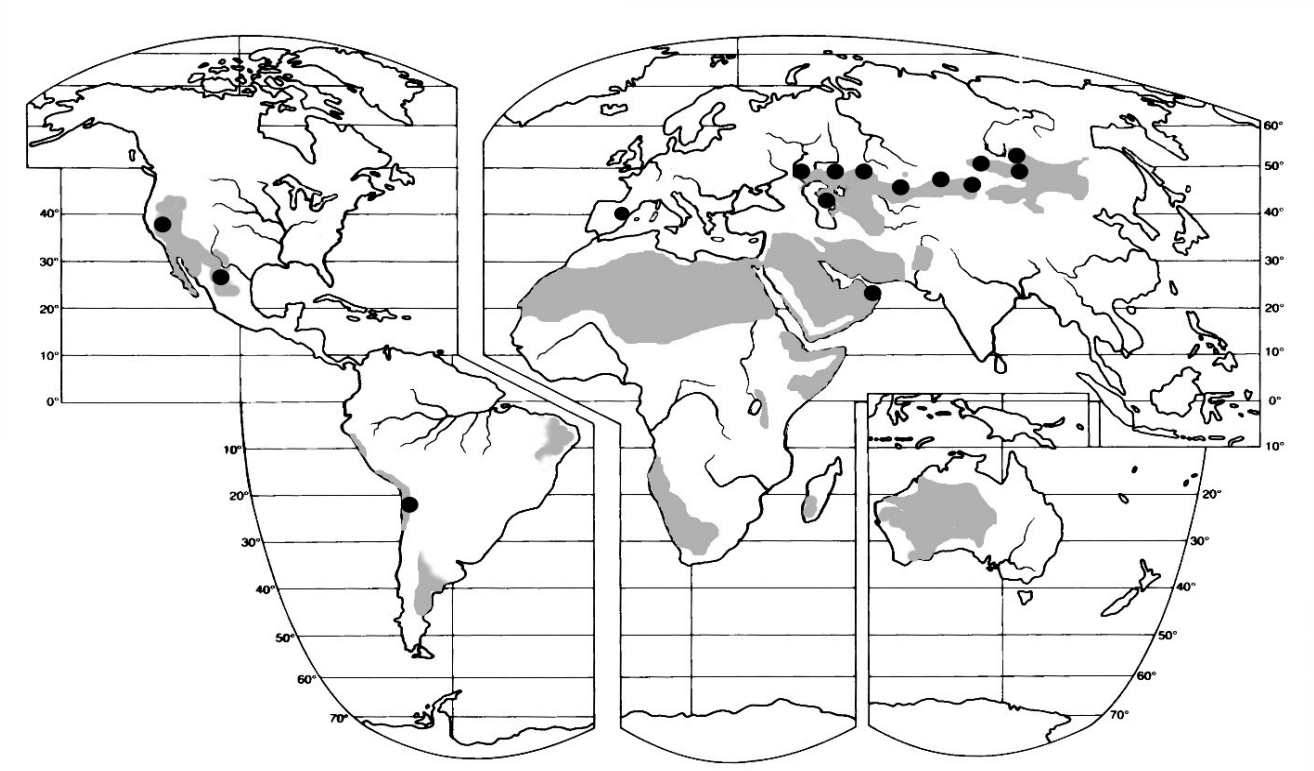


Figure 10. Collection places of the isolates used in this study. Arid regions of the world are colored gray.

4.2. Moist chamber experiments

The moist chamber method implies collecting substrate samples from soils on which Myxomycetes can possibly grow (Gilbert, Martin, 1933). Substrate samples were placed in Petri dishes on filter paper soaked in water. Samples were incubated for 2 months (for herbivore dung – 3 months) at room temperature and ambient light. Dishes were screened under a light binocular microscope on a regular basis.

4.3. Microscopy

Dried sporocarps were studied by light microscopy with a Stemi 2000 binocular microscope, a Zeiss Axio Imager A1 microscope with differential interface contrast. The study of spore ornamentation was carried out using scanning electron microscopy (SEM) on a JSM-6390 LA electron microscope. For the microscopy, sporocarps were preserved as permanent slides in polyvinyl-lactophenol, the measurements of spore diameter and sporocarp sizes were conducted with the Axio Vision 4.8 software (http://microscopy.zeiss.com/microscopy/en_de/products/microscope-

software/axiovision-for-biology.html).The spore diameter was measured in 25 spores of one herbarium sample. For the SEM, samples were fixed on a copper plate and sputter-coated with gold.

4.4. Morphological analysis

Non-metric multidimensional scaling (NMS) was used to assess morphological patterns of 85 samples of the representatives of the *Ph. notabile* species complex: arid form of *Ph. notabile* (39 samples), *Ph. notabile s.str.* (11), *Ph. album* (10), *Ph. leucophaeum* (10), *Ph. nivale* (9) and *Ph. pusillum* (6). The analysis was carried out with the PC-ORD 5 software (McCune, Mefford, 1999; Holland, 2008). The software was run in autopilot mode with default search settings. 17 morphological features were involved in the analysis; quantitative features were measured (m), qualitative were evaluated according to a scale (s). Measurements were performed either on 3 well-developed sporocarps (+), or on the full collection of fruiting bodies (++).

Below is a list of all studied features (roman numerals) and their possible values (Arabic numerals):

I - sporotheca shape (s++; 1 = depressed to lenticular, 2 = subglobose or globose, 3 = reniform, 4 = obovoid);

II- sporotheca width (m+) and III height (m+);

IV - peridium color at bottom of sporotheca (s+; 1 = black, 2 = brownish, 3 = reddish, 4 = white);

V – stalk color (s+; 1 = black, 2 = brownish, 3 = reddish, 4 = white);

VI - dominant sporophore type in colony (s++; 1 = sessile, 2 = subsessile, 3 = stipitate);

VII - stalk length (m+);

VIII - ratio stalk length to sporotheca height min (m+) and IX max (m+);

X - capillitium network (s+; 1 = scanty, 2 = dense, abundant);

XI - capillitium shape (s+; 1 = reticulate, 2 = branching dichotomously);

XII - capillitium nodes (s+; 1 = absent or poorly differentiated from threads, 2 = spherical, 3 = elongate, 4 = branched and various);

XIII - nodes size (s+; 1 = nodes large, 2 –5 times wider than an adjacent part of filament; 2 = nodes delicate, 1.5 –2 times wider than an adjacent part of filament; 3 = absent);

XIV - spore mass color (s++; 1 = brown, 2 = dark brown, 3 = black);

XV - spore diameter (m+; 15–25 spores per sporocarp);

XVI - length of spore ornaments (spinulae, m+; 5–10 spores per sporocarp);

XVII - density of spore ornaments (m+; spinulae per 9 μm^2 spore surface, 3 spores per

sporocarps). The data used for the NMS analysis is presented in Supplementary Table S2.

4.5. DNA extraction from myxomycete spores

DNA was extracted directly from the spores that were obtained from sporocarps. For this, sporocarps were shock-frozen with liquid nitrogen, and the spore mass was disrupted with a pestle in a 1.5 ml centrifuge tube. Alternatively, Retsch MM200 and Qiagen TissueRaptor laboratory mills were used for the disruption. The resulting protoplasts were subjected to CTAB-Buffer (2% CTAB , 2%PVP, 5% SDS, 100mM TrisHCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol) for 2 hours at 65° C for cell lysis. DNA was extracted with chloroform precipitation and then precipitated using isopropyl alcohol and 5 M ammonium acetate. The washing step was performed with 70% alcohol, and then the DNA was eluted with 15 µl of the elution buffer from the Quiagen QIAquick PCR Purification Kit. Alternatively, after spore wall disruption, the Axygen Genomic DNA Miniprep Kit was used for DNA extraction according to the manufacturer's protocol, except that 50 µl of the elution buffer were used in the elution step.

Later during the study, an alternative method of spore wall disruption was developed, that does not require liquid nitrogen. Fruiting bodies were subjected to a complex of natural chitinases from the fungus *Trichoderma* that were diluted in BFLE buffer (0.1 M sodium citrate pH=6.0, 20 mM EGTA, 5% BSA) to a concentration of 2 mg/ml. Lysis occurred at room temperature for 1 hour. This treatment thinned the spore wall that contains galactosamine complexes (McCormic et al., 1970), and facilitated further detergent treatment on the cell membrane. After chitinase treatment, samples were centrifuged at 12000 rpm for 10 minutes for the cells to precipitate; supernatant was removed and the spore mass was washed twice with 0.1 M PBS buffer with subsequent centrifugation at 12000 rpm for 5 minutes. Samples obtained this way were then used in DNA extraction with the Axygen DNA extraction kit as described above.

4.6. Primer design

A new primer pair was designed to amplify a part of the translation elongation factor gene (tef1). Primer design was carried out in the web-based primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) on the NCBI web portal. The tef1 gene sequence of *Physarum polycephalum* (AF016243) was used as a template. Primer search was done with the following parameters: amplicon length – 600-900 bp, difference in melting temperature - < 3°C, number of oligonucleotides – 2, not more than 2 mismatches to the template sequence.

4.7. PCR

For the amplification of the partial 18S SSU rDNA gene the S1-SU19R primer pair was used (Fiore-Donno et al., 2008). The 18S gene can include up to 6 introns; the presence of an intron in the studied sequence significantly lowers its phylogenetic informativity because intron sequences vary in length and cannot be aligned properly. Moreover, the introns hamper PCR and sequencing through tandem repeats. The S1-SU19R primer pair used in this study amplifies the beginning of the 18S gene, appr. 660 bp long, which is free of introns. This part of the 18S gene contains the V2 helix that has increased variability; this additionally increases the resolution of phylogenetic methods. Together these benefits allow us to use the phylogenetic information the best possible way and sequence many samples to increase the informativity of the overall analysis. Still, to further improve the reliability of the trees constructed based on the marker mentioned above we obtained the full sequence of the 18S SSU gene for several specimens. For those samples, the 18S gene was amplified in three PCR runs with the primers S1-SU19R, S3Dark-SR14, S12'-SRBE (Fiore-Donno et al., 2008). For two of these samples, 4 and 5 runs were performed, respectively. Here, the primer pairs S1-SU19R, S3Dark-753Rn, 718Fl-SR13, S12'-SRBE, S12'-SR15, S14,5a-SRBE were used (Fiore-Donno et al., 2008). All primer sequences and their respective melting temperatures are listed in Table 4.

Primer	Sequence	Melting temperature, °C
S1	AACCTGGTTGATCCTGCC	62
S3Dark	GTGCTTCTGACCTATCAACTAG	58
718FL	GTAATTCCAGCTCTAATAGCATACG	58
S12'	CAGATACCGTCGTAGTCTTAAC	58
S14.5A	TAATTTGACTCAACACGGGA	57
SU19R	TCGAGTAACAATTAGAGGACA	56
753Rn	GGTTAAAACGCTCGTAGTCGGC	65
SR13	GGAGTATGGTCGCAAGGCTG	65
SR14	GGAAGGGCACACAAAGAGT	62
SR15	GTAAGTGGTGGTGCATGG	60
SRBE	GCAGAAGTCGTAACAAGGTA	58
PB1F	ACCCGTGAGCACGCTCTCCT	59,9
PB1R	CGCACATGGGCTTGGAGGGG	60,0

Table 4. Primers used in this study, their sequences and melting temperatures (all except the last two taken from Fiore-Donno, 2008).

The PCR reagents set-up is explained in Table 5. Besides the reagents used, in some reactions

the iQ-Supermix (Bio-Rad) PCR kit was used, which already contains polymerase buffer, polymerase, magnesium chloride and DNTP. The reagent proportions for these cases are shown in Table 6.

Reagent	Initial concentration	Volume, µl
Deionised water	-	19,4
Polymerase buffer	10x	3,0
Magnesum Chloride	50 mM	1,8
Forward primer	3 µm/ml	0,3
Reverse primer	3 µm/ml	0,3
DNA	-	2,0
DNTPs	2 mM	3,0
Polymerase	5000 u/ml	0,3

Table 5. Contents of the PCR mix used in amplification. Reagent volumes for one sample (30 µl) are given.

The polymerase buffer (SibEnzyme) included 20 mM Tris-Hcl (pH 7.6), 100 mM Kcl, 0.1 mM EDTA, 0.5% Triton-X100, 1 mM DTT, 50% glycerin.

Reagent	Initial concentration	Volume, µl
Deionised water	-	12,4
Forward primer	3 µm/ml	0,3
Reverse primer	3 µm/ml	0,3
DNA	-	2,0
iQ Supermix	2x	15,0

Table 6. Contents of the PCR mix when using iQ-Supermix kit. Reagent volumes for one sample (30 µl) are given.

The following PCR protocol was used for the amplification (primer annealing temperatures can be found in Table 4):

1. 95°C – 3 min (initial denaturation)
2. 95°C – 30 sec (denaturation)
3. XX°C – 30 sec (primer annealing)
4. 72°C – 48 sec (elongation), go to step 2, 40 times
5. 72°C – 5 min (final elongation)

For the amplification of the tef1 gene, the primers PB1F and PB1R were used that were designed in this study. The resulting sequence is about 700 bp long and can include one intron. All PCRs were carried out on a Bio-Rad C1000 thermocycler.

PCR results visualization was carried out with agarose gel electrophoresis. For this, 5 µl of the PCR sample were mixed with 1 µl of loading dye (Fermentas) and loaded onto a 1% agarose gel (70 ml) containing 2 µl of the GelRed dye (alternatively, ethidium bromide or SYBR-Green). The electrophoresis was run under the following conditions: 70 volts, 1 hour. Band visualization was performed in the Bio-Rad GelDoc XR gel documentation system using the software Quantity One (Bio-Rad) with a 100 bp DNA ladder (Axygen).

PCR products purification was performed with the Qiagen QIAquick PCR Purification Kit or with the Axygen PCR Purification Kit. In some cases, if non-specific products were obtained in the PCR and several bands were visible on the gel, the product was loaded onto an agarose gel and separated by electrophoresis. The fragment in question was then cut out of the gel and purified with the Qiagen MinElute Gel Extraction Kit according to the manufacturer's protocol.

4.8. Sequencing

Sequencing PCR was set up with the purified PCR products together with the BigDye Xterminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). In the sequencing PCR, the same primers were used as during the amplification. Samples obtained this way were purified with the BigDye Xterminator purification Kit (Applied Biosystems) or by means of alcohol precipitation. For the latter, samples were centrifuged at 10000 g for 20 minutes, then the supernatant was removed and the samples were washed with 100 µl of 75% isopropanol. Then samples were centrifuged again for 2 minutes, the supernatant was removed and the samples were air-dried at 60°C for 2 minutes. Sequencing was performed on a ABI 3130 Genetic Analyzer (Applied Biosystems). Raw sequence data was analyzed in the ABI Sequencing Analysis 5.3.1 software or in Sequencher 4.7.

4.9. Computational analysis

Sequence alignment. Editing of the chromatograms was conducted in the Chromas Lite 2.1.1 software (http://technelysium.com.au/?page_id=13). The forward and reverse sequences were aligned in MEGA 5.2 (http://megasoftware.net) using the Muscle algorithm. In order to avoid contamination, each sequence was analyzed with BLAST (Basic Local Alignment Search Tool) on the NCBI web-portal to check for homologs in possible contaminants

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) (default search settings). Sequences checked this way were aligned with different algorithms: ClustalW (Larkin et al., 2007), Muscle (Edgar, 2004), MAFFT (Katoh et al., 2002), Tcoffee (Notredame et al., 2000). Full 18S SSU as well as partial tef1 gene sequences were submitted to the NCBI GenBank database (Table 7).

Sample	Species	SSU	tef1
LE 255721	*Ph. pusillum*	JX035984	KC473819
LE 255719	*Ph. pusillum*	JX035983	KC473815
LE 204508	*Ph. pusillum*		KC473821
LE 255714	*Ph. pseudonotabile*	JX035987	KC473818
LE 255438	*Ph. pseudonotabile*	JQ478431	KC473817
LE 255437	*Ph. pseudonotabile*	JX035986	KC473813
LE 255712	*Ph. pseudonotabile*	JX035985	KC473816
LE 47300	*Ph. leucophaeum*		KC473814
LE 47431	*Ph. leucophaeum*	JX035989	
LE 255710	*Ph. notabile*	JX035988	KC473820

Table 7. A list of nucleotide sequences of the 18S SSU gene (full sequence) and tef1 (partial sequence) submitted to the NCBI GenBank database. GenBank accession numbers are given in the third and fourth columns.

Tree reconstruction. The phylogenetic trees were reconstructed using the Maximum Likelihood (ML) and Bayesian Inference (BI). When applying the ML approach, RaxML 7.0.3 (www.exelixis-lab.org) and IQ-Tree 0.9.5 were used (www.cibiv.at/software/iqtree). In the RaxML software, 200 trees were reconstructed first, of which the tree with the highest log likelihood was selected. Then 1,000 bootstrap replicates were performed based on the initial alignment. The bipartition information from bootstrapped topologies was then drawn on the best tree to obtain a topology with support values. This approach is recommended by the software author as the most reliable (Stamatakis, 2006). When working with the IQ-Tree software, the tree was reconstructed using the program's default settings, then 10,000 ultra-fast bootstrap iterations were performed on the alignment (Minh et al., 2013). The IQ-Tree software supports several evolutionary models. Before each tree reconstruction the best-fit model among 88 models for DNA was selected for the alignment with the ModelTest as implemented in IQ-Tree. When analyzing protein-coding sequences, they were translated into amino-acid sequences. For these data, the best-fit amino-acid model was selected among possible 112 models in IQ-Tree.

The Bayesian Inference was performed in the MrBayes 3.2.2 software (www.mrbayes.sourceforge.net) with the following settings: GTR+Gamma as the evolutionary model, 1 million generations, 2 independent chains. If the standard deviation of split frequencies

between runs did not reach the value of 0.01 after the given number of generations, another 1 million of generations was run. After the runs finished, the trees that were sampled before the runs reached a deviation value of 0.01 were not taken for consensus tree reconstruction. When summarizing the trees to a consensus, the *allcompat* option was used that kept all the branches independently of their support values as long as they do not contradict each other, thus minimizing multifurcations.

For combining the ML and BI topologies the IQ-Tree software was used. For this, the bipartition information from bootstraped trees obtained during the ML search was drawn onto the BI tree. Thus, the resulting tree had support values from both bootstrap tests and Bayesian posterior probability.

For a two-gene based phylogenetic analysis, the partition model options of IQ-Tree and MrBayes were used. The two data sets were aligned separately and then concatenated ("super-matrix"). Evolutionary models and all parameters were estimated separately for the two data sets, as well as the bootstrap in IQ-Tree. All alignments used in this study as well as resulting trees were submitted to the TreeBASE repository (www.treebase.org) under the accession number 15723. For a list of additional sequences taken from the NCBI GenBank database see Table 8.

Species	**18S SSU**	**tef1**
Badhamia melanospora	KC759110	
Badhamia panicea		FJ546661
Badhamia utricularis	HE614597	
Badhamia versicolor		FJ546663
Diderma globosum	DQ903677	EF513191
Didymium melanospermum		FJ546667
Didymium nigripes	AF239230	FJ546668
Physarum album	DQ903681	EF513196
Physarum cinereum	HE614599	
Physarum compressum	HE614600	
Physarum didermoides	HE614602	FJ546684
Physarum flavicomum	HE614611	
Physarum melleum	KC759095	
Physarum nivale	DQ903680	EF513184
Physarum polycephalum	X13160	AF016243
Physarum rigidum	HE614604	
Physarum roseum	HE614605	

Physarum vernum	KC759103	
Physarum virescens		FJ546687
Physarum viride		FJ546688

Table 8. Accession numbers of sequences taken from the NCBI GenBank database and used in this study.

Phylogeographic analysis. Partitioning the data set into OTUs for the phylogeographic analysis was performed as described in Zarrei et al. (2012). Sequences were referred to a certain cluster based on the genetic distance with a threshold value given. The rule was that no sequence in a cluster had a distance larger than the threshold to its closest neighbor. In order to assess the threshold distance, plots were drawn that show the relations of cluster number and the mean genetic diversity in one cluster (standardized to zero) to the threshold value. With the increase of the threshold the number of clusters decreases to a limit of 1, whereas the mean genetic diversity increases. The point where these two graphs intercept is the optimal threshold value: the data set is divided into clusters in a most efficient way without losing too much phylogenetic information. The respective calculations were carried out in Microsoft Excel with the PopTools plugin (www.poptools.org). The evaluation of study completeness was performed using the Chao2 estimator (Chao 1987) and the hyperbolic model (de Caprariis et al., 1976).

We also tried to apply the GMYC method (Generalized Model Yule-Coalescent) (Fujisawa & Barraclough, 2013) to our data to obtain phylogeographic clusters. Ultrametric trees for the GMYC analysis were produced as follows. First, an ML tree was constructed in IQ-Tree. Then the branch lengths were computed in the Tree-Puzzle software (www.tree-puzzle.de) according to the molecular clock hypothesis. The obtained ultrametric tree was analyzed by the GMYC approach using single and multiple threshold settings.

Alternatively, the PTP software (Poisson Tree Process, www.species.h-its.org/ptp) was used to try to split the data set into clusters. This software handles all tree types as input.

For the 69 sporocarp samples of the arid form of *Ph. notabile* belonging to a transect going through the regions of Caspian Lowland, Kazakhstan, western Mongolia and China, as well as for the total of 91 samples a test of isolation by geographical distances was carried out (Slatkin, 1987). This approach makes use of the Mantel test statistics to search for a correlation between genetic distances (the matrix of genetic distances was obtained in the BioEdit software) and geographical distances. The obtained empirical correlation coefficient r was then compared to values obtained in randomization tests. These were conducted in the PopTools Excel plugin, and their 95% confidence interval was compared to the coefficient r.

Chapter 5. Results

5.1. Optimization of DNA extraction and PCR protocols

In addition to the CTAB (hexadecyl-trimethyl-ammonium bromide), commonly used in DNA extraction protocols, we also used the BRIJ58 detergent in order to digest the plasma membrane, and as an alternative, the widely used SDS. Lysis buffers from commercial reagent kits also handled this task. Still, the spore wall disruption was a major problem throughout the whole study.

In literature, many methods of spore wall disruption are mentioned, including deep freezing with following physical destruction, lysis etc. We decided to stick to freezing the samples in centrifuge tubes with liquid nitrogen and crushing with a laboratory ball mill or a pestle directly in the tube. Later on, a novel lysis approach has been established that includes spore wall digestion with the *Trichoderma* chitinase complex. While being generally more time-consuming, this approach requires less hands-on time and does not require liquid nitrogen. Commercial DNA extraction kits (like the Qiagen DNAeasy Plant Mini Kit) did not handle this task: of the four attempts none were successful. The protocols of these manufacturers have to be modified appropriately to apply them to Myxomycetes.

The PCR protocols also were subjected to modifications. Traditional PCR protocols include the dilution of the matrix DNA prior to the reaction set-up. In our experiments, we achieved the best results when using undiluted DNA.

5.2. Molecular analysis of the “classical” and arid morphotypes of *Physarum notabile*

A phylogenetic analysis involving the 18S SSU gene sequences was carried out that included the samples of the arid and boreal forms of *Ph. notabile*. A total of 103 partial SSU sequences of the representatives of the arid morphotype and 33 – of the boreal form (*Ph. notabile* s.str.) were obtained. For the sake of better visualization, a tree of only 15 samples is presented here. This tree (Figure 11) clearly shows that the samples form two separate clusters with high support that correspond to the two studied morphotypes. It can also be seen that the arid morphotype is characterized by a larger genetic variability than the boreal form. Thus, the two forms may actually represent two separate species, artificially combined in one morphospecies, *Ph. notabile*. These results were confirmed by trees based on all sequences obtained (data not shown).

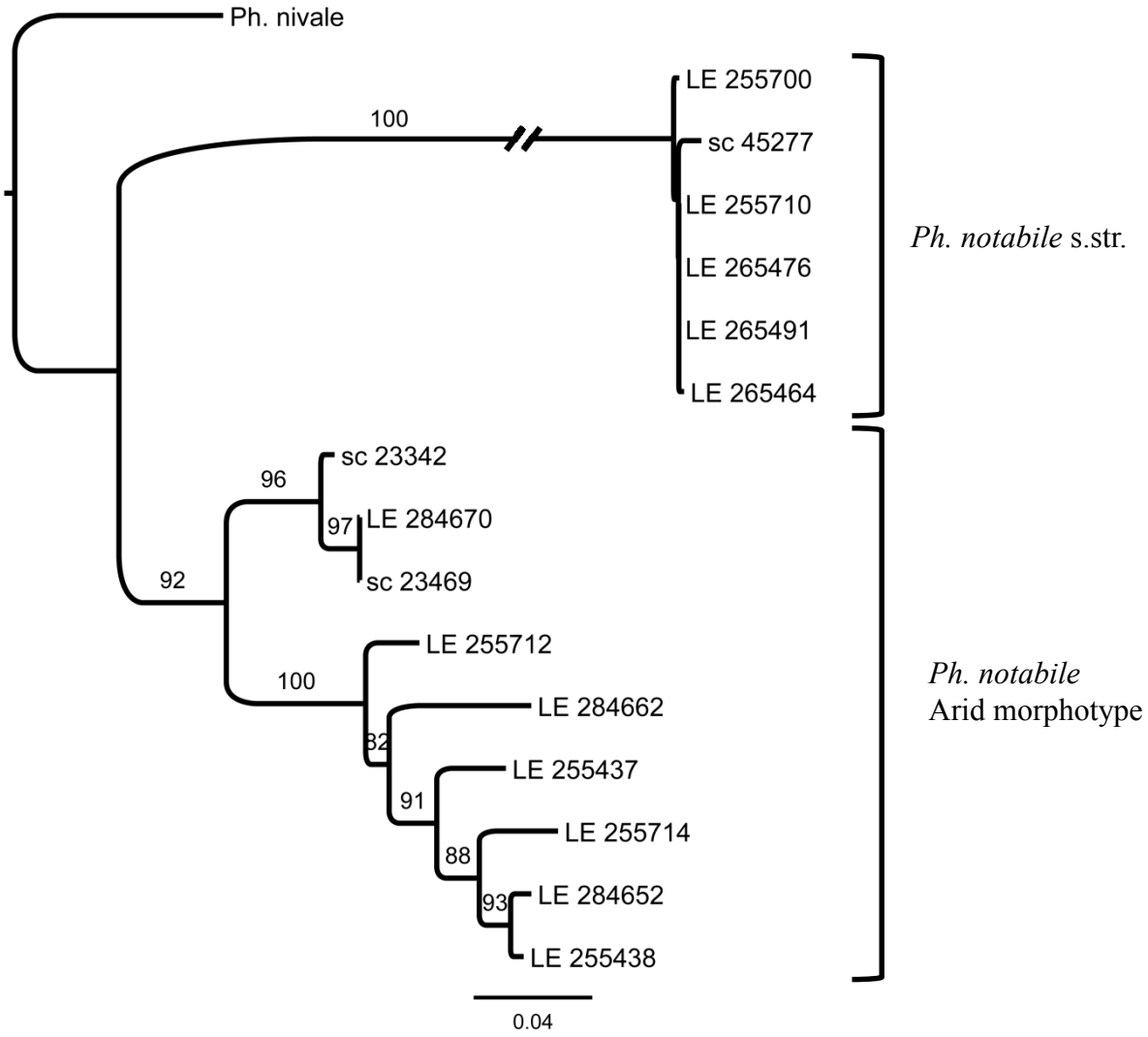


Figure 11. Phylogenetic tree based on the partial 18S SSU sequence (646 bp) from 16 samples. ML analysis: IQ-Tree software, TNe + G4 evolutionary model, 10000 ultra-fast bootstraps. Bootstrap values over 80 are shown for the respective nodes. *Ph. nivale* is used as an outgroup. The herbarium accession numbers (LE) are given for each sample. Samples marked with sc were taken from the private collection of Prof. M. Schnittler (Greifswald University, Germany). Scale bar equals 0.04 nucleotide substitutions per site. The branch length leading to samples of *Ph. notabile* s.str. has been shortened for visual convenience. Real length 0.309.

5.3. Phylogenetic analysis of the arid samples of *Ph. notabile*

In order to increase the informativity of the phylogenetic analysis carried out we obtained full 18S SSU sequences as well as partial tef1 sequences for several specimens – representatives of the arid form of *Ph. notabile* (4 full 18S SSU sequences, 4 partial tef1 sequences) – to compare them with the respective sequences from the boreal samples (1 full SSU, 1 – tef1). We also obtained 5

partial SSU sequences for the species *Ph. album*, 7 – *Ph. leucophaeum*, 3 – *Ph. pusillum* and 2 – *Ph. nivale*, and one full SSU sequence for each of these species, respectively (3 for *Ph. pusillum*). We used representatives of the family *Didymiaceae* as the outgroup; this family forms the order Physarales together with the family *Physaraceae*. Figure 12 shows the result of the phylogenetic reconstruction based on combined full SSU and tef1 sequences of four arid samples of *Ph. notabile* and closely related species. Since the topologies of the ML and Bayesian trees were identical, the ML tree is shown with posterior probability values from the BI analysis as well as bootstrap support for each branch. As shown previously, isolates of the arid form of *Ph. notabile* form a separate clade from *Ph. notabile* s.str. The same results were obtained when analyzing the partial 18S SSU gene sequences (Okun et al., 2011). Arid samples of *Ph. notabile* form a monophyletic clade with high support. This topology was also confirmed in a tree produced in RAxML with a rapid bootstrap support value of 90 for the clade of arid *Ph. notabile* samples (data not shown). However, the branches inside this group also have high support values, indicating a high level of genetic divergence among these specimens. This divergence will be further discussed in the phylogeographic part of this study.

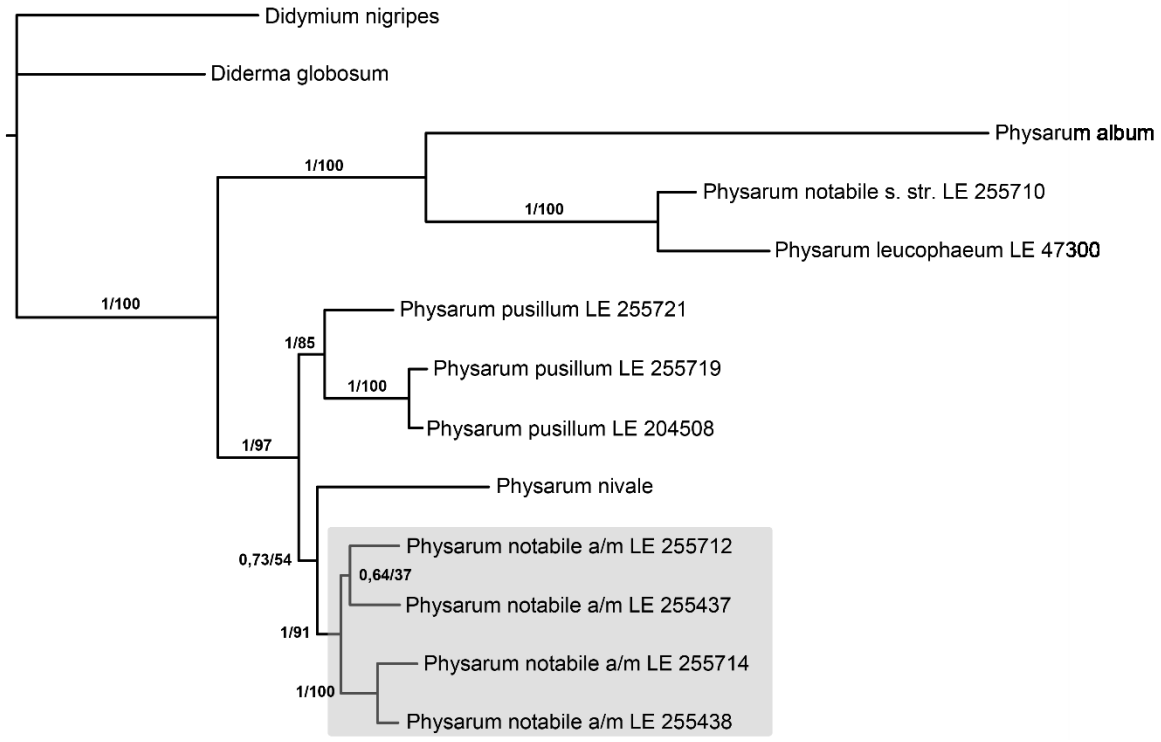


Figure 12. Phylogenetic tree based on the full 18S SSU sequence and the partial tef1 sequence (2008 bp + 237 a/a) from 12 samples of 8 species. ML Analysis: IQ-Tree software, evolutionary models – TNe + G4 (SSU), VT + G4 (tef1), 10000 ultra-fast bootstraps. BI analysis: MrBayes software, evolutionary models – TNe + G4 (SSU), VT + G4 (tef1); 1 mio tree generations. Bootstrap (0-100)

and Bayesian posterior probability values (0-1) are given for each node. *Physarum notabile* (a/m) - arid morphotype of *Ph. notabile*; *Didymium nigripes* and *Diderma globosum* were used as the outgroup. For sequences obtained in this study, herbarium numbers are given. For other taxa, see GenBank sequence accession numbers in Table 8. Samples belonging to the arid morphotype of *Ph. notabile* are shaded gray. Scale bar indicates 0.03 substitutions per site (average between nucleotide and amino-acid).

5.4. Morphological analysis of the *Ph. notabile* species complex

A preliminary morphological analysis has demonstrated that there are differences in sporotheca shape and size as well as spore ornamentation size and density between the boreal *Ph. notabile* and its arid morphotype. Sporocarp type (sessile or stalked) varies more in arid form then in the boreal one. The latter is characterized by a dense spore ornamentation with spinulae, whereas isolates of the arid morphotype have spores ornamented with warts, that are more dispersed and not that uniform (Figure 13, E-G). The spinulae in isolates of the boreal form are 0.60 ± 0.11 μm long with a density of 41.0 ± 11.9 over 9μm^2 spore surface. The warts in the arid form are 0.40 ± 0.18 μm long; ornamentation density is 14.5 ± 5.8, respectively.

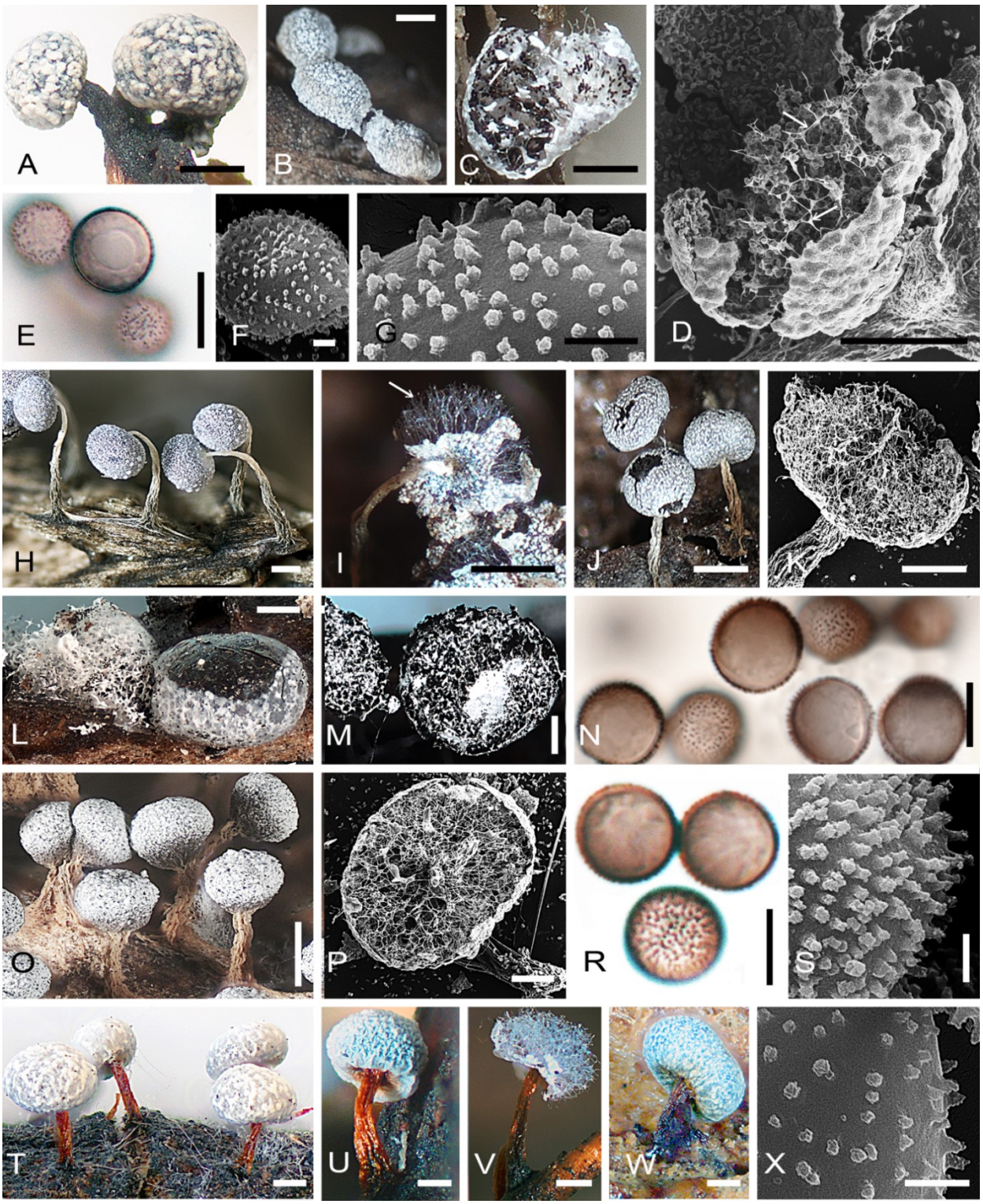


Figure 13. Morphological features of the arid morphotype of *Ph. notabile* (A-G, LE 255438), *Ph. album* (H,I, LE 259241), *Ph. leucophaeum* (J, K, LE 45842), *Ph. nivale* (L–N, LE 285902), *Ph. notabile* s.str. (O–S, LE 255700) and *Ph. pusillum* (T-X). **A.** Two closed sporocarps of the arid morphotype of *Ph. pseudonotabile* under a binocular microscope (BM); scale = 1000 µm. **B.** Plasmodiocarps; scale = 1000 µm. **C.** open sporocarp with white lime nodes in the capillitium (arrow, BM); scale = 1000 µm. **D.** Scanning electronogram (SEM) of an open sporocarp with capillitium

threads and lime nodes; scale = 100 μm. **E.** Spores under a light microscope (LM, immersion oil, 100x lens, view from top and side); scale = 10 μm. **F.** SEM of a spore. **G.** SEM with spore ornamentation details. **H.** Sporocarps of *Ph. album* (BM); scale = 500 μm. **I.** Open sporocarp with radial capillitium (arrow, BM); scale = 1000 μm. **J.** Sporocarps of *Ph. leucophaeum* (BM); scale = 500 μm. **K.** Open sporocarp with an isodiametric network of capillitium threads and lime nodes (SEM); scale = 500 μm. **L.** Sporocarps of *Ph. nivale* (BM); scale = 500 μm. **M.** Open sporocarp (BM); scale = 500 μm. **N.** Spores (LM, immersion oil, 100x lens, view from top and side); scale = 10 μm. **O.** Sporocarps of *Ph. notabile* (BM); scale = 500 μm. **P.** Open sporocarp with capillitium threads and lime nodes (SEM); scale = 100 μm. **R.** Spores (LM, immersion oil, 100x lens, view from top and side); scale = 10 μm. **S.** Details of spore ornamentation (SEM); Scale = 1 μm. **T-W.** Sporocarps from samples LE 255721 (T), LE 204508 (U,V), LE 255719 (W)(BM); scale = 500 μm. **X.** *Ph. pusillum*, details of spore ornamentation (SEM, LE 204508); scale = 1 μm.

We performed a multi-dimensional nonmetric scaling analysis to assess if the studied species intersect in terms of their morphological characteristics. This analysis attempts to illuminate the structure hidden in the data matrix by representing each entry with a corresponding dot in an n-dimensional space. This is done in an iterative design; the best positions for entries are searched for that minimize the stress (sum over all differences from observed data) of the n-dimensional configuration. The NMS carried out on 17 morphological features of 85 sporocarps of *Ph. album*, *Ph. notabile* (both boreal and arid forms), *Ph. leucophaeum*, *Ph. nivale* and *Ph pusillum* resulted in a solution with a final stress value of 7.6 after 60 iterations in a 2D-solution (final instability = 0.00001, Figure 14, A). Reducing the number to eight quantitative characters changed these values only marginally (final stress 7.5, 92 iterations, final instability = 0.00001, Figure 14, B). According to McCune et al. (2002), these values speak for a good ordination with no real risk of drawing false inferences. The data used for the NMS analysis is presented in Supplementary Table S2.

The NMS analysis finds three main groups that show little or no intersection with other taxa: the first one is formed by the samples of the arid morphotype of *Ph. notabile*, the second – *Ph. nivale*, the third – *Ph. album*. The other three species (*Ph. notabile*, *Ph. leucophaeum*, *Ph. pusillum*) are not well separated from each other. However, when analyzing all 17 characters studied these three species can be distinguished by the color and shape of the sporotheca and stalk. Thus, NMS results confirm that most of the isolates of the arid form of *Ph. notabile* can be distinguished from similar representatives of the genus *Physarum* despite a considerable amount of intra-specific morphological diversity. Also, the arid form distinctly differs from *Ph. notabile* s.str. by length and density of spore ornaments, although spore ornamentation and type of sporocarp (sessile or stalked) in the arid from

is generally more variable than in *Ph. notabile* s.str.

We assessed the overall stability of morphologic traits on the example of three studied specimens. The microscopic features are mostly stable (Table 9). The capillitium in sporocarps obtained from cultural experiments is often poorly developed, as are lime nodes in the capillitium and lime flakes in the peridium. At the same time, sporotheca and spore size as well as spore ornamentation seem to be more stable. These data agree with the results of the NMS analysis (Figure 14 A, B).

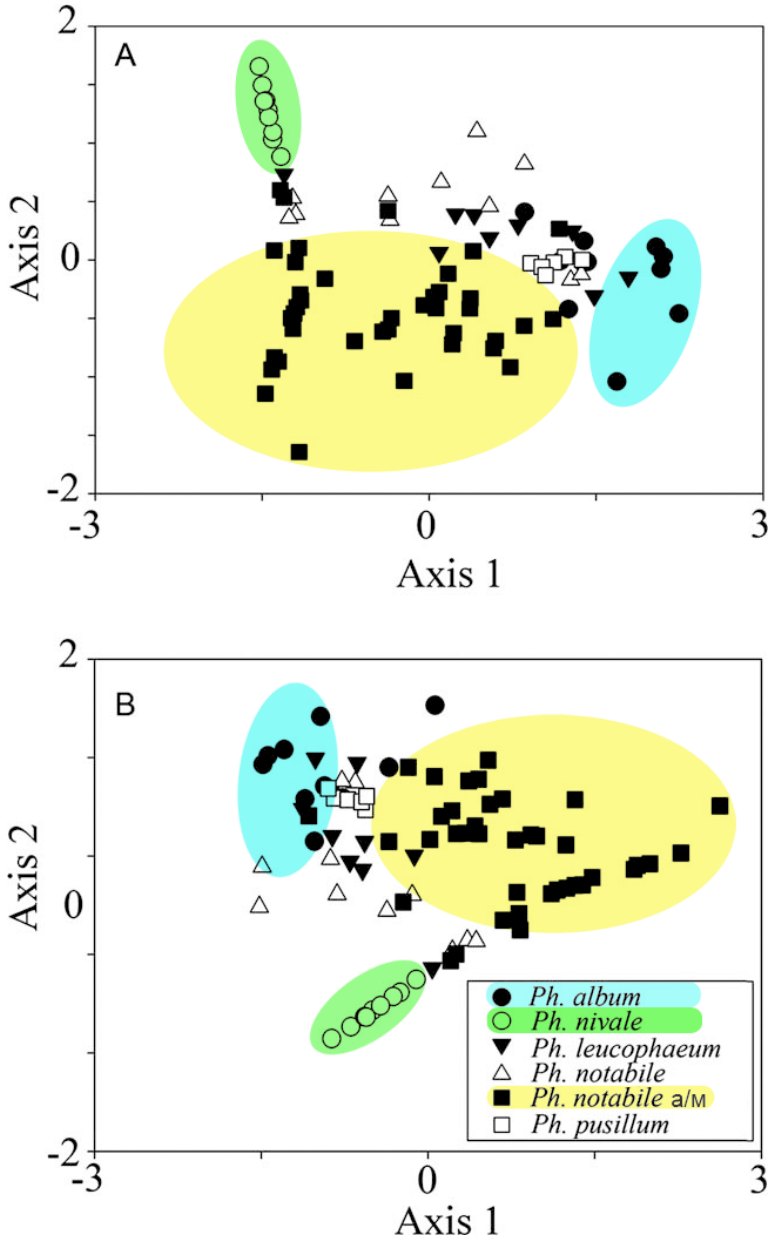


Figure 14. Ordination plot obtained by NMS for the arid morphotype of *Ph. notabile* (39 samples), *Ph. album* (10), *Ph. leucophaeum* (10), *Ph. notabile* (11), *Ph. nivale* (9), *Ph. pusillum* (6). For the characters analyzed, see Chapter **4.4. Morphological analysis A.** Ordination plot of quantitative and qualitative characters. **B.** Ordination plot of qualitative characters.

Sample	LE 220408	LE 253652	LE 255721
I. Sporotheca shape	2 / 2	2 / 2	2 / 2
II. Sporotheca width	290 ± 24 / 294 ± 23	378 ± 52 / 409 ± 88	400 ± 48 / 409 ± 61
III. Sporotheca height	279 ± 39 / 249 ± 43	320 ± 20 / 344 ± 37	294 ± 54 / 267 ± 50
IV. Peridium color	3 / 1	4 / 1	1 / 1
V. Stalk color	1 / 1	0 / 0	1 / 1
VI. Sporocarp type	3 / 2	1 / 1	1 / 2
VII. Stalk length	393 ± 58 / 366 ± 72	0 / 0	402 ± 40 / 431 ± 55
VIII. Ratio VII/III (min.)	1,1 / 0,0	0 / 0	0,5 / 0,0
IX. Ratio VII/III (max.)	1,4 / 1,2	0 / 0	1,0 / 0,0
X. Capillitium net-work	2 / 1	2 / 1	2 / 1
XI. Capillitium type	1 / 0	1 / 0	1 / 0
XII. Capillitium node shape	2 / 1	2 / 1	2 / 1
XIII. Capillitium node size	4 / 0	4 / 0	4 / 0
XIV. Spore mass color	3 / 1	3 / 3	3 / 1
XV. Spore diameter	10.5 ± 1.3 / 10.6 ± 1.5	10.9 ± 1.2 / 9.6 ± 0.4	10.8 ± 1.3 / 10.3 ± 1.0
XVI. Height of spore or-namentation	0.3 ± 0.1 / 0.5 ± 0.2	0.3 ± 0.2 / 0.2 ± 0.1	0.3 ± 0.2 / 0.3 ± 0.2
XVII. Density of spore ornamentation	8.5 ± 2.8 / 9.0 ± 2.6	6.9 ± 1.7 / 11.1 ± 3.0	7.6 ± 2.3 / 8.6 ± 2.9

Table 9. The stability of morphological characters in three specimens of the arid form of *Ph. notabile*. The values before the slash are for first generation (moist chambers), after – for the second one (cultures). For character codes, see the Chapter **4.4. Morphological analysis**

5.5. Ecological analysis of species from the *Ph. notabile* complex

During the present study, Myxomycete samples from arid regions of Eurasia, South and North America were analyzed, as well as morphotypes from broad-leaved and boreal forests in North America, Germany, and the Novosibirsk region in Russia. Nine regional studies on Myxomycete biodiversity were analyzed, from which 5 were conducted in arid regions (Table 10). A comparative analysis of the abundance of species from the *Ph. notabile* complex in different plant communities has shown that *Ph. album*, *Ph. leucophaeum* and *Ph. notabile* s.str. prefer coarse woody debris in temperate deciduous and boreal coniferous forests, and can also be occasionally found in forest plantations and scattered forest pockets in the desert and steppe zones (Table 10). On the contrary, the arid morphotype of *Ph. notabile* was not discovered in temperate and boreal forests; the habitat of this form was limited to treeless communities in steppes and desert regions. At the same time, this group inhabits several substrate types, including grass litter, herbivore dung, decaying wood, the bark of living trees and shrubs. In moist chambers, they form sporocarps on substrates with a very wide range of possible pH values (Novozhilov et al., 2006). The only species close to the arid form of *Ph. notabile* in terms of areal and habitat preferences is *Ph. pusillum*, however, it is mostly found on grass litter in grasslands (Rollins, Stephenson, 2013) in steppe regions and rarely inhabits true deserts. *Ph. nivale*, as mentioned above, has a very specific ecological niche, producing sporocarps at the edge of the melting snow in alpine herb communities.

	PHYnot a/m	PHYalb	PHYlph	PHYniv	PHYnot	PHYpus
Vegetation type						
Desert and steppe (a-d, i)	278	0	18	0	0	31
Intrazonal arboreal communities in desert and stepe zones (a, b, d)	124	33	33	0	3	4
Coniferous boreal forest (e-g)	0	118	19	0	54	0
Subalpine and alpine communities (h)	0	0	0	14	0	0
Substrate group						
Coarse woody debris	31	134	39	0	47	0
Bark	185	10	14	0	5	4
Litter	141	6	17	14	2	31
Dung	57	0	0	0	0	0

Table 10. The occurrence of the arid morphotype of *Ph. notabile* (PHYnot a/m), *Ph. album* (PHYalb), *Ph. leucophaeum* (PHYlph), *Ph. nivale* (PHYniv), *Ph. notabile* s.str. (PHYnot) and *Ph. pusillum* (PHYpus) in different plant communities and on various substrates. a – Caspian Lowland, Russia,

steppe and semi-desert (Novozhilov et al. 2006); b – Altai Mountains, southwestern Siberia, Russia, intermountain basins with steppe (Novozhilov et al. 2010); c – Western Mongolia, semi-desert, mountain steppe (Novozhilov and Schnittler 2008); d – Tarim Basin and eastern Tian-Shan, China, poplar gallery forests in desert and mountain steppe (Schnittler et al. 2012a); e – Ural Mountains, Sverdlovsk region, Russia, boreal forest (Novozhilov and Fefelov 2001); f – Altai Mountains, Russia, boreal forests in mountains (Novozhilov et al. 2010); g – Novosibirsk region, western Siberia, Russia, lowland boreal forests (Vlasenko and Novozhilov 2011); h – Northwestern Caucasus, Russia, subalpine shrub communities and alpine meadows (Novozhilov et al. 2012); i - Western and central United States, grasslands (Rollins and Stephenson 2013).

5.6. Description of *Physarum pseudonotabile*

Myxomycete samples assigned to the arid morphotype of *Ph. notabile* were often found in collections from Central Asia. The NMS analysis based on 17 characters showed that arid samples of *Ph. notabile* differ from other taxa even more than *Ph. notabile* s. str. differs from *Ph. leucophaeum*. According to NMS, the distinctive features of the arid form of *Ph. notabile* are quantitative characters, especially details of spore structure. In addition, the phylogenetic analyses performed show the monophyly of this group on phylogenetic trees. Thus, both morphological and phylogenetic species definitions for this group of organisms are satisfied. Together with the deviating ecology, these differences seem to justify the description of the specimens from arid regions as a separate species.

Physarum pseudonotabile Novozh., Schnittler et Okun, MycoBank MB800893 (Novozhilov et al., 2013). Figure 13, A-G.

Sporocarps usually gregarious or clustered, rarely solitary, typically stalked, 0.2–1.0 mm high, rarely subsessile (especially if fruiting on the lid of a moist chamber culture), in this case sometimes merging into small short plasmodiocarps up to 3 mm long. Hypothallus inconspicuous, membranous. Stalk, if present, dark gray to black, up to 0.5 mm high, appearing stout, rarely tapering conspicuously toward the apex, rugulose, outer side appearing limeless but may contain some lime because bubbles develop if stalks are placed in polyvinyl lactophenol. Sporotheca 0.15–0.8 mm diam, globose to globose-depressed, rarely slightly umbilicate, light to medium gray but at base much darker gray to nearly black. Peridium single, thin, membranous, dehiscence irregular, densely covered with light gray to white lime flakes; these usually isolated, never forming a homogenous layer. Columella absent. Capillitium abundant, an isodiametric network of hyaline filaments. Lime nodes varying in size (20–80 mm) and shape, light gray to white. Occasionally the lime accumulates toward the center of the sporocarp, in this case the capillitium appears badhamioid. Spore mass dark

brown to brownish black. Single spores in transmitted light yellowish brown to moderate yellowish brown, globose, (8.0–)10–11(–11.5) mm diam., including the ornamentation of irregularly distributed warts. Warts 4–40 per 9 mm2 spore surface, in SEM appearing conical with a tuberculate obtuse apex, 0.10–1.48 mm in height , sometimes forming small clusters but typically solitary. Phaneroplasmodium light gray to yellowish white.

Holotype: Kazakhstan. Atyrau region, 20 km SO Inderborskii settlement, desert steppe at the southern bank of Inder lake, 48°23'36"N 51°51'32"E, on litter of small twigs in moist chamber culture, isolation date 20-IX-2006, Yu. Novozhilov & I. Zemlyanskaya, (LE 255438, MycoBank MB800893, GenBank JQ478431 for complete SSU sequence and KC473817 for partial tef1a).

104 specimens of this species were studied by means of molecular and/or morphological analyses. Etymology: From the Latin *pseudo* (seeming), referring to the similarity of this species to *Ph. notabile*. Habitat: On ground litter but also on bark of living trees, shrubs and dwarf shrubs, occasionally on weathered dung of herbivorous animals. Distribution: In arid regions of Eurasia, Central and South America, likely to occur in other arid regions around the world.

5.7. Phylogenetic analysis of the *Ph. notabile* species complex

In order to conduct a phylogenetic analysis of the species belonging to the *Ph. notabile* complex besides taking the full 18S SSU sequences of the respective species we also included sequences from other representatives of the genera *Physarum* and *Badhamia* not belonging to the morphocomplex. The main goal of the analysis was to elucidate the taxonomic position of the species complex within the family *Physaraceae*.

The result of the analysis – a phylogenetic tree – is presented on Figure 15. The representatives of the species complex (marked with arrows) do not form a mono- or paraphyletic group inside the *Physaraceae* family, thus not satisfying the phylogenetic criterion for a taxonomic rank in the concept of evolutionary systematics.

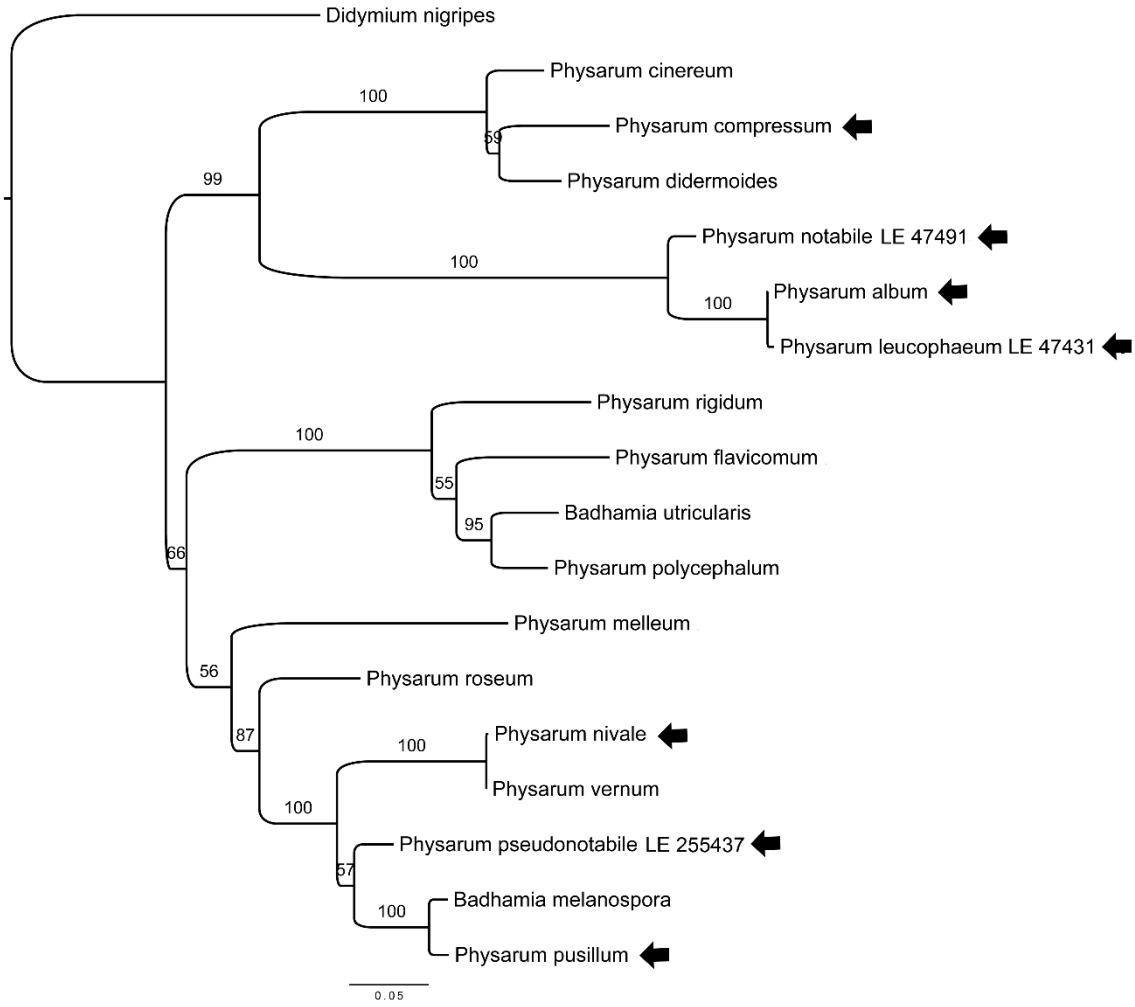


Figure 15. Phylogenetic tree based on the full 18S SSU gene sequence (2270 bp) of 18 taxa. ML analysis: IQ-Tree software, TIM2+G4 model of evolution, 10000 ultra-fast bootstraps. Bootstrap support values are given for each node. *Didymium nigripes* is used as the outgroup. Representatives of the *Ph. notabile* species complex are marked with arrows. Herbarium numbers are given for sequences obtained in this study. For GenBank accession numbers of other sequences, see Table 8. Scale bar indicates 0.05 nucleotide substitutions per site.

We have also shown the expediency of using the full SSU gene sequence compared to the partial one based on the data set used in Figure 15. The statistical support of the branches can be seen as an important criterion for tree resolution. We calculated the mean genetic distance of our partial and full SSU alignments using the Maximum Composite Likelihood method implemented in MEGA 5.2. Then the tree was reconstructed with the same evolutionary model and 10000 ultra-fast bootstrap replicates were conducted. The resulting branch support values were compared. For the partial SSU data set (635 bp), the mean genetic distance was 0.105±0.021, and the mean branch support was 76%. When using the full SSU data set, the genetic distance increased to 0.162±0.024 and the mean

support – to 85%. Thus, using the full SSU data set increased the genetic distance by 54.3%, and the tree support – by 11.75%.

A phylogenetic analysis based on the partial amino-acid sequence of the elongation factor gene (Figure 16) gives similar results to the ones obtained from full SSU data. In this case, the studied DNA sequence is shorter and the mean genetic distance lower, which results in an overall lower tree support values. The mean genetic distance in the tef1 alignment for the species from the complex (*Ph. notabile*, *Ph. album*, *Ph. leucophaeum*, *Ph. nivale*, *Ph. pseudonotabile*, *Ph. pusillum*) is 0.052±0.012, versus 0.128±0.008 for the full SSU data. Still, even despite the lower informativity it can be seen, that representatives from the *Ph. notabile* morphocomplex (marked with an arrow) do not form a monophyletic cluster on the tree – the branch with 88% support combines *Ph. album* and *Ph. leucophaeum*, and another one with 75% support - *Ph. nivale* и *Ph. pseudonotabile*. Moreover, this marker gene also supports the polyphyly of the genera *Physarum* and *Badhamia*.

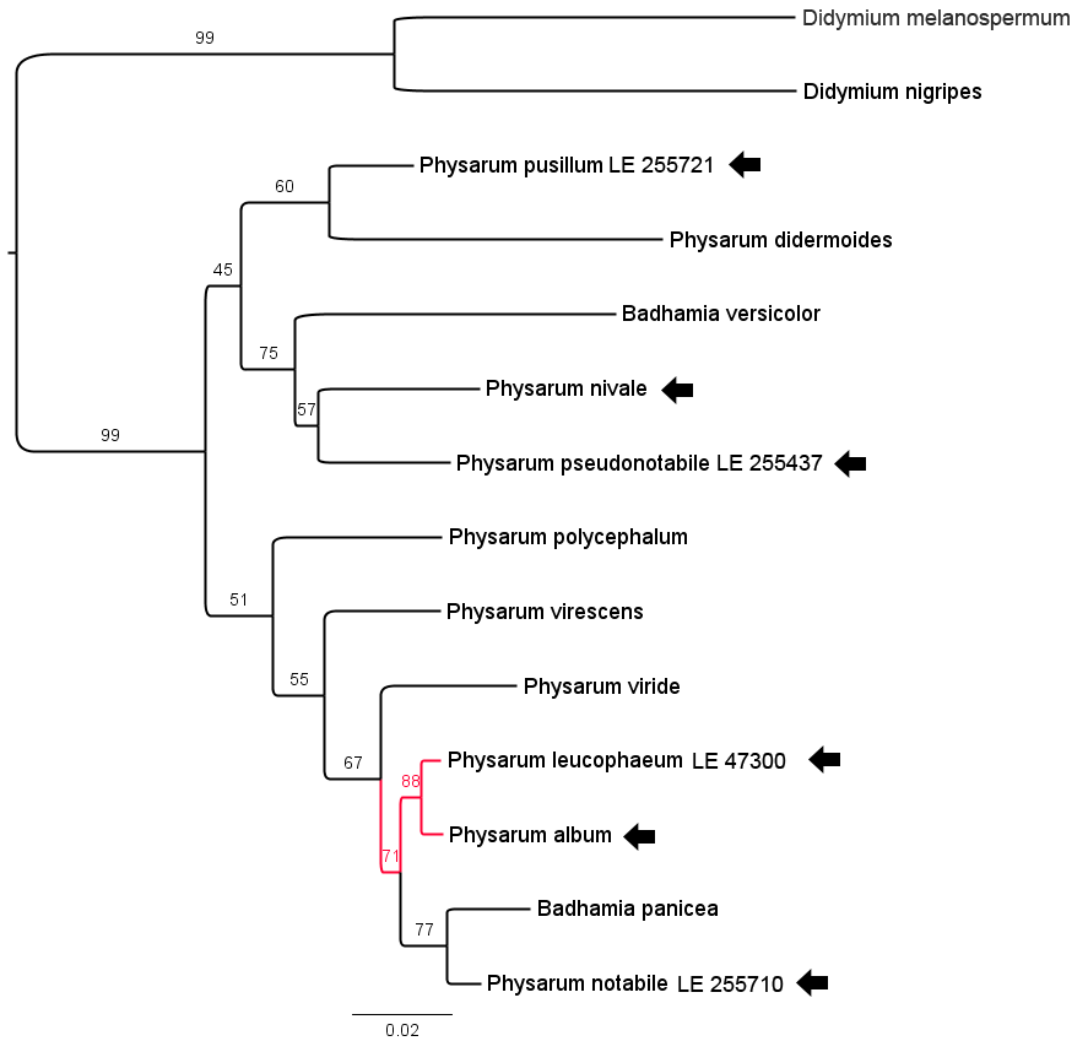


Figure 16. Phylogenetic tree based on a partial amino-acid sequence of the tef1 gene (211 a/a) from

14 samples. Ml analysis: IQ-Tree software, WAG+I+G4 model of evolution, 10000 ultra-fast bootstraps. Support values are given for each node. *Didymium nigripes* and *Didymium melanospora* are used as the outgroup. Herbarium numbers are given for sequences obtained in this study. For GenBank accession numbers of other sequences, see Table 8. Representatives of the *Ph. notabile* morphocomplex are marked with arrows. Branches marked red were artificially extended from 0 to 0.04 for better clarity. Scale bar indicates 0.02 amino-acid substitutions per site.

5.8. Phylogeographic analysis of *Ph. pseudonotabile*

5.8.1. Partitioning into clusters

For the phylogeographic analysis, 89 partial SSU sequence were selected. As the first step, these had to be split into groups for further study. 69 of of those samples belong to a transect going through the territory of Central Asia (13 – China, 8 – Mongolia, 39 – Kazakhstan, 8 – Russia), and 20 – to other regions of the world (5 – USA, 1 – Spain, 7 – Oman, 7 – Argentina) (Figure 17). The list of studied isolates with sampling coordinates is given in Supplementary Table S1.

In classical phylogeography during the analysis of mitochondrial markers haplotypes are studied – groups based on genetic information that are inherited maternally. In our case it was not possible to separate haplotypes of ribosomal DNA, thus the sample were grouped to clusters based on sequence similarity. The analyzed alignment has a length of 551 bp, of which 87 positions are variable. The majority of nucleotide substitutions occurs in the variable helix E8_1 (positions 92-128 in the alignment), as well as in helices 10 and E10_1 (positions 180-315, see Wuyts et al., 2002 for RNA secondary structure nomenclature). The maximal difference between two sequences was 46 mismatches (genetic distance 0.083), the mean was 37 mismatches (0.067). A threshold has to be set that divides the clusters according to a given mismatch value. For a threshold value of 0 or 1 mismatch we obtain 45 clusters for the 69 transect samples, but this number quickly drops to 32, 26 and 21 when increasing the threshold to 2, 3 and 4 positions (Figure 18, A). The first plateau on the graph showing the relation of cluster numbers to the threshold appears in the range between 13 and 17 nucleotide mismatches (genetic distance of 0.024 – 0.030). On this stage, a clear objective criterion for threshold selection is needed. A too high threshold value would result in loss of phylogenetic information, with the extreme case of obtaining one cluster including all studied sequences. On the other hand, a too low value would over-estimate the number of clusters, thus hampering the phylogeographic analysis and lowering its informativity.

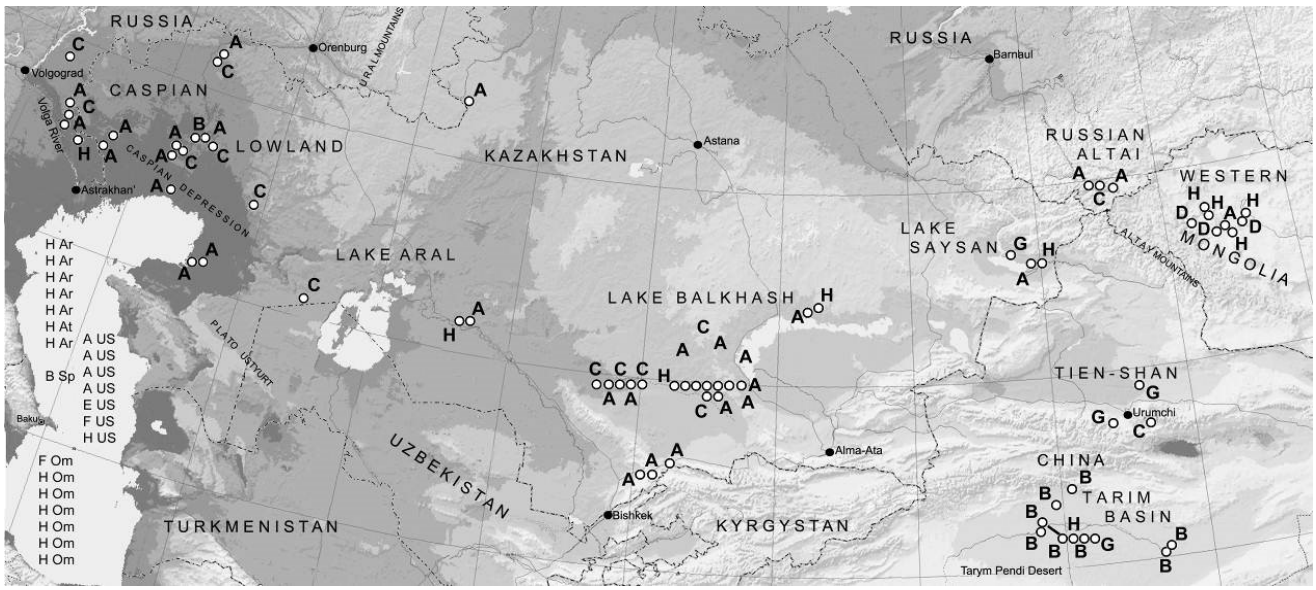


Figure 17. The geographical distribution of *Physarum pseudonotabile* clusters belonging to 69 samples from 8 regions, studied in Central Asia. Samples from other regions are given in a list on the left side. Clusters (threshold value of 0.024-0.030) are denoted with Latin letters from A to H.

For the estimation of the threshold value, we used a method described in a work dedicated to the phylogeographic study of the genus *Gagea* (fam. Liliaceae) (Zarrei et al., 2012). In our case, this value was in the range between 0.024 – 0.030 and corresponded with the clear plateau on the cluster number graph (Figure 18, A). With this value the data set is split into 8 clusters; China is represented by 4 clusters (13 samples), Mongolia – 3 clusters (8 samples), Kazakhstan – 5 clusters (39 samples) and Russia – 3 clusters (8 samples).

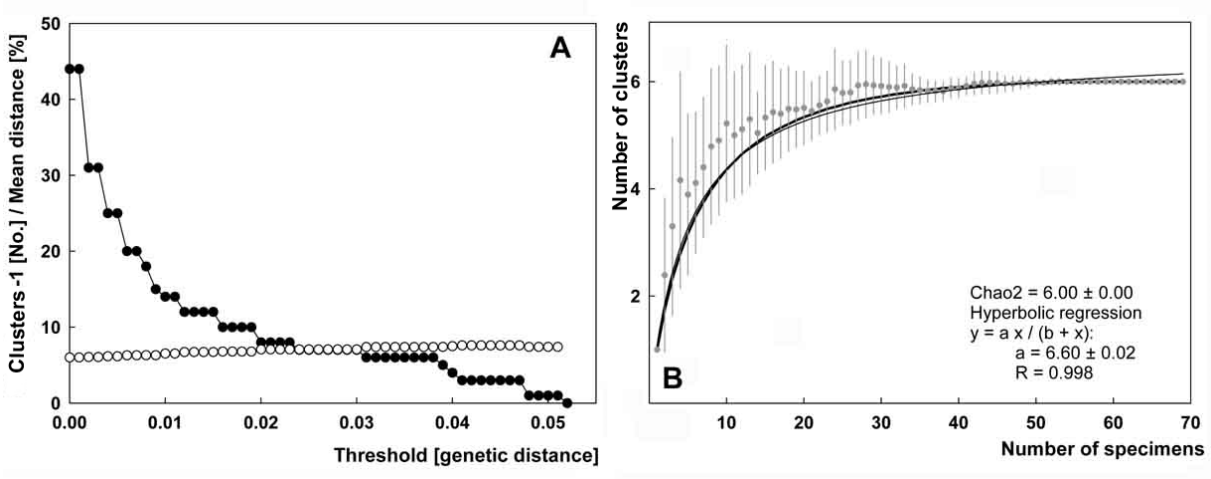


Figure 18. A. Plot of the number of clusters defined by thresholds of genetic distances from 0 to 0.052, equaling 0-30 polymorphic sites in the alignment of 552 aligned positions of the partial SSU

of the rRNA gene (dark circles). Overlaid is the mean difference between sequences assigned to one cluster for these thresholds (open circles). **B.** Accumulation curve of six major clusters found for the Central Asian transect with a threshold between 0.024 and 0030 (13 to 17 polymorphic sites) with the diversity estimator Chao2 and its standard deviation (grey dots and bars, final value 6.00). The fit with a hyperbolic regression (thin line) runs close to the accumulation curve.

We also applied the GMYC method as an alternative way to split the data into clusters. This method involves testing of several speciation models on an ultrametric tree. It is mostly used for detection of cryptic biodiversity based on environmental sampling studies. The GMYC method can only be applied to ultrametric trees – trees, where the branch length of all taxa is equal. Such a tree assumes the molecular clock model was accepted for the data used, which means all the taxa studied evolved with the same speed. However, tree reconstruction and testing in TreePuzzle have shown that the molecular clock model is rejected on the 5% confidence interval: LogL (w/o clock) = -2106.98; LogL (clock) = -2205.96. Thus, the GMYC method could not be used to obtain the clusters from the data set. This also could mean that SSU sequences cannot be used for molecular dating and for OTU delimitation since this locus evolved with an unequal rate in different taxa. These conclusions agree with results obtained in studies aimed to use ribosomal RNA sequences for molecular dating in higher plants (Kay et al., 2006).

Another method of OTU delimitation – PTP – does not require for ultrametric trees. We analyzed a tree containing only unique sequences of *Ph. pseudonotabile*; groups obtained this way a shown on Figure 19. Groups marked as 1-3 as well as sample LE 255713 make up cluster A (according to the threshold method). Group 4 exactly represents cluster B, and group 5 – cluster C. Sample sc 21106 is put into a separate cluster by both methods (cluster D), similarly to sample LE 255712 (Cluster E). Group 6 together with samples sc 23291 and LE 274212 makes up cluster H. Thus, the results of the two delimitation methods differ only in two clusters (A and G). At the same time, taking into account that the program was initially designed for cryptic diversity studies and that only the first version of the software is available, we decided to use the results of the threshold approach in further analysis.

Evaluation of study completeness. The Chao2 estimator calculated for the cluster accumulation curves assumes a final value of 6.00 ± 0.00 clusters for six major clusters from 69 specimens sequenced from the Central Asian transect (Figure 18, B). For the 8 clusters differentiated all 91 specimens, the figure for Chao2 is 8.0 ± 0.25 clusters (accumulation curve not shown). Comparing these figures with the real number of 15/18 species retrieved, the survey should be complete to 100% for both the transect and all specimens, i.e. cannot expect to find more major

clusters. The hyperbolic model fits the data well, but this diversity estimator assumes slightly higher values (6.6 ± 0.02 species for the specimens from the transect, R=0.998; 10.00 ± 0.21 for all specimens analysed, R=0.995), estimating the survey to be to 91/80 % complete, respectively.

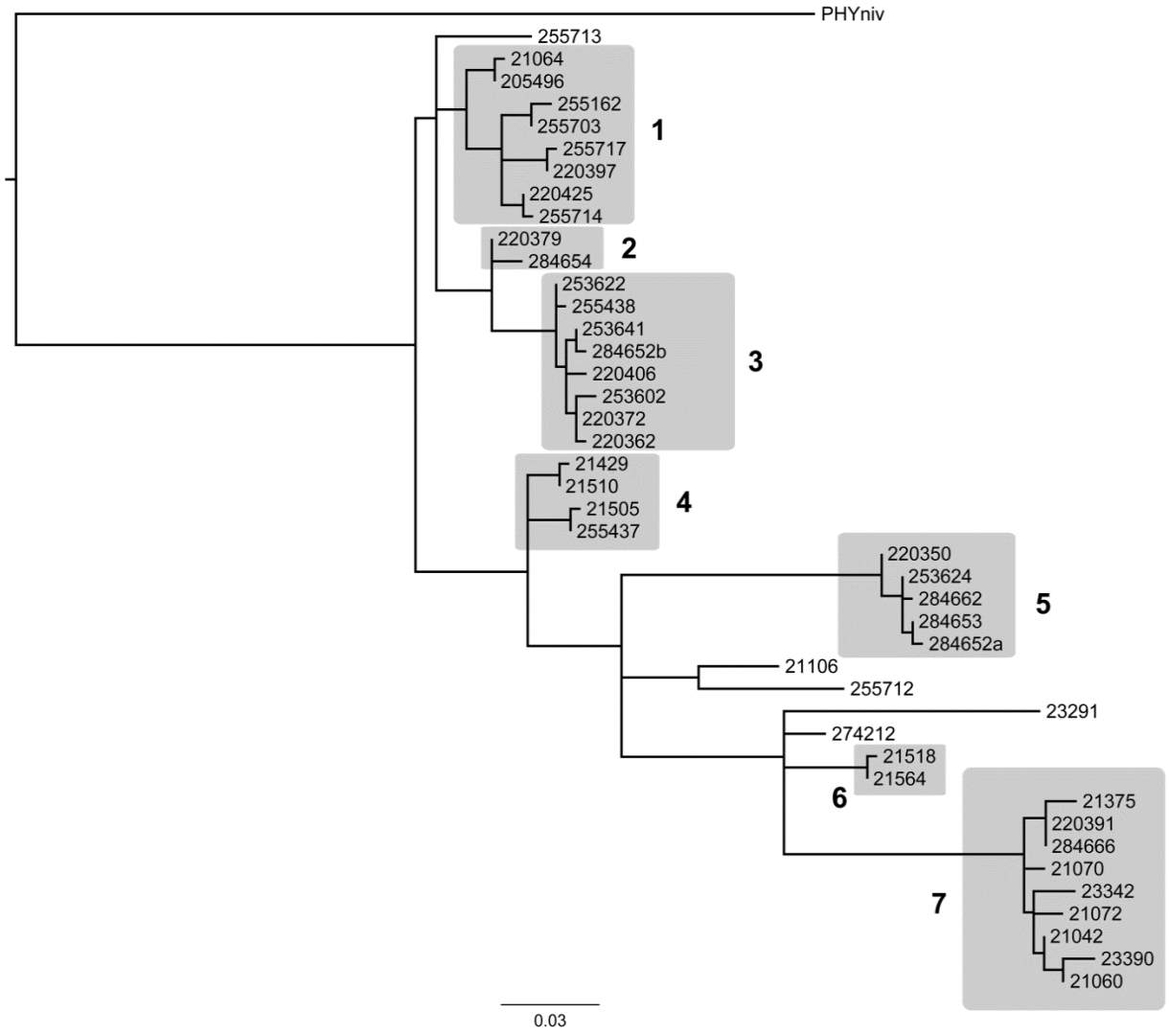


Figure 19. Results of OTU delimitation by the PTP software on a phylogenetic tree of 45 unique 18S SSU sequences of *Ph. pseudonotabile* (552 bp). OTU's discovered are shaded grey. Non-shaded samples represent separate clusters. *Ph. nivale* (PHYniv) was used as the outgroup. Scale bar indicates 0.03 substitutions per site. Herbarium numbers are given for each specimen.

Geographic distribution of clusters. Figure 17 shows the geographic distribution of 6 major clusters (according to the threshold approach) over the territory of Central Asia. Cluster A is the mostly distributed group found in samples in Central Asia and the US. Cluster B is mostly found in the area of the Tarim basin; except two samples, cluster D is limited by the Great Lake depression

(Mongolia). Clusters C, G and H can be found all over Central Asia, however, the latter has a wider distribution, being found among the samples from Patagonia (Argentina) and Oman. Cluster E is represented by one sample from Texas (USA) and, finally, cluster F was discovered once in South Dakota (USA) and once in Northern Oman.

Mantel test. In order to test for a possible correlation between the geographical localities of the studied isolates and genetic distances between them we performed a Mantel test. A significant level of correlation was discovered between the distance matrices for the transect data (correlation coefficient r=0.124), whereas randomisation tests after 1,000 iterations resulted in r=-0.049 (5% confidence interval), r=0.000 (mean value) and r=0.039 (95% confidence interval). A weaker but still significant correlation was discovered for the complete data set: r=0.110, whereas the randomization test resulted in r = -0.043 (5%), 0,000 (mean) and 0,035 (95%).

5.8.2. Phylogenetic analysis of clusters

We also performed a phylogenetic analysis of the studied isolates of *Ph. pseudonotabile* that allowed us to assess phylogenetic relationships of the obtained clusters. For the tree reconstruction based on 89 sequences of the partial 18S SSU gene of *Ph. pseudonotabile* three alignments were prepared: full-length sequences (552 bp), a more conservative alignment (504 bp) and the most conservative (464 bp). The masking of alignment sites was performed based on the topology of hyper-variable regions of the rRNA (Wuyts et al., 2002). All three alignments resulted in almost equal tree topologies. The Robinson-Foulds distance between the more conservative tree and the two others is 14 and 16, respectively. Still, the likelihood values and branch support were different for the trees. With increasing conservativity the support decreased and the likelihood increased (from -2127.4 to -1736.6). However, it is known, that the likelihood value is inversely proportional to the alignment length. At the same time, the support value plays the major role in the informativity of the clusters. Masking of specific alignment patterns decreased this informativity; thus, the unmasked tree seems the most probable to us. This tree is given in Figure 20.

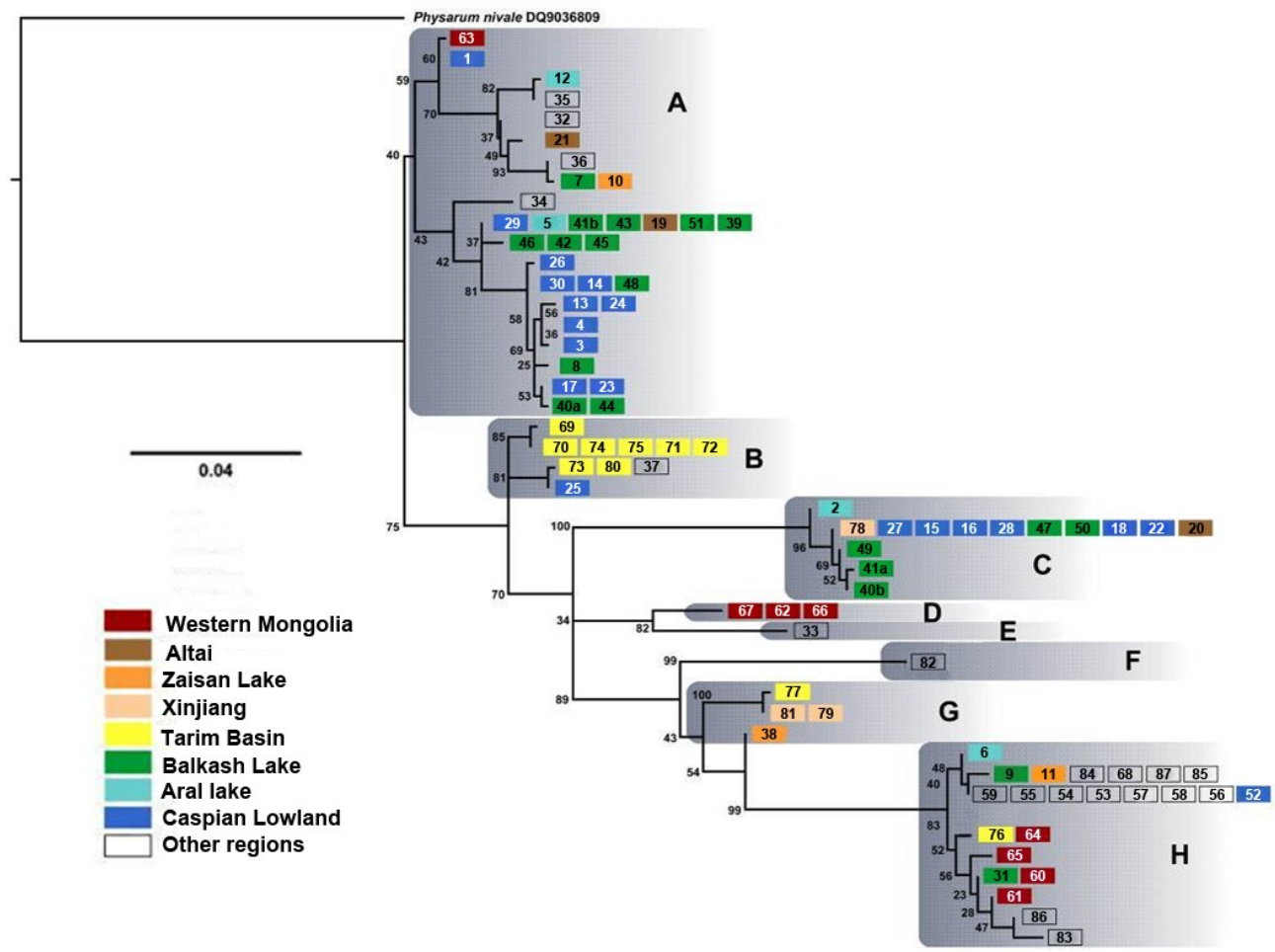


Figure 20. Phylogenetic tree based on the partial sequence of the 18S SSU gene (552 bp) from 89 samples of *Ph. pseudonotabile*. Ml analysis: IQ-Tree software, TrNef+G8 evolutionary model, 1000 bootstrap iterations. Support values are given for each node. Identical sequences are grouped to rows for visual clarity. For samples corresponding with numbers see Supplementary Table S1. Regions belonging to the Central Asian transect are marked by colours. Clusters are denoted with A-H and marked grey. Scale bar indicates 0.04 nucleotide substitutions per site.

The clades obtained in the phylogenetic analysis mostly correspond to the clusters obtained with the threshold method. The bootstrap support for the clusters is in most cases high: cluster B – 75, C – 100, D, E – 82, F – 99, H – 99. Cluster A and G have lower support: 40 and 43, respectively. Moreover, since we had 4 full SSU sequences of *Ph. pseudonotabile*, we could confirm the obtained partial SSU topology with the respective tree based on full SSU sequences (see Figure 12).

For 17 specimen of *Ph. pseudonotabile* from the phylogeographic data set we also obtained partial tef1 sequences. The resulting alignment had a genetic variability of 0.112. Based on the translated amino-acid alignment and the partial 18S SSU data we reconstructed a combined tree

(Figure 21). The clades on this tree also represent the clusters obtained before; the involvement of a second marker raises the support values for the groups. Clusters A, C, and H have 100% support. Due to amplification difficulties not all clusters could be represented on the tree.

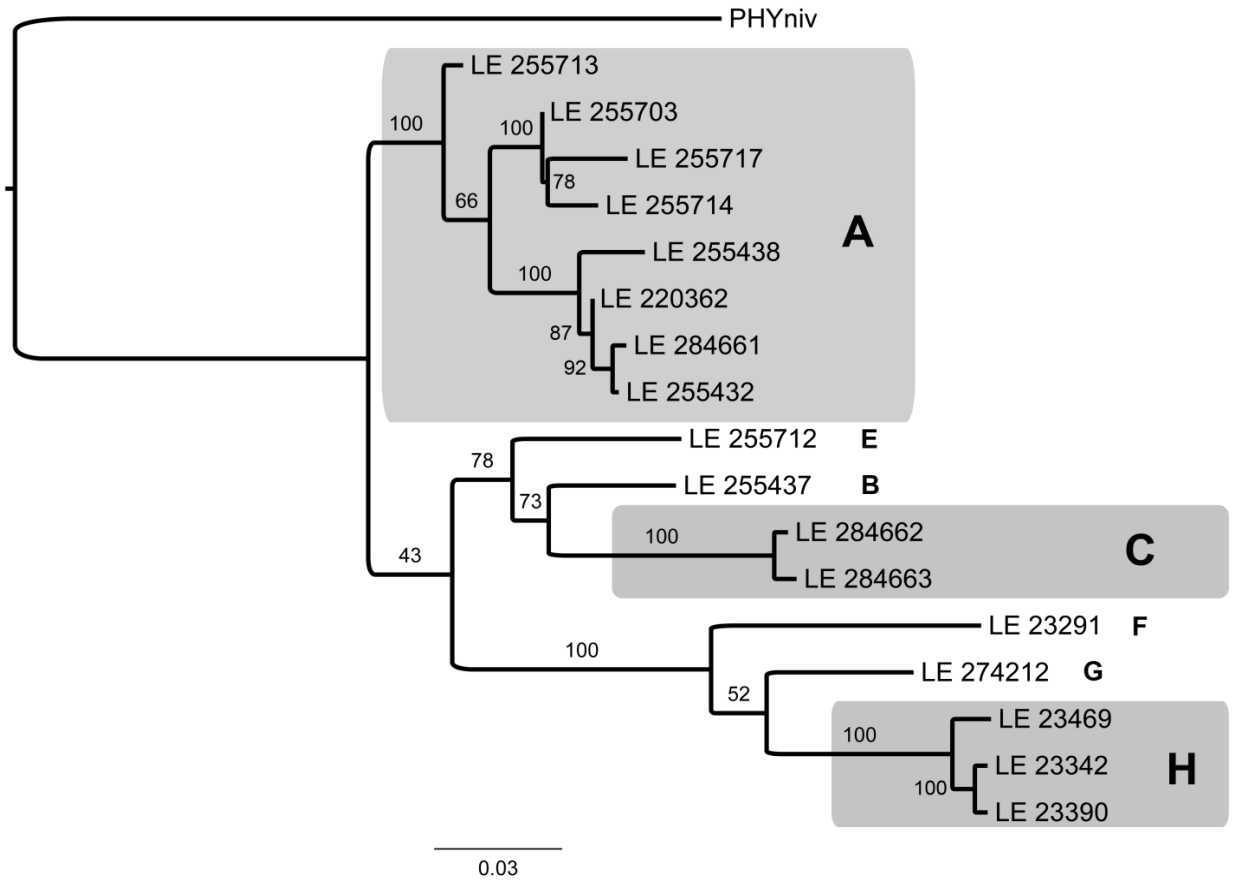


Figure 21. Phylogenetic tree based on the partial tef1 amino-acid sequence (211 a/a) and partial SSU sequence (552 bp) from 17 isolates of *Ph. pseudonotabile*. ML analysis: partition model, TrNef+G8 evolutionary model for the SSU partition, WAG+I+G4 model for tef1 partition. 1000 bootstrap iterations. Support values are given for each node. Herbarium numbers are given for each sample. Clusters are marked gray and denoted with A-H. *Physarum nivale* was used as the outgroup. Scale bar indicates 0.03 substitutions per site (average between nucleotide and amino-acid).

Chapter 6. Discussion

6.1. Optimization of DNA extraction protocols

Molecular research in the field of myxomycete phylogeny is still in its beginnings. Standard DNA extraction, PCR and sequencing protocols typical for bacterial and eukaryotic model objects were absent when this works began. For example, most of DNA extraction methods include cell treatment with CTAB buffer. DNA is usually extracted from fruiting bodies, since they contain DNA in sufficient concentrations, and can be used for precise morphological species identification (in contrast to the plasmodial stage). CTAB binds DNA, providing its purification from other cell components; however, plasma membrane lysis and especially spore wall disruption are both still major problems during DNA extraction in Myxomycetes. The surfactant features of CTAB alone might not be strong enough to perform these tasks. A proper mechanical disruption of the spore wall plays a key role in the DNA extraction process, ensuring that the surfactants can act upon the plasma membrane. The usage of ball mills, pestles on deep frozen material seems to be an adequate approach to this task. Alternatively, treatment with a chitinase complex perforates the spore wall in an enzymatic way; this approach may prove useful in field studies since no extra lab machinery (except a standard centrifuge) is required.

During the PCR, best amplification results were achieved when using undiluted DNA; this can be explained by lower concentrations of DNA obtained after extraction when compared to plant or animal tissues. This especially applies to samples where the sporocarp number is small. The usage of the commercial iQ-Supermix (BioRad) allowed us to make use of the Protocol AutoWriter option of the Bio-Rad C1000 thermocycler. With this option enabled, the primer annealing temperature was automatically adapted for the polymerase in the kit and the primers used. Thus we could avoid additional reactions (e.g. gradient PCRs) aimed to optimize the annealing temperature.

6.2. Phylogeny of the arid form of *Ph. notabile*

In order to study the phylogeny of the Ph. notabile species complex we first had to elucidate the taxonomic status of the arid form of *Ph. notabile*. For this, genetical differences between the representatives of this morphotype and *Ph. notabile sensu stricto* had to be figured out.

Due to the effects of morphological variability described in previous chapters as well as certain features of the reproductive system (connected with a necessity to perform mating type tests) we have chosen to apply the phylogenetic species concept in this study. This approach should have a high resolution in Myxomycetes: it has been shown in phylogenetic studies of 18S SSU myxomycete

sequences that the number of genetical clusters can be almost twice as high as the number of morphospecies (Novozhilov et al., 2013b). This fact evidently demonstrates the genetic diversity in myxomycete morphospecies that were described based only on morphological features and raises the question if multiple biological species may be present within one morphospecies. Moreover, for specimens obtained in moist chambers it is known that the expression of morphological traits depends on the environmental conditions during fruiting body development. For example, sporocarps growing upside down on the cover of the moist chamber often have reduced stalks, thus decreasing the number of traits available for a morphological analysis. This phenomenon can severely hamper a morphological species description, as it was evidently shown in the work of Fiore-Donno et al. (2012) on the genus *Diacheopsis*, which is differentiated from the closely related genus *Lamproderma* by sessile sporangia. At the same time, molecular studies based on the 18S SSU gene have shown the respective sequences of *Lamproderma* and *Diacheopsis* are often strikingly similar or even identical, thereby questioning the existence of the genus *Diacheopsis* as a separate taxonomical unit. This example demonstrates how morphological features can be misleading, destabilizing the borders between natural taxa. This can be especially dangerous when describing a new species based only on one or few samples due to morphological aberrations, as it was shown in Schnittler & Mitchell (2000).

An analysis involving a number of isolates from both morphotypes of *Ph. notabile* has shown that the arid and boreal form are grouped in highly supported clusters (Figure 11). However, to describe the arid form of *Ph. notabile* as a separate species according to the phylogenetic concept one has to show its monophyly on the phylogenetic tree among its taxonomic surroundings (Mayr & Bock, 2002). For this, other representatives of the genus *Physarum* must be involved in the analysis that are closely related to *Ph. notabile*. In a tree produced with such a data set, all the arid samples of *Ph. notabile* must have one ancestor. Thus, taking into account the taxonomic complexity of the *Ph. notabile* species complex, which was already mentioned by Hagelstein (1943), we used several representatives of this complex in our tree reconstructions. The latter are common for boreal and moderate areas and include *Ph. notabile* s. str., *Ph. album*, *Ph. pusillum* and *Ph. leucophaeum*. Moreover, we included the nivicolous species *Ph. nivale* in our analysis since molecular studies carried out before have demonstrated its phylogenetic kinship to *Ph. notabile* (Okun et al., 2011, 2013; Novozhilov et al., 2013a). The obtained phylogenetic tree shows that specimens belonging to the arid morphotype of *Ph. notabile* are always represented by a separate cluster with high statistical support, thus being separated not only from the classical boreal form of *Ph. notabile*, but also from the other species of the *Ph. notabile* complex (Figure 12).

6.3. Morphological analysis of the *Ph. notabile* species complex

We performed a NMS analysis of morphological data from 85 samples of representatives from the studied species complex in order to visualize the limits of morphological variability for these taxa. One of the better-defined groups is formed by samples of *Ph. nivale*. This species was included in the morphological analysis since the phylogenetic analysis based on 18S SSU and tef1 genes has demonstrated it to be a close relative to the arid form of *Ph. notabile*. A detailed study of the morphological traits of these taxa displayed their differences. Indeed, sessile sporocarps of *Ph. notabile* may represent small fruiting bodies of *Ph. nivale*; both species have a capillitium consisting of a network of hyaline tubes with large white lime nodes (Figure 13, A-G, L-N). However, in addition to different ecological preferences (*Ph. nivale* is a nivicolous species, forming sporocarps on the edge of the melting snow in alpine communities), this species is characterized by large sessile sporocarps (942 ±108 μm diameter), whereas arid representatives of *Ph. notabile* ale smaller (435 ± 17 μm) and are usually stalked. The latter are also ornamented with scattered blunt warts, while *Ph. nivale* has a dense ornamentation (Figure 13, N) similar to *Ph. leucophaeum* and *Ph. notabile* s.str. (Figure 13, J-K, O-S). *Ph. nivale* was just recently separated from *Badhamia panicea*, where it was considered as var. *nivale*. This species demonstrates morphological similarity with another nivicolous species, *Ph. vernum* Sommerf. (Poulain et al., 2011). The authors compare in to *Ph. vernum* as the only nivicolous species with a similar morphology of fruiting bodies. The distinguishing features between them are sporocarp types (usually plasmodiocarps in *Ph. vernum* and sporocarps in *Ph. nivale*) (Figure 22), the structure of the lime layer in the peridium (a contiguous layer in *Ph. vernum*, separate flakes in *Ph. nivale*) and spore morphology (pale- and darker colored spore hemispheres in *Ph. vernum*, uniformly colored spores in *Ph. nivale*). Moreover, a molecular analysis based on partial 18S SSU sequences has demonstrated that these two taxa are clearly separated (Novozhilov et al., 2013).

Specimens of *Ph. album* also form a better-defined group on the NMS ordination. Indeed, *Ph. album* is a distinctive species in terms of morphology, especially considering that the most prominent features of this species – the radial dichotomously branching capillitium and the nodding sporothecae have not been coded for in this analysis.

Finally, the samples of the arid morphotype of *Ph. notabile* form a group showing less intersection with other specimens. Close to this group is *Ph. pusillum*, which, however, can be easily distinguished from the latter by a slender reddish, always limeless stalk and often a reddish base of the sporocarp (Figure 13, T-X). At the same time, spore ornamentation of *Ph. pusillum* indeed may represent the one of arid *Ph. notabile* forms: in both cases spores are covered by blunt scattered warts. The red pigment in the stalk has a tendency to discolor over time during herbarium storage; thus, the

two specimen studied (LE 255719 and LE 255721) were initially erroneously assigned to the arid morphotype of *Ph. notabile*. Later, according to a more detailed morphological study of the specimen's sporocarps as well as the molecular data, however, these samples were assigned to *Ph. pusillum*.

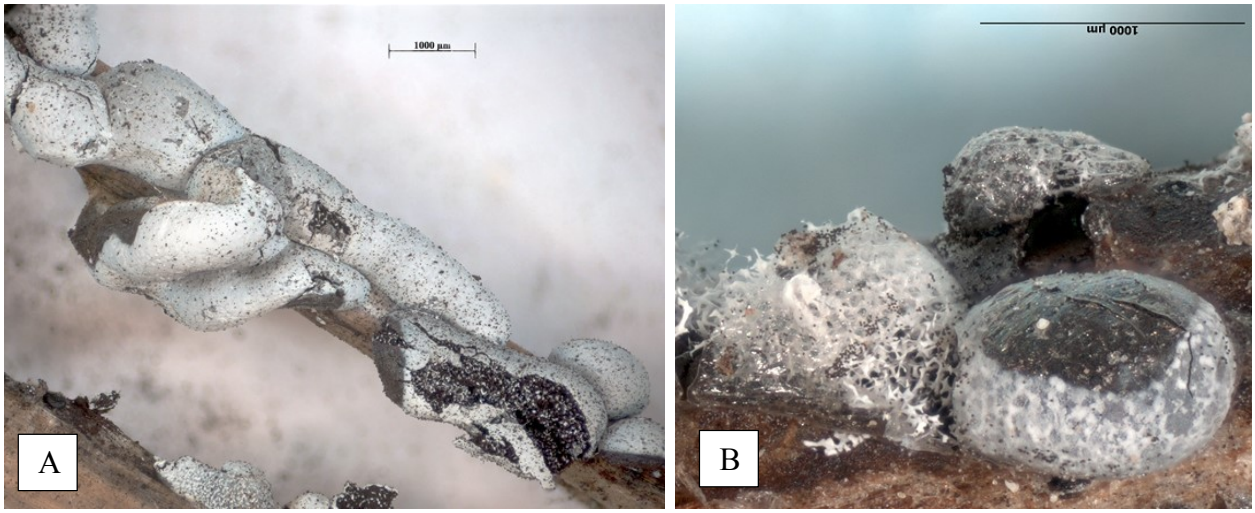


Figure 22. A morphological comparison of fruiting bodies of *Ph. vernum* and *Ph. nivale*. Scale bar = 1 mm. **A.** Plasmodiocarps of *Ph. vernum*. **B.** Sporocarps of *Ph. nivale*.

Altogether, the arid morphotype of *Ph. notabile* satisfies ecological, morphological and phylogenetic species definitions and therefore was described as a separate species, *Ph. pseudonotabile*. A detailed description of this new species is given in 5.6. Description of *Physarum pseudonotabile*

6.4. Phylogenetic analysis of the species from the *Ph. notabile* complex

A phylogenetic analysis of the representatives of the *Ph. notabile* species complex including also closely related species from the genera *Diderma* and *Physarum* has shown that these do not form a mono- or paraphyletic group within the order Physarales, thus not satisfying the phylogenetic criterion for a taxonomic rank in the concept of evolutionary systematics (Figure 15). The species *Ph. compressum*, *Ph. notabile*, *Ph. album* and *Ph. leucophaeum* form one clade with high support, but this clade also includes *Ph. cinereum* and *Ph. didermoides*. The first species can be distinguished from *Ph. notabile* by mostly sessile sporangia and plasmodiocarps, as well as by paler spores with a less dense ornamentation. The latter species has a double-layered peridium (Figure 23). The second group from the species complex - *Ph. nivale*, *Ph. pseudonotabile* and *Ph. pusillum* – form a clade with *Ph. vernum* and *Badhamia melanospora* (= *B. gracilis*). As mentioned before, the border

between the genera *Badhamia* and *Physarum* is largely artificial (Nandipati et al., 2012); still, *B. melanospora* can be easily told apart from species from the *Ph. notabile* complex by the absence of nodes in the capillitium (badhamioid capillitium) and a polygonal shape of the spores. *Ph. vernum* is similar to *Ph. nivale*, but can be distinguished by the sessile sporangia and the spore color (Figure 22). Thus, there is no phylogenetic reason to combine the species from the *Ph. notabile* complex mentioned above to a systematic group; the similar morphology of these species is most likely due to convergent evolution and cannot be used in the creation of a taxonomic system.

The second marker gene, tef1, also confirms these results (Figure 16), although with lower support values due to the lower variability of the chosen marker gene. Both trees also agree on the polyphyly of the genera *Physarum* and *Diderma*.

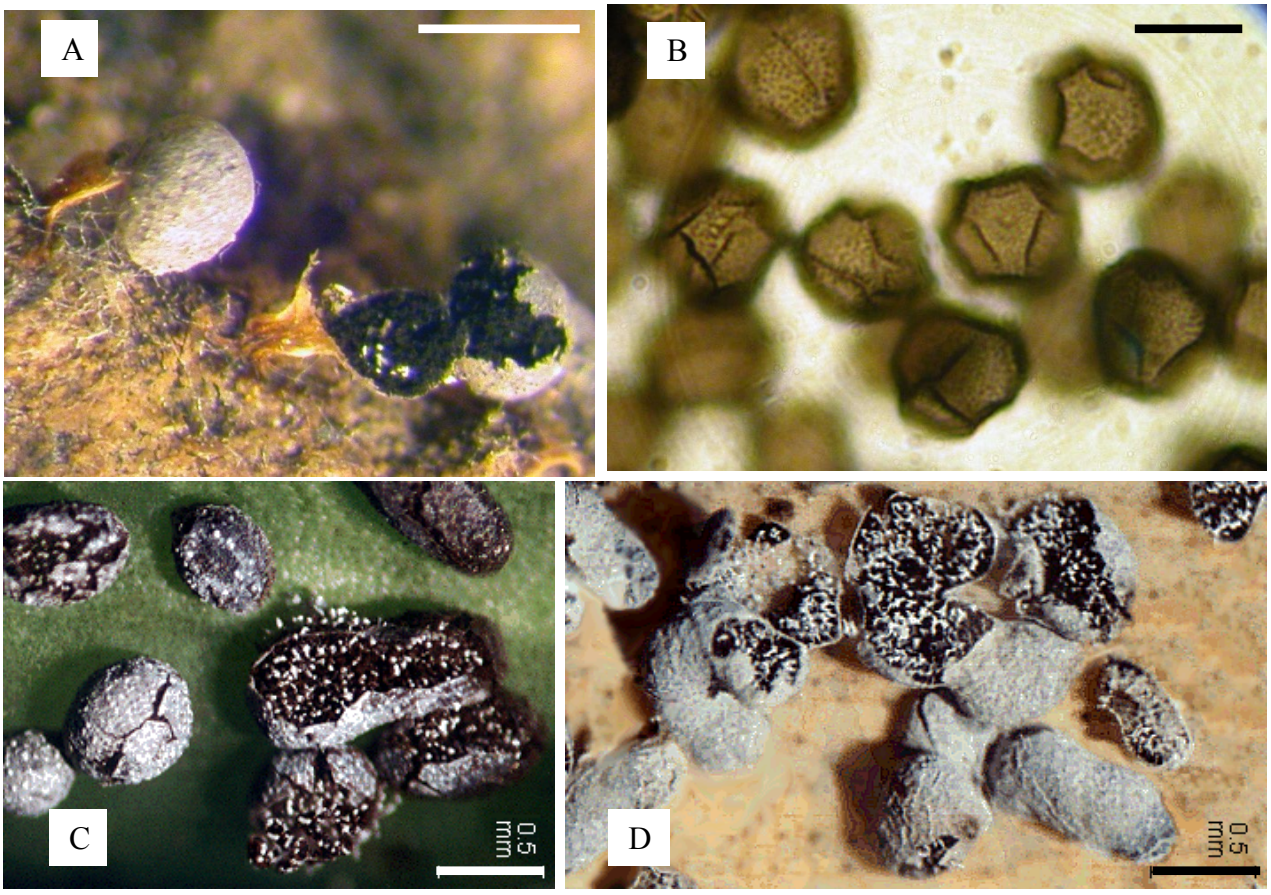


Figure 23. Morphological features of *Badhamia melanospora* and *Physarum cinereum*. **A.** Sporocarps of *B. melanospora*. Scale bar = 1 mm. **B.** Spores of *B. melanospora*. Scale bar = 10 µm. **C-D.** Sporocarps of *Ph. cinereum*. Sale bar = 0.5 mm.

The closely related species *Ph. album* (syn. *Ph. nutans*), *Ph. leucophaeum* and *Ph. notabile* are worth additional mentioning. Lister (1894) and Hagelstein (1944) describe the first two species

as centers surrounded by innumerable morphological transitional forms that create a "taxonomic continuum", where identification of separate species is limited. At the same time, the authors see *Ph. leucophaeum* as a variety of *Ph. nutans* and as the form that connects the species mentioned above in the continuum. Hagelstein mentions that spores of *Ph. nutans* and its varieties are smaller and paler than in typical *Ph. notabile* forms, but the latter still may form colonies with paler and smaller spores as well. In our phylogenetic analysis, the three species form a monophyletic group with maximal support; a more detailed analysis may reveal *Ph. leucophaeum* and *Ph. notabile* to be one species described on different continents: in Europe and North America, respectively.

6.5. Phylogeography of *Ph. pseudonotabile*

We performed a phylogeographic analysis of 89 isolates of Ph. pseudonotabile from various arid regions of the world. 69 of those samples belong to a transect going through the territory of Central Asia (Figure 17). We delimited the sequences to clusters based on the genetic distance and evaluated he distribution of these groups over the studied area. Besides this, a Mantel test was performed to assess possible correlation between geographical and genetic distances of the studied samples. Altogether, the results of cluster delimitation and Mantel test show that despite the high mobility of myxomycete spores and microcysts and the high probability of being transferred over large distances these factors still cannot create a fully random distribution of isolates that would be independent of geographical barriers. These data agree with the theory of moderate endemism in regard to Myxomycetes. Still, further research may reveal the discovered clusters of *Ph. pseudonotabile* to represent separate biological species. It is the morphological similarity of biological species that often leads to an artificial increase of the habitat areal and thus to an illusion of cosmopolitism.

Moreover, from the data obtained conclusions can be made about the predominance of a certain type of reproductive strategies in Myxomycetes. As it can be seen from Figure 20, the data set includes a decent amount of identical sequences, representing putative clones. These clones are often associated with one habitat. The presence of clonal populations is the consequence of some of strains switching to asexual reproduction; apparently, in the short term asexual reproduction is beneficial when occupying new territories since the need to search for a cytogamy partner is thereby avoided. These data agree with previous research in this field conducted by Schnittler, Tesmer (2008). The authors have shown that the density of the "spore rain" (amount of spores falling on the substrate on a given territory) has to be at least 2.4 times larger for sexual strains when compared to asexual ones. At the same time, sexual reproduction is a source of genetic diversity due to recombination events occurring during meiosis. A "shuffling" of genetic material can be a source of selective

benefits required for occupying new habitat areas with sub-optimal environmental conditions. This is of special importance for taxa inhabiting arid regions that are often characterized by an uneven distribution of vegetation. Thus, cycles of sexual and asexual reproduction in Myxomycetes can be connected with the processes of occupying and inhabiting new territories.

Chapter 7. Summary

In this thesis, we used a complex morphological and molecular approach to study isolates of the arid morphotype of *Ph. notabile*; based on phylogenetic trees and the results of the NMS analysis these were described as a new species – *Ph. pseudonotabile*. The closest relative from the species complex to the newly described species seems to be *Ph. pusillum*; the two taxa, while having distinctive features, share an overall similar morphology but occupy different ecological niches. Another species phylogenetically close to *Ph. pseudonotabile* – *Ph. nivale* – has a distinctive morphology and is a nivicolous myxomycete. The phylogenetic analysis based on full 18S SSU and partial tef1 gene sequences also showed that the *Ph. notabile* species complex is polyphyletic and therefore cannot be seen as a taxonomic entity. It has been shown that the species *Ph. notabile* and *Ph. leucophaeum* have a similar morphological structure and form monophyletic clades on trees with maximum support. Additional studies may reveal the synonymy of these taxa. According to both morphological and phylogenetic analyses, *Ph. album* is the most distinctive species from the *Ph. notabile* species complex. Finally, the phylogeographic analysis has shown the presence of a weak correlation between geographic localities and genetic distances of studied isolates, as according to the theory of moderate endemism in microorganisms.

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Supplementary material

Supplementary Table S1. List of specimens used in this work. In the "Herbarium number" column, specimens from the Komarov Botanical Institute herbarium are marked with LE; specimens from the private collection of Prof. Dr. Martin Schnittler (Greifswald, Germany) – sc. Numbers of *Ph. pseudonotabile* samples from the tree on Figure 20 are given in brackets. Acronyms used: PHYalb – *Physarum album*, PHYlcp – *Ph. leucophaeum*, PHYniv – *Ph. nivale*, PHYnot – *Ph. notabile*, PHYpse – *Ph. pseudonotabile*, PHYpus – *Ph. pusillum*. NMS – a morphological analysis was carried out, SSU –partial 18S SSU gene sequence obtained, FSSU – full 18S SSU gene sequence obtained, tef1 – partial tef1 gene sequence obtained. For the list of sequences submitted to GenBank and their respective accession numbers, see Table 7.

Herbarium number	**Species (acronym)**	**Country**	**Region**	**Latitude**	**Longitude**
LE 227199 (NMS)	PHYalb	Russia	Volgograd region	85°80'02"N	44°42'19"E
LE 227204 (NMS)	PHYalb	Russia	Volgograd region	48°35'18"N	44°39'32"E
LE 228018 (NMS)	PHYalb	Russia	Sverdlovsk region	56°36'00"N	61°05'00"E
LE 259241 (SSU)	PHYalb	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259348 (SSU)	PHYalb	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259660 (SSU)	PHYalb	Russia	Altai region	53°19'19"N	84°06'13"E
LE 259701 (SSU)	PHYalb	Russia	Altai region	53°19'19"N	84°06'13"E
LE 265462 (SSU)	PHYalb	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265801 (SSU)	PHYalb	Russia	Altai region	53°19'19"N	84°06'13"E
LE 265807 (SSU)	PHYalb	Russia	Altai region	53°19'19"N	84°06'13"E
LE 265919 (SSU)	PHYalb	Russia	Novosibirsk region	55°06'25"N	82°49'11"E
LE 266069 (SSU)	PHYalb	Russia	Novosibirsk region	55°06'24"N	82°49'53"E
LE 266093 (SSU)	PHYalb	Russia	Altai region	52°38'35"N	84°25'00"E
LE 266149 (SSU)	PHYalb	Russia	Altai region	53°19'19"N	84°06'13"E
LE 45889 (SSU)	PHYalb	Russia	Kursk region	51°36'00"N	37°16′00′′ E
LE 45900 (NMS)	PHYalb	Belarus	Minsk region	54°25'00"N	28°11'00"E
LE 45902 (NMS)	PHYalb	Belarus	Minsk region	54°52'00"N	26°56'00"E
LE 45920 (NMS)	PHYalb	Russia	Leningrad region	59°31'34"N	30°21′42′′ E
LE 45935 (NMS)	PHYalb	Russia	Leningrad region	59°31'34"N	30°21′42′′ E
LE 46916	PHYalb	Belarus	Minsk region	53°41'00"N	101°40'00" E
LE 48910 (NMS)	PHYalb	Russia	Krasnoyarsk region	71°30'50"N	103°05'00" E
LE 204042 (NMS)	PHYlcp	USA	Virginia	37°25'00"N	80°30'00"W

LE 218252 (SSU)	PHYlcp	Russia	Volgograd region	49°30'35"N	43°32'33"E
LE 224689 (NMS)	PHYlcp	Russia	Volgograd region	49°30'35"N	43°32'33"E
LE 225022 (SSU)	PHYlcp	USA	Michigan	44°53'00"N	84°18'00"W
LE 227129 (NMS)	PHYlcp	Russia	Leningrad region	59°45'00"N	30°18'00"E
LE 228082 (NMS)	PHYlcp	Russia	Leningrad region	56°39'00"N	59°17'00"E
LE 259225 (SSU)	PHYlcp	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259351 (SSU)	PHYlcp	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259424 (SSU)	PHYlcp	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259593 (SSU)	PHYlcp	Russia	Novosibirsk region	55°08'28"N	82°36'53"E
LE 259611 (SSU)	PHYlcp	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259780 (SSU)	PHYlcp	Russia	Altai region	53°49'28"N	83°46'26"E
LE 265456 (SSU)	PHYlcp	Russia	Novosibirsk region	55°08'28"N	82°36'53"E
LE 265858 (SSU)	PHYlcp	Russia	Novosibirsk region	55°08'28"N	82°36'53"E
LE 266011 (SSU)	PHYlcp	Russia	Novosibirsk region	54°49'44"N	83°07'31"E
LE 266012 (SSU)	PHYlcp	Russia	Novosibirsk region	54°49'43"N	83'06'31"E
LE 266017 (SSU)	PHYlcp	Russia	Novosibirsk region	52°37'32"N	84°27'27"E
LE 266049 (SSU)	PHYlcp	Russia	Novosibirsk region	54°48'59"N	83°06'10"E
LE 45842 (NMS)	PHYlcp	Russia	Leningrad region	59°31'34"N	30°21'42"E
LE 46905 (NMS)	PHYlcp	Russia	Stavropol region	44°03'03"N	43°05'18"E
LE 47013 (NMS)	PHYlcp	Switzerland	Zurich	46°42'00"N	80°00'00"E
LE 47014 (NMS)	PHYlcp	Switzerland	Zurich	46°42'00"N	80°00'00"E
LE 47300 (NMS, FSSU)	PHYlcp	Russia	Karelia	66°17'32"N	33°39'40"E
LE 47431 (NMS, FSSU, tef1)	PHYlcp	Russia	Leningrad region	60°18'05"N	28°36'00"E
LE 45879 (SSU)	PHYlcp	Russia	Kursk region	51°21'35"N	37°09'35"E
LE 285146 (NMS, SSU)	PHYniv	Russia	Karachay-Cherkessiya	43°26'18"N	41°40'54"E
LE 285157 (NMS, SSU)	PHYniv	Russia	Karachay-Cherkessiya	43°26'00"N	41°41'42"E
LE 285180 (NMS)	PHYniv	Russia	Karachay-Cherkessiya	43°26'10"N	41°41'16"E
LE 285190 (NMS, SSU)	PHYniv	Russia	Karachay-Cherkessiya	43°26'01"N	41°41'38"E
LE 285193 (NMS)	PHYniv	Russia	Karachay-Cherkessiya	43°25'58"N	41°41'52"E
LE 285194 (NMS)	PHYniv	Russia	Karachay-Cherkessiya	43°26'00"N	41°41'42"E
LE 285195 (NMS)	PHYniv	Russia	Karachay-Cherkessiya	43°26'00"N	41°41'42"E
LE 285196 (NMS)	PHYniv	Russia	Karachay-Cherkessiya	43°26'01"N	41°41'38"E
LE 285902 (NMS, SSU, tef1)	PHYniv	Russia	Karachay-Cherkessiya	43°26'18"N	41°40'54"E
LE 228079 (NMS)	PHYnot	Russia	Sverdlovsk region	56°60'00"N	59°50'00"E
LE 255700 (NMS, SSU)	PHYnot	Russia	Pskov region	57°51'23"N	28°44'58"E
LE 255701 (SSU)	PHYnot	Lithuania	Birzai	56°16'00"N	24°58'00"E

LE 255704 (SSU)	PHYnot	USA	Colorado	39°53'26"N	105°30'31" W
LE 255705 (NMS, SSU)	PHYnot	USA	Colorado	39°38'00"N	105°35'00" W
LE 255706 (NMS, SSU)	PHYnot	USA	Colorado	39°53'26"N	105°30'31" W
LE 255707 (NMS, SSU)	PHYnot	USA	Colorado	39°53'26"N	105°30'31" W
LE 255708 (NMS, SSU)	PHYnot	USA	Colorado	39°53'26"N	105°30'31" W
LE 255709 (NMS, SSU)	PHYnot	USA	Colorado	39°53'26"N	105°30'31" W
LE 255710 (FSSU, tef1)	PHYnot	Lithuania	Birzai	56°16'00"N	24°58'00"E
LE 255711 (NMS, SSU)	PHYnot	Lithuania	Birzai	56°16'00"N	24°58'00"E
LE 259177 (SSU)	PHYnot	Russia	Novosibirsk region	54°29'09"N	83°03'39"E
LE 259209 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259560 (SSU)	PHYnot	Russia	Novosibirsk region	54°48'54"N	83°06'07"E
LE 259640 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259642 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259651 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259652 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259654 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 259750 (SSU)	PHYnot	Russia	Altai region	53°37'55"N	83°43'31"E
LE 259778 (SSU)	PHYnot	Russia	Altai region	54°50'20"N	83°06'21"E
LE 259813 (SSU)	PHYnot	Russia	Altai region	53°37'55"N	83°43'31"E
LE 265457 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265461 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265463 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265464 (NMS, SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265465 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265476 (NMS, SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265491 (NMS, SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265493 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 265496 (SSU)	PHYnot	Russia	Novosibirsk region	54°49'36"N	83°06'18"E
LE 266190 (SSU)	PHYnot	Russia	Altai region	53°37'55"N	84°07'13"E
LE 282136 (SSU)	PHYnot	Russia	Novosibirsk region	54°48'59"N	83°06'10"E
LE 45277 (NMS, SSU)	PHYnot	USA	Colorado	41°21'00"N	75°00'00"W
LE 205496 (1) (NMS, SSU)	PHYpse	Russia	Astrakhan region	48°04'48"N	46°29'24"E
LE 219913 (NMS)	PHYpse	USA	Arizona	35°46'26"N	110°10'00" W

LE 220337 (SSU)	PHYpse	Kazakhstan	Western Kazakhstan region	50°18'14"N	51°01'37"E
LE 220350 (2) (NMS, SSU)	PHYpse	Kazakhstan	Mangystau region	45°35'34"N	57°16'50"E
LE 220362 (3) (SSU)	PHYpse	Kazakhstan	Atyrau region	45°35'33"N	53°09'09"E
LE 220372 (4) (NMS, SSU)	PHYpse	Kazakhstan	Atyrau region	45°35'33"N	53°09'09"E
LE 220379 (5) (SSU)	PHYpse	Kazakhstan	Kyzylorda region	45°45'45"N	62°52'34"E
LE 220391 (6) (NMS, SSU)	PHYpse	Kazakhstan	Kyzylorda region	45°45'45"N	62°52'34"E
LE 220397 (7) (SSU)	PHYpse	Kazakhstan	Almaty region	42°30'07"N	71°06'04"E
LE 220406 (8) (SSU)	PHYpse	Kazakhstan	Karagandy region	47°00'21"N	76°15'49"E
LE 220408 (9) (NMS, SSU)	PHYpse	Kazakhstan	Karagandy region	47°00'59"N	76°26'36"E
LE 220416 (10) (NMS, SSU)	PHYpse	Kazakhstan	Eastern Kazakhstan region	47°56'59"N	85°04'09"E
LE 220418 (11) (NMS, SSU)	PHYpse	Kazakhstan	Eastern Kazakhstan region	47°56'59"N	85°04'09"E
LE 220425 (12) (SSU)	PHYpse	Kazakhstan	Kostanay region	51°36'09"N	61°37'17"E
LE 253574 (SSU)	PHYpse	Russia	Volgograd region	49°12'21"N	46°31'08"E
LE 253579 (SSU)	PHYpse	Russia	Volgograd region	46°49'44"N	46°53'50"E
LE 253602 (13) (SSU)	PHYpse	Kazakhstan	Atyrau region	48°18'20"N	51°28'60"E
LE 253622 (14) (NMS, SSU)	PHYpse	Kazakhstan	Western Kazakhstan region	50°27'47"N	51°31'33"E
LE 253624 (15) (SSU)	PHYpse	Kazakhstan	Western Kazakhstan region	50°27'21"N	51°33'04"E
LE 253626 (NMS, SSU)	PHYpse	Russia	Volgograd region	46°49'44"N	46°53'50"E
LE 253638 (16) (SSU)	PHYpse	Kazakhstan	Western Kazakhstan region	48°18'41"N	52°00'10"E
LE 253641 (17) (NMS, SSU)	PHYpse	Kazakhstan	Atyrau region	48°18'48"N	51°28'31"E
LE 253652 (18) (NMS, SSU)	PHYpse	Russia	Volgograd region	49°12'21"N	45°35'25"E
LE 255130 (19) (NMS, SSU)	PHYpse	Russia	Altai region	50°10'21"N	87°54'32"E
LE 255134 (20) (NMS, SSU)	PHYpse	Russia	Altai region	50°07'03"N	88°20'41"E
LE 255162 (21) (NMS, SSU)	PHYpse	Russia	Altai region	50°07'03"N	88°20'41"E
LE 255230 (NMS, SSU)	PHYpse	Russia	Altai region	50°05'00"N	88°54'27"E
LE 255404 (22) (NMS, SSU)	PHYpse	Russia	Astrakhan region	48°09'44"N	46°32'06"E

LE 255406 (23) (SSU)	PHYpse	Russia	Astrakhan region	48°16'23"N	46°53'51"E
LE 255432 (24) (NMS, SSU)	PHYpse	Kazakhstan	Western Kazakhstan region	47°31'32"N	52°04'59"E
LE 255437 (25) (NMS, FSSU, tef1)	PHYpse	Kazakhstan	Western Kazakhstan region	48°23'36"N	51°51'32"E
LE 255438 (26) (NMS, FSSU, tef1)	PHYpse	Kazakhstan	Western Kazakhstan region	48°23'36"N	51°51'32"E
LE 255445 (NMS, SSU)	PHYpse	Mongolia	Khovd aimag	48°21'55"N	92°17'12"E
LE 255447 (27) (SSU)	PHYpse	Kazakhstan	Atyrau region	48°14'01"N	51°30'48"E
LE 255448 (28) (SSU)	PHYpse	Kazakhstan	Western Kazakhstan region	47°23'14"N	54°26'21"E
LE 255451 (29) (NMS, SSU)	PHYpse	Kazakhstan	Atyrau region	47°40'47"N	48°34'02"E
LE 255455 (SSU)	PHYpse	Kazakhstan	Atyrau region	47°40'46"N	48°34'01"E
LE 255465 (30) (NMS, SSU)	PHYpse	Kazakhstan	Atyrau region	47°24'17"N	48°20'25"E
LE 255545 (31) (SSU)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E
LE 255703 (32) (SSU)	PHYpse	USA	Wyoming	43°41'47"N	104°49'43" W
LE 255712 (33) (NMS, FSSU, tef1)	PHYpse	USA	Texas	29°18'02"N	103°35'42" W
LE 255713 (34) (NMS, SSU)	PHYpse	USA	Texas	29°20'23"N	103°30'35" W
LE 255714 (35) (NMS, FSSU, tef1)	PHYpse	USA	Texas	29°12'08"N	103°02'06" W
LE 255717 (36) (NMS, SSU)	PHYpse	USA	Wyoming	44°04'31"N	105°02'37" W
LE 255718 (SSU)	PHYpse	USA	Wyoming	44°04'23"N	104°38'17" W
LE 255722 (37) (SSU)	PHYpse	Spain	Granada	37°25'00"N	03°00'00"W
LE 256562 (SSU)	PHYpse	Russia	Karachay-Cherkessiya	43°26'38"N	41°43'54"E
LE 274211 (SSU)	PHYpse	Kazakhstan	Eastern Kazakhstan	48°13'40"N	83°56'53"E
LE 274212 (38) (SSU)	PHYpse	Kazakhstan	Eastern Kazakhstan	48°13'40"N	83°56'53"E
LE 284651 (39) (SSU)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E
LE 284652 (40a, b) (SSU, tef1)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E
LE 284653 (41a, b) (SSU)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E
LE 284654 (42) (SSU, tef1)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E
LE 284655 (43) (SSU)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E

LE 284656 (44) (SSU)	PHYpse	Kazakhstan	Jambyl region	44°45'01"N	71°09'56"E
LE 284657 (45) (SSU)	PHYpse	Kazakhstan	Jambyl region	42°31'16"N	70°31'33"E
LE 284659 (46) (SSU)	PHYpse	Kazakhstan	Jambyl region	42°31'16"N	70°31'33"E
LE 284660 (47) (SSU)	PHYpse	Kazakhstan	Jambyl region	44°46'24"N	69°52'11"E
LE 284661 (48) (SSU)	PHYpse	Kazakhstan	Kyzylorda region	44°46'24"N	69°52'11"E
LE 284662 (49) (SSU, tef1)	PHYpse	Kazakhstan	Kyzylorda region	44°46'24"N	69°52'11"E
LE 284663 (50) (SSU, tef1)	PHYpse	Kazakhstan	Kyzylorda region	44°46'24"N	69°52'11"E
LE 284664 (51) (SSU)	PHYpse	Kazakhstan	South Kazakhstan region	44°27'44"N	69°31'15"E
LE 284666 (52) (SSU, tef1)	PHYpse	Russia	Astrakhan region	47°34'00"N	47°04'50"E
LE 284668 (53) (SSU)	PHYpse	Argentina	Patagonia	43°49'58"S	70°55'12"W
LE 284669 (54) (NMS, SSU)	PHYpse	Argentina	Patagonia	47°34'32"S	71°22'42"W
LE 284670 (55) (NMS, SSU)	PHYpse	Argentina	Patagonia	47°34'32"S	71°22'42"W
LE 284671 (56) (SSU)	PHYpse	Argentina	Patagonia	44°07'60"S	70°29'34"W
LE 284672 (57) (NMS, SSU)	PHYpse	Argentina	Patagonia	47°13'01"S	70°53'43"W
LE 284673 (58) (SSU)	PHYpse	Argentina	Patagonia	46°07'57"S	70°35'51"W
LE 284674 (59) (NMS, SSU)	PHYpse	Argentina	Patagonia	46°39'42"S	71°36'28"W
sc 21042 (60) (SSU)	PHYpse	Mongolia	Khovd aimag	48°21'55"N	92°17'12"E
sc 21060 (61) (SSU)	PHYpse	Mongolia	Khovd aimag	48°16'27"N	92°31'33"E
sc 21061 (62) (NMS, SSU)	PHYpse	Mongolia	Khovd aimag	48°16'26"N	92°31'41"E
sc 21064 (63) (SSU)	PHYpse	Mongolia	Khovd aimag	48°16'27"N	92°31'33"E
sc 21070 (64) (NMS, SSU)	PHYpse	Mongolia	Khovd aimag	48°18'49"N	93°05'42"E
Sc 21072 (65) (SSU)	PHYpse	Mongolia	Khovd aimag	48°21'55"N	92°17'12"E
sc 21095 (SSU)	PHYpse	Mongolia	Khovd aimag	48°30'48"N	91°34'22"E
sc 21106 (66) (SSU)	PHYpse	Mongolia	Khovd aimag	48°20'43"N	93°07'18"E
sc 21154 (67) (SSU)	PHYpse	Mongolia	Khovd aimag	48°19'27"N	92°10'54"E

sc 21375 (68) (SSU)	PHYpse	Oman	Northern Oman	23°08'13"S	58°55'01"E
sc 21429 (69) (SSU)	PHYpse	China	Xinjiang	41°58'13"N	85°21'75"E
sc 21442 (70) (SSU)	PHYpse	China	Xinjiang	40°49'93"N	84°17'32"E
sc 21453 (71) (NMS, SSU)	PHYpse	China	Xinjiang	40°07'40"N	88°22'28"E
sc 21455 (72) (NMS, SSU)	PHYpse	China	Xinjiang	40°07'40"N	88°22'28"E
sc 21505 (73) (SSU)	PHYpse	China	Xinjiang	41°11'16"N	84°15'93"E
sc 21510 (74) (NMS, SSU)	PHYpse	China	Xinjiang	41°14'77"N	84°26'92"E
sc 21512 (75) (NMS, SSU)	PHYpse	China	Xinjiang	41°14'77"N	84°26'92"E
sc 21514 (76) (SSU)	PHYpse	China	Xinjiang	41°14'13"N	84°26'31"E
sc 21518 (77) (SSU)	PHYpse	China	Xinjiang	43°53'30"N	88°07'23"E
sc 21559 (78) (NMS, SSU)	PHYpse	China	Xinjiang	43°50'35"N	88°10'40"E
sc 21564 (79) (SSU)	PHYpse	China	Xinjiang	43°53'30"N	88°07'23"E
sc 21633 (80) (NMS, SSU)	PHYpse	China	Xinjiang	41°20'31"N	84°25'01"E
sc 21689 (81) (SSU)	PHYpse	China	Xinjiang	43°53'30"N	88°07'23"E
sc 23290 (SSU, tef1)	PHYpse	Oman	Northern Oman	18°39'16"S	54°04'23"E
sc 23291 (82) (SSU, tef1)	PHYpse	Oman	Northern Oman	23°08'13"S	58°55'01"E
sc 23342 (83) (SSU, tef1)	PHYpse	Oman	Central Oman	23°11'07"S	57°08'15"E
sc 23362 (84) (SSU)	PHYpse	Oman	Central Oman	17°54'01"S	53°47'21"E
sc 23363 (SSU)	PHYpse	Oman	Central Oman	17°54'01"S	53°47'21"E
sc 23366 (85) (SSU)	PHYpse	Oman	Central Oman	17°03'34"S	54°26'31"E
sc 23390 (86) (SSU)	PHYpse	Oman	Central Oman	19°57'02"S	56°24'16"E
sc 23469 (87) (SSU)	PHYpse	Oman	Central Oman	19°44'03"S	56°05'11"E
sc 23471 (SSU)	PHYpse	Oman	Central Oman	19°37'01"S	55°45'04"E
sc 23475 (SSU)	PHYpse	Oman	Central Oman	17°54'01"S	53°47'21"E
sc 23477 (SSU)	PHYpse	Oman	Central Oman	17°47'32"S	53°56'11E
LE 204508 (NMS, SSU, tef1)	PHYpus	Germany	Sachsen - Anhalt	51°12’58’’N	11°46’32’’E
LE 255719 (FSSU, tef1)	PHYpus	USA	South Dakota	44°02'38"N	104°22'58" W

LE 255721 (NMS, FSSU, tef1)	PHYpus	USA	South Dakota	29°06'21"N	102°34'17" W
LE 270172 (NMS, SSU)	PHYpus	Russia	Kalmykia	46°00'53"N	45°53'16"E
LE 270215 (NMS)	PHYpus	Russia	Astrakhan region	45°54'14"N	47°03'00"E
LE 46929 (NMS)	PHYpus	USA	Georgia	31°22'00"N	81°26'00"W

Supplementary Table S2. Morphological data used in the NMS analysis. Herbarium numbers are given for all samples used. For the codes of morphological characters, see Chapter 4.4.

	LE 227199	LE 227204	LE 228018	sc 45899	sc 45900	sc 45902	sc 45920	sc 45935	sc 46916	sc 48910
I	2	1	3	1	1	1	3	2	1	2
II	265	496	287	430	430	527	462	464	612	466
III	147	254	182	238	217	298	267	235	430	332
IV	1	4	4	4	4	4	4	3	4	4
V	1	1	1	1	1	1	1	1	1	1
VI	3	3	2	3	3	3	1	1	3	3
VII	426	655	704	511	881	714	989	939	613	651
VIII	19	21	17	11	33	28	29	23	9	17
IX	31	30	66	85	55	29	49	85	25	23
X	2	2	2	2	2	2	2	2	2	2
XI	2	2	2	2	2	2	2	2	2	2
XII	1	1	1	1	1	2	1	1	1	2
XIII	2	2	2	2	2	2	2	2	2	2
XIV	1	1	1	1	1	1	1	1	1	1
XV	9	9	9	8	8	8	8	9	9	8
XVI	1	1	3	2	3	3	1	1	1	3
XVII	25	34	31	17	40	54	31	20	15	29

	LE 285146	LE 285157	LE 285180	LE 285190	LE 285193	LE 285194	LE 285195	LE 285196	LE 285902	LE 204042	LE 224689
I	2	2	2	2	2	2	2	2	2	2	2
II	960	870	798	1100	1115	845	967	894	932	607	545
III	720	610	567	789	945	670	789	723	823	455	408
IV	4	4	4	4	4	4	4	4	4	4	1
V	0	0	0	0	0	0	0	0	0	4	1
VI	1	1	1	1	1	1	1	1	1	3	3
VII	0	0	0	0	0	0	0	0	0	439	463
VIII	0	0	0	0	0	0	0	0	0	8	7
IX	0	0	0	0	0	0	0	0	0	16	19
X	2	2	2	2	2	2	2	2	2	2	2
XI	1	1	1	1	1	1	1	1	1	1	1
XII	4	4	4	4	4	4	4	4	4	2	4
XIII	1	1	1	1	1	1	1	1	1	2	1
XIV	3	3	3	3	3	3	3	3	3	2	2
XV	12	11	12	11	12	11	12	12	11	7	8
XVI	7	6	7	8	7	9	8	7	7	1	1
XVII	47	44	38	42	39	53	43	51	46	24	23

	LE 227129	LE 228082	sc 45842	sc 46905	sc 47013	sc 47014	sc 47300	sc 47431	LE 228079	LE 255700	LE 255705
I	2	1	1	2	1	1	1	1	1	3	2
II	515	757	466	634	599	465	587	466	625	741	641
III	421	507	173	445	384	290	287	173	426	542	425
IV	4	4	4	2	4	1	1	4	1	1	4
V	4	0	1	2	2	4	4	1	2	2	0
VI	2	1	3	3	3	3	3	3	1	3	1
VII	309	0	680	388	574	664	673	548	203	719	0
VIII	5	0	27	6	12	25	23	15	4	12	0
IX	11	0	52	16	16	35	33	32	6	16	0
X	2	2	2	2	2	2	2	2	2	2	2
XI	1	1	1	1	1	1	1	1	1	1	1
XII	4	1	1	1	1	1	1	1	1	4	4
XIII	1	2	1	2	1	2	2	1	1	1	1
XIV	2	2	2	2	2	2	2	2	3	3	3
XV	8	9	8	9	9	9	8	9	11	9	9
XVI	1	1	2	3	3	1	2	2	8	4	5
XVII	20	39	18	23	16	40	35	32	35	40	30

	LE 255706	LE 255707	LE 255708	LE 255709	LE 265464	LE 265476	LE 265491	sc 45277	LE 205496	sc 21061	sc 21070
I	1	2	3	2	2	3	2	2	2	2	2
II	663	565	670	582	686	415	866	407	409	467	457
III	510	497	463	499	532	305	704	315	302	439	312
IV	4	4	4	4	4	1	1	1	1	1	1
V	2	0	0	2	4	4	2	4	2	1	1
VI	2	1	2	2	3	3	3	3	3	2	3
VII	219	0	0	509	370	618	543	687	318	725	347
VIII	0	0	0	0	6	21	7	17	77	0	4
IX	4	0	0	10	10	30	10	29	139	24	15
X	2	2	2	2	2	2	2	2	1	2	2
XI	1	1	1	1	1	1	1	1	1	1	1
XII	4	4	3	4	2	4	2	4	1	2	2
XIII	1	1	1	2	1	1	1	1	2	1	1
XIV	3	3	3	3	3	3	3	3	3	3	3
XV	10	9	12	9	10	10	9	9	11	9	10
XVI	5	5	5	5	10	4	5	6	4	4	5
XVII	30	40	40	30	58	65	37	35	18	19	15

	sc 21453	sc 21455	sc 21510	sc 21512	sc 21559	sc 21633	sc 219913	LE 220350	LE 220372	LE 220391	LE 220408
I	2	2	2	1	3	2	1	1	2	1	2
II	372	359	298	418	323	372	771	672	471	574	411
III	340	316	280	347	328	340	425	405	388	273	366
IV	3	1	1	3	3	4	4	2	1	3	1
V	1	1	1	0	1	1	0	1	1	1	1
VI	2	3	3	1	3	3	1	3	2	2	3
VII	159	292	174	0	294	159	0	176	50	297	246
VIII	0	7	3	0	8	6	0	37	0	8	0
IX	5	13	10	0	9	9	0	51	1	16	12
X	2	2	2	2	2	2	2	2	2	2	1
XI	1	1	1	1	1	1	1	1	1	1	0
XII	4	4	2	2	1	4	2	4	2	4	1
XIII	2	1	1	1	2	1	1	1	2	2	0
XIV	3	3	3	2	3	3	3	3	3	3	3
XV	10	10	10	9	11	10	10	12	12	10	10
XVI	4	6	5	5	5	3	6	5	3	3	3
XVII	8	13	13	15	15	15	15	13	10	10	10

	LE 220416	LE 220418	LE 253622	LE 253626	LE 253641	LE 253652	LE 255130	LE 255134	LE 255162	LE 255230	LE 255404
I	3	2	2	2	2	2	2	2	2	3	2
II	447	297	478	306	321	251	409	463	520	389	343
III	519	252	321	232	266	177	340	315	399	336	270
IV	3	2	4	4	4	1	1	4	4	4	4
V	0	1	1	1	2	2	0	0	1	0	0
VI	1	2	1	1	1	1	1	2	3	1	2
VII	0	347	0	0	0	40	0	0	408	0	0
VIII	0	11	0	0	0	0	0	0	7	0	0
IX	0	19	0	0	0	6	0	0	12	0	0
X	2	2	2	2	2	2	2	2	2	2	2
XI	1	1	1	1	1	1	1	1	1	1	1
XII	1	2	2	3	2	4	4	2	2	4	2
XIII	2	2	2	2	1	1	1	2	2	1	1
XIV	3	3	2	3	2	3	3	3	3	3	3
XV	9	10	10	13	11	12	13	11	14	9	11
XVI	3	3	4	2	6	3	3	3	7	3	5
XVII	27	20	17	10	18	15	12	20	10	28	12

	LE 255432	LE 255437	LE 255438	LE 255445	LE 255451	LE 255465	LE 255711	LE 255712	LE 255714	LE 255717	LE 284669
I	2	2	1	2	1	1	1	2	2	2	2
II	492	329	696	395	454	396	560	445	448	421	405
III	415	294	461	234	256	302	380	200	341	332	216
IV	1	4	4	3	4	4	4	4	4	4	2
V	0	0	0	1	2	0	0	1	1	1	1
VI	1	1	1	1	2	1	1	1	3	3	3
VII	0	0	0	486	74	0	0	396	258	261	336
VIII	0	0	0	12	0	0	0	11	8	6	8
IX	0	0	0	31	60	0	0	13	10	9	10
X	2	1	1	2	1	2	2	2	2	2	2
XI	1	1	1	1	1	1	1	1	1	1	1
XII	4	1	1	3	1	4	3	2	4	4	4
XIII	1	2	2	2	2	1	1	2	1	1	1
XIV	2	3	3	3	3	3	3	3	2	2	3
XV	13	11	12	11	10	11	10	9	9	10	10
XVI	5	6	7	5	5	5	5	3	4	3	6
XVII	12	12	8	12	16	19	26	15	22	22	14

	LE 284670	LE 284672	LE 284674	LE 204508	LE 255719	LE 255721	LE 270172	LE 270215	sc 46929
I	1	1	3	2	2	2	2	2	2
II	430	471	429	448	430	487	428	410	400
III	311	339	308	368	350	344	379	390	371
IV	4	2	2	4	4	4	4	4	4
V	1	1	1	3	3	3	3	3	3
VI	3	3	3	3	3	3	3	3	3
VII	162	276	336	680	723	548	634	590	590
VIII	4	7	8	18	20	18	15	13	13
IX	6	10	13	27	35	27	22	19	19
X	2	2	2	2	2	2	2	2	2
XI	1	1	1	2	2	2	2	2	2
XII	4	4	4	1	1	1	1	3	3
XIII	1	1	1	2	2	2	2	2	2
XIV	3	3	3	1	1	1	1	1	1
XV	10	9	11	7	8	8	8	8	8
XVI	6	7	5	3	3	4	3	4	3
XVII	10	10	12	10	9	10	8	8	10

Curriculum Vitae

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