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DIPLOMARBEIT

Titel der Diplomarbeit

„Genetic variability of geographically widely separated mediterranean population of *Sphaerosyllis sp.* (Syllidae, Polychaeta, Annelida)“

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Contents

1: Abstract

Interstitial Fauna inhabits the pore space between sand grains. To live in such an environment special adaptations like a vermiform body type occur. This habitat is rich in representatives of like Nematoda, Mollusca and Polychaeta. The polychaete family Syllidae consists of 70 genera with over 700 species, are a very diverse family and have many interstitial species. Their dispersal potential is low because of their reproductive modes and, therefore, a high differentiation between widely separated populations is expected. In this study the genetic variability between two geographically separated populations of *Sphaerosyllis sp.* in the Mediterranean Sea is investigated and to clarify if these populations are conspecific or represent cryptic species. Animals from the Adriatic Sea (Croatia) and the Tyrrhenian Sea (Italy) were collected. 16S and ITS genes were used as genetic markers and with this data-set phylogenetic and statistical analyses were computed. The results showed two clades, one from each sampling site. Pairwise distances between populations varied from 2.4% to 14%; F_{st} values range from 0.928 to 0.783, with calculated 0.4 migrants per generation. The analysis points towards conspecific populations rather than to cryptic species. This could be explained with the Last Glacial Maximum 11.000 years ago, when gene flow was facilitated by the decreased sea level. Additional sequences with other genes and sampling location from Albania, France and Spain would help to further clarify the status of *Sphaerosyllis sp.* and its relationships within the genus *Sphaerosyllis*.

Deutsche Zusammenfassung

Der Zwischenraum von Sandkörnern wird von interstitieller Fauna bewohnt. Um in so einem Habitat zu leben, müssen einige Anpassungen getroffen werden, wie zum Beispiel einen wurmförmigen Körperbau zu haben, um sich besser durch den Lebensraum bewegen zu können. Nematoden, Mollusken und Polychaeten sind nur einige Vertreter in diesem artenreichen Habitat. Syllidae (Polychaeta) sind weit verbreitet in der interstitiellen Fauna. Da das Ausbreitungspotenzial durch ihre geringe Körpergröße und verschiedenen Fortpflanzungsstrategien gering ist, ist anzunehmen, dass weit verbreitete Populationen eine hohe genetische Differenz aufweisen. Diese Studie befasst sich mit der Frage, ob die geographisch weit voneinander getrennten Populationen von *Sphaerosyllis sp.* konspezifisch oder kryptisch sind. Dafür wurden an der adriatischen Küste (Kroatien) sowie im Tyrrhenisches Meer bei Neapel (Italien) Individuen gesammelt. Mit 16S und ITS wurden phylogenetische und statistische Analysen durchgeführt. Es zeigen sich zwei voneinander separierte Kladen, eine von jedem Sammelorten. Der F_{st} Wert liegt zwischen 0.92798 und 0.78308 und die berechneten genetischen Distanzen reichen zwischen den Populationen von 0.024 bis 0.14. Die damit berechneten Migranten pro Generation beträgt 0.4. Die Analysen deuten eher auf eine konspezifische, weitverbreitete Art im Mittelmeer hin und nicht auf kryptische Arten. Dies könnte mit der Würmeiszeit erklärt werden, denn hier sank der Meeresspiegel drastisch und ein Genfluss war möglich; dazu kommt, dass seitdem nur 11.000 Jahre vergangen sind. Zusätzliche Sequenzen mit anderen Primern als auch weitere Standorte wären hilfreich, um die Position und Verwandtschaft von *Sphaerosyllis sp.* innerhalb von *Sphaerosyllis* zu klären.

2: Introduction

2.1: Habitat

Meiobenthos, term was created by Molly F. Mare in 1942, describes benthic metazoans with a small body size, living in soft substrates like sand and mud. These animals do not belong to "macrobenthos" or to the "microbenthos". Remane (1933) first used the term "Sandlückenfauna", Nicholls (1935) introduced the term "interstitial fauna". It is a rather young biological field, but in the last decades the importance of this habitat got recognized.

Key factors for the interstitial habitat are sand grains, water flow, and permeability just to name a few (Giere, 2009). Sand grains play an important role for its inhabitants and determine the space between the individual sand grains. Well sorted, coarse sediment has more pore volume, up to 45%, than unsorted they are tightly packed and have only 20% pore space. Round shaped grains offer more pore space than irregular grains. Most samples in the field of unsorted freshwater sand had 40% of pore volume (Ruttner-Kolisko, 1962). The space needed for interstitial fauna are estimated between 20% and 45% pore volume. In experiments a correlation of pore dimension and body size could be demonstrated (Williams, 1972). But the surface of the sediment plays a vital role for the development of biofilms (mucus secretion of bacteria, fungi, diatoms and fauna). Mayer and Rossi (1982) calculated the amount of total surface area. One gram of fine, dry sand with a particle diameter of 63 – 300 µm has a total surface area of 8 – 12.5 m^2, 1 g of coarse sand has just 1.8 m^2 (Suess, 1973). With different grain structures, different bacterial colonies are found, which attracts different meiofauna (Meadows & Anderson, 1968). Specific sediment has specific inhabitants (Vanreusel, 1991; Schratzberger et al., 2004).

It has been estimated, that "the critical grain size" for mesopsammon is about 200 µm in diameter (Wieser, 1959). The anatomy and the function of the organs of the meiobenthic animals depend on the structure and the dimension of the pore system (Ax, 1966).

The water itself plays an important role for the interstitial habitat. Wave action and water currents have an impact on sediment sorting (Giere, 2009). The exposure of the habitat can be calculated (Hummon, 1989), important factors are current velocity, sediment agitation,

wave height, and many more. All this plays an important role for deposition und packing of the sediment. Are the currents and waves too strong, a downward migration can be observed (Sedlacek and Thistl, 2006; McLachlan, 1977). But the occurrence of tubes and mucus by bacteria and animals can increase the stability of the sediment (Wiltshire, 2000). The flow speed of water through the sediment and, therefore, the supply of oxygen, dissolved and particulate nutrients is called permeability and is important for the control of the life conditions. Under a diameter of 200 µm it water flow decreases.

Bioturbation is inevitable in sediments and has positive effects as the supply with oxygen and nutrients, but also negative effects on its inhabitants like destabilization of the substrate (Koller et al., 2006). The occurrence of species and their composition depends on the sediment and its other characters (Richmond et al., 2007).

The mesopsammon are aquatic animals, that are small, motile and are marine or limnic. Their habitat ranges from the Polar Regions to tropical coral reefs. Almost every phylum has at least one member in the interstitial fauna. Prominent members of the interstitial habitat are Nematoda, Annelida, Arthropoda, Mollusca and Tardigrada.

Life in such an extreme habitat requires various adaptations. The most noticeable adaption, in case of metazoans, is miniaturization and their vermiform body shape. It helps to navigate through tight habitats and gives more mobility and flexibility. There are different kinds of methods for adhesion to the sediment like adhesive glands, a tail for anchoring in the sediment and solid external structures like scales, spicules and plates. These external structures can be also reinforcements for protection from mechanical powers. Often there are statocysts for orientation, eyes and pigments are reduced because of the dark environment. Due to their small size they only produce one or just a few eggs that are often attached to sand grains. Adaptations like direct sperm transfer, internal fertilization, spermatophores, hermaphroditism, brood care, brood protection, viviparie and the reduction of pelagic larvae can be observed.

The adaptations and different reproduction modes in Polychaetes and especially in syllids are detailed described in the following chapter.

2.2: Systematics

Phylum Annelida

Class Polychaeta

Subclass Aciculata

Order Phyllodocida

Suborder Nereidiformia

Family Syllidae

Subfamily Exogoninae

Polychaeta (Grube, 1850):

Polychaetes belong to the Annelida, they are segmented worms, ranging from under 1 mm up to 3 meters, but in general have a length of less than 10 centimeters. Each segment has parapodia bearing chitin chaetae. The prostomium bears of two to four of eyes, a pair of antennae and palps. Occurrence is worldwide, from the cold polar ice to warm tropical regions to hydrothermal vents. They consist of about 80 families (Rouse & Fauchald, 1997) and more than 14.000 marine species. Only 168 species of polychaetes are living in freshwater (Glasby & Timm, 2008). Polychaetes have adapted to many different ecological niches, like tube dwelling or parasitism, with the corresponding modifications. It is a class with great morphological diversity. Polychaetes are divided into three subclasses: Aciculata, Canalipalpata and Scolecida.

Aciculata (Rouse & Fauchald, 1997):

Together with the Canalipalpata they form the former subclass of Palpata, it is characterized by a pair of palps, which are missing in Scolecida. Aciculatia represents more than half of all polychaetes and is equivalent to the “Errantia”, an old taxonomic group, as “Sedentaria” is for Canalipalata. Errantia was an old decryption for free moving animals, whereas Sedentaria stood for tube dwelling worms. They are mostly deposit or filter feeders. Aciculata are divided in the orders Phyllodocida and Eunicida.

Phyllodocida:

Phyllodocida consist of 3.500 species, mostly marine and benthic, with a few species found in brackish water. The have two pair of eyes, frontal or frontolateral palps, a peristomium often hidden by the prostomium and the first segment and an eversible pharynx. There are five subgroups Nereidiformia, Aphroditiformia, Glyceriformia, Phyllodociformia and Phyllodocida incertae sedis.

Nereidiformia:

They have at least one pair of antennae and a pair of tentacular cirri, short and blunt palps, an eversible pharynx and the first parapodia is lateral. Syllidae, Antonbruuniidae, Hesionidae, Nereidae and Pilargidae are the five families in this suborder.

Syllidae (Grube, 1850):

Syllidae are small to medium-sized polychaetes with four eyes, three antennae, simple and sometimes fused palps, two pairs of tentacular cirri and a nuchal organ. They have a proventricle and an eversible pharynx that bears a single tooth or smaller teeth in a circlet. The Parapodia a uniramous, setae are simple or composite. The common habitat for syllids is shallow-water with hard substrates or seagrass beds, but they can also be found in the deep sea. They are mostly benthic or interstitial, and only have a pelagic phase during reproduction (Rouse & Pleijel, 2001). Syllids consists of 70 genera with over 700 species and have a worldwide occurrence from the Arctic to the Antarctica, but they are more diverse in the tropic and subtropical regions. It is a big and diverse family, with five subfamilies, Anoplosyllinae, Exogoninae, Eusyllinae, Autolytinae and Syllinae (Aguado & San Martin, 2009). They show a lot of specializations, the most noticeable are the reproduction specializations.

Epitoky is a “metamorphosis” from a benthic to a pelagic form. The animals undergo modification, morphological, physiological and behavioural alike (Franke, 1999). The most common modifications are the locomotory and the visual system. In Nereididae the modifications are more extreme, because they die after reproduction. Syllids survive reproduction and reverse the modification to a point. There are two types of epitoky, epigamy and schizogamy. Epigamy is the total transformation of the animal into pelagic species, in schizogamy only the posterior end changes into a stolone, which has a small specialized head and is used to distribute their sexual products. The stolons break free and have a short pelagic life. There are also various other reproductive modes as external brooding, direct sperm transfer, internal fertilization, viviparity, parthenogenesis, and simultaneous as well as successive hermaphroditism.

The 5 subfamilies can be distinguished by their reproduction modes. Anoplosyllinae, Exogoninae, Eusyllinae have epigamy, Autolytinae & Syllinae are characterized by schizogamy.

Exogoninae (Langerhans, 1879):

Exogoninae are small individuals with just a few segments, living interstitial in sand, mud, between fissures in rocks and corals, and are found on most shallow substrates but some species a found also in the deep sea. Most lack pigments, have three antennae, fused or partial fused palps, four lensed eyes, a nuchal organ and two pairs of tentacular cirri. The pharynx is armed with a single tooth, the parapodia are uniramous with dorsal and ventral cirri and have a single aciculum. Antennae, dorsal and tentacular cirri are short in Exogoninae. The most common reproduction mode is epigamy. The eggs are attached either on the ventral or dorsal side. But some animals have also viviparity. All these reproduction modes show parental care. This is interpreted as a modification due to their small body size and the interstitial habitat (Westheide, 1984).

***Sphaerosyllis* (Claparède, 1863):**

The genus is diagnosed by a small body with papillae on the dorsal and ventral side. Prostomium has three antennae, four eyes, fused or partial fused palps and a single pair of tentacular cirri. Antennae, tentacular and dorsal cirri are short, with a bulbous- or flask-shaped form, dorsal cirri usually absent on chaetiger two. Parapodial glands are present, parapodia have on the ventral and dorsal side simple chaetae and thick aciculae. The

pharynx is slender with a single conical tooth, the proventricle is short with few but large muscle rows. Reproduction by epigamy, males have long natatory chaete for brooding eggs on the ventral side.

Sphaerosyllis sp. **(Figure 1):**

Body with small papillae, prostomium has four eyes, arranged in a trapezium, three flask-shaped antenna, where the median antenna is slightly longer, and slim, partial fused palps Prostomium and palps are clearly separated, the first segment and prostomium are fused. Tentacular and dorsal cirri have the same size and are bulbous-, flask-shaped as well, as are the anal cirri. The proventricle has 12 to 15 muscle rows and stretches over 2 or 3 segments. Parapodial glands are onion shaped and the aciculae are distal pointed and angular. Chaetae are simple. The females have the eggs and embryos on their ventral side.

The present *Sphaerosyllis* species closely resembles, and can easily be confused with, *Sphaerosyllis hystrix*. On close examination, the presence of dorsal cirri on the chaetiger 2 and the partially fused, oval palps set it apart from *S. hystrix* (no dorsal cirrus on chaetiger 2, fused, slender and triangular palps). Sequences of the 18S rDNA gene match more closely that of *Sphaerosyllis sp.* in Aguado et al. (2007), and not those of *S. hystrix*.

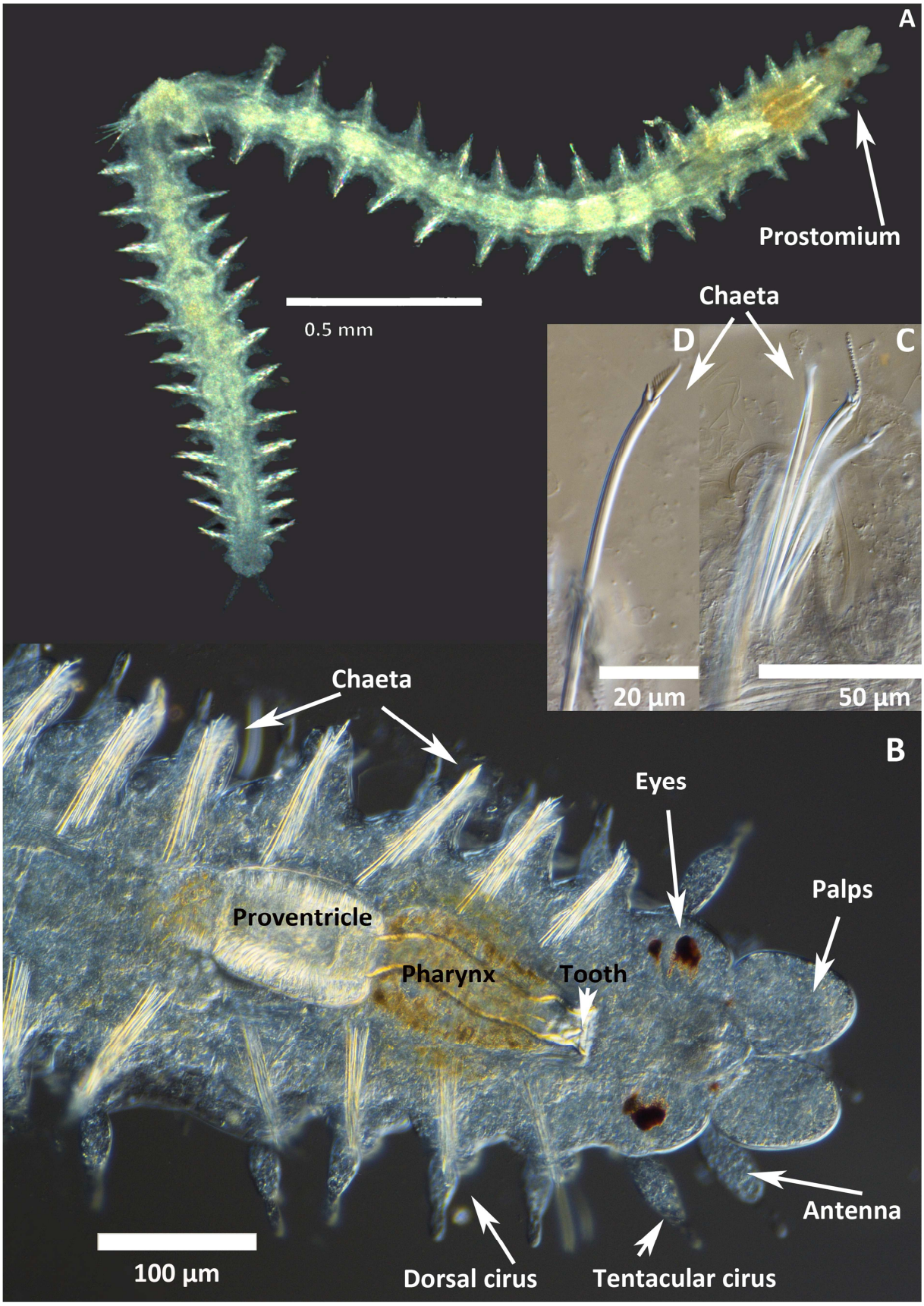

Figure 1 ***Sphaerosyllis sp.*** **A: dorsal view, total; B: View of prostomium with dorsal cirrus on the second chaetiger; C: magnification of chaetae; D: Single chaeta which are identical to** ***Sphaerosyllis hystrix***

2.3: Aim of this study

The aim of this study is to clarify, if the two populations of *Sphaerosyllis sp.*, one from the Adriatic Sea and the second from the Tyrrhenian Sea, are conspecific or separate species. How are they related, how much genetic difference is between this 2 populations? Because of their small body size, brood care, just a short time as a pelagic form and therefore limited dispersal potential, a high differentiation and separated species are expected. The two sampling sites are separated by over 2.500 km of coast line. The specimens found on both sampling sites seem to be morphological identical. Are they conspecific or cryptic species? Cryptic species are specimens that are morphological identical but differ in DNA sequence or in behavior or in chemical or acoustic signals. Another question is, if *Sphaerosyllis sp* could be *S. hystrix.*

Werth (2008) studied *Pontohedyle milaschewitchii* (Acochlidia, Gastropoda) also from the Croatian and West-Italian coast and the Gastropoda specimens showed low genetic variability ranging from 0.7% to 8.1%. She explained the low genetic variability with the glacial cycles and the contraction and expansion of interstitial populations because of habitat shift. The last glacial maximum was about 18 thousand (kyr) years before present (BP), during which the sea level reached its lowest position of about minus 120 meters relative to the present level. Sandy habitats on the ocean floor were shifted and interstitial populations of the northern Adriatic Sea contracted in the south. In this time gene flow could be possible between both sides of Adriatic Sea. After the last glacial maximum, ice melted and the sea level rose again and flooded the north Adriatic shelf again. Populations migrated northwards again. This could be the reason for the low genetic differentiation of *P. milaschewitchii*, because the separation happened 15 to 5.5 thousand years before present, were the last glacial maximum more or less ended. Could this be also the case in *Sphaerosyllis* sp.? Or are they genetically strongly differentiated populations, or even separate, cryptic species, as would be expected from their limited dispersal abilities.

3: Material and methods

3.1: Generating sequences

3.1.1: Sampling

Animals were collected at three different locations: the Croatian Coast (Isle of Pag) and the Italian east coast for the Adriatic Sea and the west Coast of Italy for the Tyrrhenian Sea. Sampling sites in Italy were between Pescara and Bari for the Adriatic part and in the area around Naples for the Tyrrhenian Sea. On the Adriatic site of Italy, no individuals were found. The coastline from Pag to Naples is about 2.500 kilometers long. In Figure 2 the sampling sites are shown and are listed in Table 1.

Figure 2: Map of the sampling sites in the Mediterranean Sea; red dots are the sampling sites, stars are the major cities in these areas; coastline from the Isle of Pag to Neaples is about 2,500 kilometers long.

Table 1: Location and information (coordinates, depth) of the sampling sites

Country	Location	Spot	Coordinates	Sea	Depth
Croatia	Isle of Pag	Lun	44°41'38" N 14°43'57" E	Adriatic Sea	1-4 m
Italy	Pescara to - Bari	Peschici	41°56'37" N 16°00'14" E		2-3 m
		Vico del Gargano	41°56'30" N 15°58'52" E		1-2 m
		Lungo-mare Lido del sole	41°55'33" N 15°51'10" E		1-2 m
		San Nicandro Garganico	41°55'37" N 15°37'02" E		2-3 m
		Vieste	41°53'40" N 16°09'40" E		2-3 m
		San Matteo	41°10'25" N 16°42'38" E		1-2 m
		Lungomare marina Italiana; Giovanazzo	41°11'32" N 16°39'26" E		6 m
		Margherita di Savoia	41°25'12" N 16°02'25" E		3-4 m
	Naples	Meta (Strada Statale della Penisola sorrentina)	40°38'52" N 14°24'19" E	Tyrrhenian Sea	3-4 m
		Marina del Cantone	40°35'00" N 14°21'27"E		4-8 m
		Laura	40°26'09" N 14°58'33" E		3 m

		Viale mare cristallo	40°17'59" N 14°57'59" E		3-5 m
		Marina diaequa	40°39'34" N 14°24'54" E		3-4 m
		Agnone	40°12'54"N 14°59'41"E		2-4 m
		Lago di Patria	40°55'40" N 14°00'58" E		5-10m

Suitable sand was collected via snorkeling in depths of 2-10 meters. The bucket of sand rested for 24h to let the animals migrate upwards, following the oxygen gradient. Afterwards the top layer was taken off with a spoon and treated with seawater-isotonic potassium chloride ($MgCl_2$) for 10 to 20 minutes to anaesthetize the animals. Then the retort with the solution was shaken so the animals would release the sand grains. The suspension was sieved through a net with a mash-size of 250 µm and washed out with seawater on a petri dish. *Sphaerosyllis sp.* was identified under a binocular (Hartmann-Schröder, 2006) and preserved in RNAlater®. The animals were put in RNAlater® for 10 minutes, afterwards the RNAlater® was changed and the tubes with the specimens were frozen in liquid nitrogen. The numbers and codes of specimen for each sampling site are shown in Table two.

Table 2: Number and codes of the collected specimens from each sampling site

Country	Croatia	Italy	
Sampling site	Pag	Bari	Naples
Sampling size	101	0	250
Code	AK	AB	AN

3.1.2: DNA Extraction

The extraction was done with peqGOLD Tissue DNA Mini Kit (PEQLAB), following the manufacture's protocol. Before the extraction, the individuals were washed with ddH_2O to get rid of the RNAlater®. Because of the small size, the whole animal was used. The elution step was done twice (once with 70µl and the second time with 40µl) to get a higher DNA concentration.

3.1.3: PCR amplifying

As molecular markers mitochondrial 16S rRNA (480 bp) and nuclear ITS1, 5.8S and ITS2 (combined 990 bp long) and 18S (1235 bp) were used (Table 3). Both primers (16S and ITS) have previously been used for phylogenetic studies (Nygren et al., 2009). Unfortunately the subunit 1 of the mitochondrial cytochrome oxydase (CO1) did not work with *Sphaerosyllis sp.*, although specially designed primers were made.

18S was used to verify the relationship of *Sphaerosyllis sp* with other syllids and with *S. hystrix* in particular.

Table 3: Primers used in this study

Gen-Region	Primer	Sequence (5'to 3')
16S	16S2_F	CCG GTC TGA CTC ARA TCA TGT A
	16S2_R	CGC CTG TTT AHC AAA AAC AT
ITS	ITS_F	GAA TTG CAG GAC ACA TTG AAC
	ITS_R	ATG CTT AAA TTC AGC GGG T
18S	600f	GCA AGT CTG GTG CCA GCA GCC
	NS8	ATG ATC CTT CCG CAG GTT CAC

For the PCR reaction, 30 µl were used, consisting of 2µl of DNA and 28 µl of a master solution mix (the contents are listed in Table 4). The PCR was carried out in the thermocyclers "Mastercycler" (Eppendorf) and "T Professional Standard Thermocycler" (Biometra). Table 5 lists the PCR protocols.

Table 4: PCR reaction reagents.

	1
H2O	20,25 µl
10x Dream Taq Buffer	2,5 µl
dNTPs (2mM)	2,5 µl
MgCl (25mM)	1,4 µl
BSA 100x	0,5 µl
Primer F (100pM)	0,3 µl
Primer R	0,3 µl
Taq (5u/µl)	0,25 µl
DNA	2 µl

Table 5: PCR-protocols

16S			
		Temperatur	Duration
	Initial denaturation	94°C	2 min
36 Cycles	Denaturation	94°C	30 sec
	Annealing	47°C	30 sec
	Extension	72°C	90 sec
	Final extension	72°C	10 min
	Storage	08°C	∞
ITS			
	Initial denaturation	94°C	4 min
45 Cycles	Denaturation	94°C	30 sec
	Annealing	48°C	30 sec
	Extension	72°C	60 sec
	Final extension	72°C	8 min
	Storage	08°C	∞
18s			
	Initial denaturation	94°C	2 min
35 Cycles	Denaturation	94°C	30 sec
	Annealing	50°C	30 sec
	Extension	72°C	45 sec
	Final extension	72°C	7 min
	Storage	08°C	∞

Table 6: Sequencing protocol

Sequencing			
		Temperature	Duration
25 Cycles	Denaturation	94°C	20 sec
	Annealing	48°C	10 sec
	Extension	60°C	4 min
	Storage	08°C	∞

3 mg of 1% agarose-powder (PeqGOLD) and 30 µl of 1x TAE-buffer were mixed, heated and filled in a tray. After the gel had dried it was covered with 1x TAE-buffer, 5 µl of the PCR product, 2 µl of DNA Loading Dye (6XMassRuler, Thermo Science) and 3 µl of SYBR Green mixed and loaded into the gel pockets together with a DNA ladder (MassRuler DNA Ladder, Low Range (50-1500 base pairs)). Gels were running for 30 minutes at 100 volt and checked with a transilluminator (PeqLab) connected to a monitor (RMB92 Rainbow CCTV) and a Mitsubishi P93 printer.

The PCR-products where purified with the peqGOLD Cycle-Pure Kit (PEQLAB), again following the manufacture's protocol.

3.1.4: Sequencing

For the sequencing, 10 µl reactions were made, consisting of 1 µl Big Dye (v.3.1), 1 µl Primer (10 pM), 1 to 2 µl of DNA, depending on the intensity of the gel electrophoresis picture, and 7 or 6 µl of ddH_2O depending on the DNA amount used. Condition for the sequencing reaction can be seen in Table 6. Sequences were read with an ABI 3130x1 capillary sequencer.

3.1.5: Alignment

Sequences were controlled, with the help of "NCBI Nucleotid BLAST search" (http://blast.ncbi.nlm.nih.gov/Blast.cgi), FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and GeneDoc v.2.7 (Nicholas & Nicholas, 1997). Mafft (Kazutaka & Hiroyuki, 2010) was used for a computer aided alignment that was checked and corrected by hand in GeneDoc. With the help of ClustalX (Thompson et al., 1997) a nexus- file, for the four

different data-sets, was created, one consisting of the 16S data, the second of the ITS data and a third combined data-set and the fourth for the 18S data-set.

To test the relationship between *Sphaerosyllis* specimens additional sequences were downloaded from NCBI. For the 16S analysis all the available 16S sequences from this study were used as from Aguado's studies (2007 (EF), 2012 (JF)) *Sphaerosyllis sp.* (EF123801), *S. austriaca* (EF123799), *S. boeroi* (EF123800), *S. densopapillata* (JF903718.1), *Prosphaerosyllis sp.*(EF123797)*, P. battiri* (JF903711.1), *P. magnoculata* (JF903712.1) and *P. multipapillata* (JF903713.1). For the 18S analysis AI31, AI32, AK31, AK32, AK33 and AK34 from this study were used and to test the relationship with *S. hystrix* the following sequences were used, again from Aguado 2007 (EF) and Nygren & Sundberg 2003 (AF), *Sphaerosyllis sp.* (EF123853), *S. austriaca* (EF123884), *S. boeroi* (EF123856) and two *S. hystrix* (EF123880, AF474288) and *Syllides convolutus* (EF123829-30) and *Syllis vivipara* (EF123848-9).

The following methods were applied for 16S, ITS and combined data-sets.

3.2: Population genetics and genealogical analyses

3.2.1: Distance method

The distance method counts the differences between sequences. In PAUP* v.4.0b10 (Swofford 2003) Neighbour-Joining (NJ) trees from all three data sets were computed using p-distances (uncorrected number of changes between two sequences). For the 18S data-set only Neighbour-Joining (NJ) trees were computed. In all PAUP* analyses the data-type is DNA, and gaps are treated as missing. The resulting p-distance matrix was imported into SPSS v.21 (IBM Corp.). The frequency distribution was calculated and visualized as a bar graph.

The haplotypes where identified using also this method. It was verified via Alter (http://sing.ei.uvigo.es/ALTER/). For all the other methods only the unique haplotypes were used for the analyses.

3.2.2: Substitutions-models

For Maximum-Likelihood and Bayesian-Inference, the optimal substitution model was determined with Modeltest v.3.7 (Posada & Crandall, 1998). Here the “Likelihood Ratio Test” (LRT) and “Akaike Information Criterion” (AIC) are included to determine the optimal model of evolution (Tabel 9). The model with the highest Likelihood score was used.

3.2.3: Maximum-Likelihood

The statistical method of maximum likelihood (ML) chooses amongst hypotheses by selecting the one which maximizes the likelihood that is, which renders the data the most plausible and in the context of molecular phylogenetics, a model of nucleotide or amino acid replacement permits the calculation of the likelihood for any possible combination of tree topology and branch lengths (Whelan, Liò & Goldman, 2001).

For the bootstrap analysis a heuristic search option, 5.000 bootstrap replicates and 10 random addition sequences were used and with stepwise addition the starting trees were calculated. Also for the parsimony analyses heuristic search option and stepwise addition was used with 5.000 replicates. Tree-bisection-reconnection (TBR) was used for the rearrangement of the trees. For a starting tree in the maximum-likelihood analysis, a parsimony consensus tree and the estimated substitution model was used. All calculations were made with PAUP* v.4.0b10.

3.2.4: Bayesian Inference

The Bayesian method creates a genealogical tree that is based upon posterior probability (Huelsenbeck & Ronquist, 2001) using the Bayes therom. It returns a tree estimate and a measure of support for the branches of the tree (Holder & Lewis, 2003).

This analysis was made in Mr. Bayes v.3.1.2 (Huelsenbeck & Ronquist, 2001). Here the Markov chain Monte Carlo is used to optimize the model. The run parameters were 2x4 chains for 10^6 and sampling frequencies was set to 100 and burn in to 300. The burn in was determined by the log likelihood curve manually. Also the estimated substitution model was used in this analysis.

3.2.5: TCS - Statistical Parsimony

TCS collapses the sequences into haplotypes and then calculates their frequencies. With these frequencies the haplotype out-group probabilities are created. After pairwise comparisons of all haplotypes, an absolute distance matrix is calculated. The probability of parsimony is calculated for pairwise differences until the probability exceeds 0.95 (Templeton et al., 1992). The number of mutational differences associated with the probability just before this 95% cut-off is then the maximum number of mutational connections between pairs of sequences justified by the "parsimony" criterion. These justified connections are then made resulting in a 95% set of plausible solutions. The program outputs the sequences, the pairwise absolute distance matrix, probabilities of parsimony for mutational steps just beyond the 95% cut-off, a test listing of connections made and missing intermediates generated (Clement, Posada & Crandall, 2000).

For TCS v.1.21 (Clement et al., 2000) all sequences were used, because it collapses the sequences to haplotypes and calculates their frequencies and thereby infers the maximum number of mutational changes. Cutoff was set to 95 and gaps are treated as missing.

3.2.6: Arlequin – Standard AMOVA

Arlequin v.3.0 is a software package integrating several basic and advanced methods for population genetics data analysis, like the computation of standard genetic diversity indices, the estimation of allele and haplotype frequencies (Excoffier et al., 2005). The standard AMOVA (Analysis of Molecular Variance) program uses information from the haplotypes and from their frequencies to calculate the population's indices from genetic structures. (Excoffier et al., 1992). 15.000 permutations, 5.000.000 steps in the Markov chain and pairwise differences were used in this analysis.

3.2.7: Beast

BEAST provides models for DNA and protein sequence evolution, highly parametric coalescent analysis, relaxed clock phylogenetics, non-contemporaneous sequence data, statistical alignment and a wide range of options for prior distributions (Drummond and Rambaut, 2007). Prior to the Beast analysis, the saturation of substitutions was assessed to check the validity of a molecular clock analysis (Wilke et al., 2009). Patristic distances are calculated from tree branch lengths describe the amount of genetic change represented by a tree and are commonly compared with other measures of mutation to investigate the substitutional processes or the goodness of fit of a tree to the raw data (Fourment & Gibbs, 2006) were compared to absolute distances (absolute number of nucleotide differences between taxa as the distance measure). These distances were calculated in PAUP* v.4.0b10 and compared to each other in SPSS v.21. To double check, DAMBE (Xia, 2013) was used to check saturation level. The sequences with all the haplotypes were used in the BEAST v.1.7.5 coalescent analysis. In BEAUTi the xml file for BEAST was created. The model used for ITS was HKY, the starting tree was an UPGMA tree, 10 million length of chain were appointed and a strict clock was chosen with a rate of 0.3544 sequence divergence pro million years obtained from Nygren (et al., 2009). This was done four times like in a Mr. Bayes analysis and afterwards was run in BEAST. With the associated programs LogCombiner v1.7.5 the resulting log and tree files were merged and then the log files were analyzed in Tracer v1.5 and the tree files were combined in TreeAnnotator v1.7.5, burn in was set to 250.

3.2.8: Visualization

The trees were viewed using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/), the TCS network was saved as PostScript and converted to pdf. Trees and networks were edited in Photoshop (1990-2010 Adobe Systems Incorporated) or CorelDRAW X5 (Corel Corporation 2010). Distance matrixes where visualized with SPSS v. 21.

4: Results

4.1: Data-set information

The 16S data-set consist of 42 sequences and is 369 base pairs (BP) long. Here only the reverse primer worked for most of the Italian specimens and therefore only 369 base pairs were usable. From these 42 sequences 23 belong to the “Croatian Clade” and consist of 5 haplotypes and 19 to the “Italian Clade” with 4 haplotypes. ITS has 41 sequences and is 967 BP long. 19, divided into 13 haplotypes, come from Croatia and 22, again collected into 19 haplotypes, com from the Italian west coast. The combined data-set combines 30 sequences with a length of 1336 BP. Here are 16 from Croatia, combined to 13 haplotypes, and 14 from Italy, all unique haplotypes. In Table 7 number of sequence an there length, haplotypes, polymorphic sites and the nucleotide composition are shown. For all the analysis only unique haplotypes were used. Haplotypes and excluded sequences are shown in Table 8.

The 18S data-set consist of 6 sequences and is 1235 base pairs long.

Table 7: Information of the three data-sets, number of sequences, base pairs and haplotypes

	16S data-set		ITS data-set		Comb data-set	
	Croatia	Italy	Croatia	Italy	Croatia	Italy
N° sequences	24	19	19	22	16	14
N° haplotypes	5	4	13	19	13	14
N° base pairs	542	519	894	952	1516	1516
N° usable loci	369	369	860	860	1336	1336
N° polym. Sites	74	74	107	107	166	166
Composition						
C	14.04%	13.39%	24.07%	23.24%	21.17%	20.50%
T	35.17%	33.28%	29.77%	29.59%	31.44%	30.69%
A	30.17%	30.85%	19.18%	20.98%	22.28%	23.62%
G	20.63%	22.48%	26.98%	26.18%	25.11%	25.19%

.

Table 8: Different haplotypes from each data-set

16S	
	K01= K03, 04, 05, 06, 07, 09, 11, 12, 16, 18, 20, 25, 27, 28, 29 I01= I02, 04, 09, 10, 11, 13, 14, 16, 17, 18, 21, 25, 29, 31 I12= I20
ITS	
	K10= K12, 15, 22, 25, 27 K11= K16 I06= I20, 26 I08= I28
Combined	
	K11= K16 K12= K25, 27

The combined data-set consists of K02, 03, 05, 06, 07, 10, 11, 12, 13, 15, 16, 18, 24, 25, 27, 28; I01, 02, 04, 07, 09, 10, 11, 12, 13, 14, 18, 20, 21, 29.

In the 16S alignment K01, 04, 09, 17, 20, 23 and 29; I16, 17, 25, 31 and 32 are also available and in the ITS it is K08, 19, and 22; I03, 05, 06, 08, 15, 24, 26, 28.

In every analysis, there is a clear separation between the two geographically separated sampling spots. In Table 9 the different substitution models for every analysis are listed, in Table 10-12 you can see distances for all three data-sets.

Table 9 Substitution models used for the analysis

Gene	Model
ITS	HKY+I+G
Comb	TVM+I+G
16S	TrN+G

Table 10: 16S uncorrected p distances, - stands for zero

16S	I01	I02	I04	I07	I09	I10	I11	I12	I13	I14	I16	I17	I18	I20	I21	I25	I29	I31	I32	K01	K02	K03	K04	K05	K06	K07	K09	K10	K11	K12	K13	K15	K16	K17	K18	K20	K23	K24	K25	K27	K28	K29
I01																																									
I02	-																																									
I04	-	-																																								
I07	0,011	0,011	0,011																																							
I09	-	-	-	0,011																																						
I10	-	-	-	0,011	-																																					
I11	-	-	-	0,011	-	-																																				
I12	0,003	0,003	0,003	0,014	0,003	0,003	0,003																																			
I13	-	-	-	0,011	-	-	-	0,003																																		
I14	-	-	-	0,011	-	-	-	0,003	-																																	
I16	-	-	-	0,011	-	-	-	0,003	-	-																																
I17	-	-	-	0,011	-	-	-	0,003	-	-	-																															
I18	-	-	-	0,011	-	-	-	0,003	-	-	-	-																														
I20	0,003	0,003	0,003	0,014	0,003	0,003	0,003	-	0,003	0,003	0,003	0,003	0,003																													
I21	-	-	-	0,011	-	-	-	0,003	-	-	-	-	-	0,003																													
I25	-	-	-	0,011	-	-	-	0,003	-	-	-	-	-	0,003	-																												
I29	-	-	-	0,011	-	-	-	0,003	-	-	-	-	-	0,003	-	-																											
I31	-	-	-	0,011	-	-	-	0,003	-	-	-	-	-	0,003	-	-	-																									
I32	0,006	0,006	0,006	0,017	0,006	0,006	0,006	0,009	0,006	0,006	0,006	0,006	0,006	0,009	0,006	0,006	0,006	0,006																								
K01	0,123	0,123	0,123	0,129	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,122	0,123	0,123	0,123	0,123	0,129																							
K02	0,135	0,135	0,135	0,141	0,135	0,135	0,135	0,135	0,135	0,135	0,135	0,135	0,135	0,134	0,135	0,135	0,135	0,135	0,141	0,043																						
K03	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043																					
K04	0,125	0,125	0,125	0,131	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,131	-	0,043	-																				
K05	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-																			
K06	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-																		
K07	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-																	
K09	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-	-																
K10	0,124	0,124	0,124	0,130	0,124	0,124	0,124	0,124	0,124	0,124	0,124	0,124	0,124	0,123	0,124	0,124	0,124	0,124	0,130	0,003	0,046	0,003	0,003	0,003	0,003	0,003	0,003														
K11	0,123	0,123	0,123	0,129	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,129	-	0,043	-	-	-	-	-	-	0,003	-												
K12	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-	-	-	0,003	-												
K13	0,111	0,111	0,111	0,120	0,111	0,111	0,111	0,112	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,117	0,098	0,112	0,098	0,098	0,100	0,098	0,098	0,098	0,099	0,098	0,098										
K15	0,126	0,126	0,126	0,132	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,126	0,132	0,003	0,046	0,003	0,003	0,003	0,003	0,003	0,003	0,006	0,003	0,003	0,098									
K16	0,123	0,123	0,123	0,129	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,122	0,123	0,123	0,123	0,123	0,129	-	0,043	-	-	-	-	-	-	0,003	-	-	0,098	0,003								
K17	0,111	0,111	0,111	0,120	0,111	0,111	0,111	0,112	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,117	0,098	0,112	0,098	0,098	0,100	0,098	0,098	0,098	0,099	0,098	0,098	-	0,098	0,098							
K18	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-	-	-	0,003	-	-	0,100	0,003	0,098	0,100						
K20	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-	-	-	0,003	-	-	0,100	0,003	0,098	0,100	0,100					
K23	0,111	0,111	0,111	0,120	0,111	0,111	0,111	0,112	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,117	0,098	0,112	0,098	0,098	0,100	0,098	0,098	0,098	0,099	0,098	0,098	-	0,098	0,098	0,100	0,100	-				
K24	0,111	0,111	0,111	0,120	0,111	0,111	0,111	0,112	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,111	0,117	0,097	0,112	0,097	0,097	0,100	0,097	0,097	0,097	0,098	0,098	0,097	-	0,098	0,097	-	0,100	0,100	-			
K25	0,123	0,123	0,123	0,129	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,123	0,129	-	0,043	-	-	-	-	-	-	0,003	-	-	0,098	0,003	0,098	0,098	-	-	0,098	0,098		
K27	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-	-	-	0,003	-	-	0,098	0,003	0,098	0,098	-	-	0,098	0,097	-	
K28	0,120	0,120	0,120	0,129	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,120	0,126	-	0,043	-	-	-	-	-	-	0,003	-	-	0,095	0,003	0,095	0,095	-	-	0,095	0,095	-	-
K29	0,122	0,122	0,122	0,128	0,122	0,122	0,122	0,123	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,122	0,128	-	0,043	-	-	-	-	-	-	0,003	-	-	0,098	0,003	0,098	0,098	-	-	0,098	0,097	-	-

Table 11: ITS uncorrected p distances, - stands for zero

ITS	K02	K03	K05	K06	K07	K08	K10	K11	K12	K13	K15	K16	K18	K19	K22	K24	K25	K27	K28	I01	I02	I03	I04	I05	I06	I07	I08	I09	I10	I11	I12	I13	I14	I15	I18	I20	I21	I24	I28	I26	I29
K02																																								
K03	0,027																																								
K05	0,024	0,011																																							
K06	0,022	0,012	0,008																																						
K07	0,025	0,014	0,011	0,008																																					
K08	0,028	0,021	0,019	0,014	0,018																																				
K10	0,019	0,008	0,006	0,005	0,007	0,013																																			
K11	0,020	0,009	0,007	0,003	0,007	0,012	0,001																																		
K12	0,019	0,008	0,006	0,005	0,007	0,013	-	0,001																																	
K13	0,023	0,025	0,023	0,022	0,023	0,026	0,017	0,018	0,017																																
K15	0,019	0,008	0,006	0,005	0,007	0,013	-	0,001	-	0,017																															
K16	0,020	0,009	0,007	0,002	0,007	0,012	0,001	-	0,001	0,018	0,001																														
K18	0,024	0,011	0,012	0,013	0,007	0,019	0,009	0,009	0,008	0,024	0,009	0,009																													
K19	0,020	0,009	0,007	0,005	0,007	0,013	0,001	0,001	0,001	0,018	0,001	0,001	0,008																												
K22	0,019	0,008	0,006	0,005	0,007	0,013	-	0,001	-	0,017	-	0,001	0,009	0,001																											
K24	0,021	0,018	0,016	0,012	0,012	0,018	0,010	0,009	0,010	0,015	0,010	0,009	0,014	0,010	0,010																										
K25	0,019	0,008	0,006	0,005	0,007	0,013	-	0,001	-	0,017	-	0,001	0,009	0,001	-	0,010																									
K27	0,019	0,008	0,007	0,005	0,007	0,013	-	0,001	-	0,017	-	0,001	0,009	0,001	-	0,010	-																								
K28	0,017	0,011	0,008	0,008	0,008	0,014	0,003	0,005	0,003	0,018	0,003	0,005	0,013	0,005	0,003	0,011	0,003	0,003																					
I01	0,038	0,040	0,034	0,034	0,031	0,041	0,031	0,030	0,032	0,035	0,031	0,030	0,034	0,031	0,031	0,026	0,031	0,031	0,031																				
I02	0,038	0,040	0,034	0,034	0,030	0,041	0,032	0,030	0,032	0,033	0,032	0,031	0,034	0,032	0,032	0,026	0,032	0,032	0,032	0,008																			
I03	0,041	0,042	0,038	0,037	0,030	0,044	0,035	0,034	0,035	0,036	0,035	0,034	0,037	0,035	0,035	0,032	0,035	0,035	0,035	0,008	0,004																		
I04	0,037	0,039	0,033	0,033	0,030	0,040	0,030	0,029	0,031	0,034	0,030	0,029	0,033	0,030	0,030	0,025	0,030	0,030	0,030	0,005	0,004	0,004																	
I05	0,038	0,040	0,034	0,034	0,031	0,041	0,032	0,030	0,032	0,035	0,032	0,031	0,034	0,032	0,032	0,026	0,032	0,032	0,032	0,004	0,003	0,005	0,003																
I06	0,041	0,042	0,038	0,037	0,031	0,044	0,035	0,034	0,035	0,036	0,035	0,034	0,037	0,035	0,035	0,029	0,035	0,035	0,035	0,008	0,002	0,002	0,002	0,003															
I07	0,044	0,044	0,040	0,039	0,034	0,047	0,037	0,036	0,037	0,041	0,037	0,036	0,040	0,037	0,037	0,032	0,037	0,037	0,037	0,008	0,011	0,011	0,007	0,010	0,011														
I08	0,038	0,040	0,034	0,034	0,031	0,041	0,032	0,030	0,032	0,035	0,032	0,031	0,034	0,032	0,032	0,026	0,032	0,032	0,032	0,006	0,003	0,005	0,001	0,002	0,001	0,012													
I09	0,037	0,039	0,035	0,033	0,030	0,042	0,031	0,030	0,031	0,034	0,031	0,030	0,033	0,031	0,031	0,025	0,031	0,031	0,031	0,009	0,005	0,008	0,001	0,004	0,003	0,010	0,004												
I10	0,045	0,045	0,041	0,041	0,034	0,047	0,038	0,037	0,038	0,042	0,038	0,037	0,041	0,038	0,038	0,033	0,038	0,038	0,038	0,004	0,009	0,008	0,007	0,005	0,007	0,008	0,008	0,010											
I11	0,038	0,040	0,034	0,030	0,030	0,042	0,032	0,030	0,032	0,035	0,032	0,031	0,034	0,032	0,032	0,025	0,032	0,032	0,032	0,005	0,005	0,008	0,006	0,005	0,007	0,007	0,005	0,006	0,008										
I12	0,043	0,044	0,040	0,039	0,035	0,047	0,037	0,036	0,037	0,041	0,037	0,036	0,040	0,037	0,037	0,032	0,037	0,037	0,037	0,006	0,008	0,010	0,002	0,003	0,004	0,012	0,004	0,005	0,009	0,010									
I13	0,038	0,040	0,034	0,034	0,031	0,041	0,031	0,030	0,032	0,035	0,031	0,030	0,034	0,031	0,031	0,026	0,031	0,031	0,031	0,001	0,004	0,003	0,003	0,001	0,003	0,007	0,002	0,004	0,003	0,004	0,003								
I14	0,040	0,042	0,037	0,036	0,033	0,044	0,034	0,033	0,034	0,038	0,034	0,033	0,036	0,034	0,034	0,028	0,034	0,034	0,034	0,010	0,006	0,006	0,004	0,005	0,004	0,013	0,003	0,005	0,011	0,008	0,008	0,005							
I15	0,039	0,041	0,035	0,035	0,031	0,042	0,033	0,031	0,033	0,036	0,033	0,032	0,035	0,033	0,033	0,027	0,033	0,033	0,033	0,005	0,008	0,006	0,004	0,003	0,004	0,012	0,004	0,007	0,008	0,008	0,005	0,001	0,006						
I18	0,040	0,042	0,037	0,036	0,034	0,044	0,034	0,033	0,034	0,037	0,034	0,033	0,036	0,034	0,034	0,028	0,034	0,034	0,034	0,011	0,009	0,010	0,003	0,007	0,005	0,014	0,008	0,007	0,012	0,010	0,009	0,007	0,008	0,009					
I20	0,040	0,041	0,037	0,036	0,030	0,043	0,034	0,033	0,034	0,035	0,034	0,033	0,036	0,034	0,034	0,028	0,034	0,034	0,034	0,004	0,001	0,003	0,002	0,002	-	0,009	0,004	0,004	0,005	0,004	0,003	0,002	0,003	0,004	0,007				
I21	0,038	0,040	0,034	0,034	0,031	0,041	0,032	0,030	0,032	0,035	0,032	0,030	0,034	0,032	0,032	0,026	0,032	0,032	0,031	0,002	0,002	0,005	0,003	0,004	0,005	0,007	-	0,007	0,004	0,003	0,003	0,002	0,003	0,003	0,004	0,001			
I24	0,039	0,040	0,035	0,035	0,030	0,042	0,033	0,032	0,033	0,036	0,033	0,032	0,035	0,033	0,033	0,027	0,033	0,033	0,033	0,006	0,003	0,006	0,001	0,002	0,001	0,011	0,001	0,003	0,007	0,005	0,004	0,002	0,003	0,004	0,008	0,003	0,004		
I28	0,037	0,039	0,033	0,033	0,030	0,040	0,030	0,029	0,030	0,034	0,030	0,029	0,033	0,030	0,030	0,025	0,030	0,030	0,030	0,002	0,002	0,003	0,001	0,001	0,001	0,007	-	0,002	0,004	0,003	0,003	0,002	0,003	0,003	0,004	0,001	0,001	-	
I26	0,040	0,041	0,037	0,036	0,030	0,043	0,034	0,033	0,034	0,035	0,034	0,033	0,036	0,034	0,034	0,028	0,034	0,034	0,034	0,007	0,002	0,004	0,002	0,003	-	0,011	0,003	0,003	0,009	0,007	0,008	0,003	0,004	0,005	0,008	0,001	0,005	0,002	0,001	
I29	0,038	0,041	0,036	0,035	0,033	0,042	0,033	0,032	0,033	0,036	0,033	0,031	0,035	0,033	0,033	0,027	0,033	0,033	0,033	0,009	0,009	0,015	0,008	0,009	0,010	0,014	0,012	0,013	0,010	0,008	0,015	0,008	0,009	0,012	0,014	0,009	0,007	0,012	0,006	0,012

Table 12: Combined uncorrected p distances, - stands for zero

Comb	K02	K03	K05	K06	K07	K10	K11	K12	K13	K15	K16	K18	K24	K25	K27	K28	I01	I02	I04	I07	I09	I10	I11	I12	I13	I14	I18	I20	I21	I29
K02																														
K03	0,031																													
K05	0,029	0,008																												
K06	0,028	0,008	0,006																											
K07	0,030	0,009	0,007	0,006																										
K10	0,027	0,007	0,005	0,004	0,006																									
K11	0,026	0,007	0,005	0,002	0,005	0,002																								
K12	0,026	0,006	0,004	0,003	0,005	0,001	0,001																							
K13	0,048	0,046	0,045	0,043	0,046	0,040	0,041	0,040																						
K15	0,026	0,007	0,005	0,004	0,006	0,002	0,002	0,001	0,040																					
K16	0,026	0,007	0,005	0,002	0,005	0,002	-	0,001	0,041	0,002																				
K18	0,030	0,008	0,008	0,009	0,005	0,007	0,007	0,006	0,045	0,007	0,007																			
K24	0,047	0,041	0,040	0,036	0,039	0,035	0,034	0,035	0,011	0,035	0,034	0,038																		
K25	0,026	0,006	0,004	0,003	0,005	0,001	0,001	-	0,040	0,001	0,001	0,007	0,035																	
K27	0,026	0,006	0,005	0,003	0,005	0,001	0,001	-	0,040	0,001	0,001	0,007	0,035	-																
K28	0,025	0,008	0,006	0,006	0,006	0,003	0,003	0,002	0,040	0,003	0,003	0,009	0,035	0,002	0,002															
I01	0,065	0,062	0,059	0,058	0,059	0,057	0,056	0,057	0,056	0,058	0,056	0,058	0,050	0,057	0,057	0,056														
I02	0,065	0,062	0,059	0,058	0,058	0,057	0,056	0,057	0,055	0,058	0,056	0,058	0,050	0,057	0,057	0,056	0,005													
I04	0,064	0,062	0,058	0,058	0,059	0,056	0,055	0,056	0,056	0,057	0,055	0,058	0,049	0,056	0,056	0,055	0,004	0,003												
I07	0,070	0,067	0,065	0,064	0,063	0,063	0,062	0,062	0,063	0,063	0,062	0,064	0,056	0,062	0,062	0,062	0,009	0,011	0,008											
I09	0,064	0,062	0,059	0,058	0,059	0,056	0,055	0,056	0,056	0,057	0,055	0,058	0,049	0,056	0,056	0,055	0,006	0,004	0,001	0,010										
I10	0,069	0,066	0,064	0,063	0,061	0,062	0,061	0,061	0,061	0,062	0,061	0,063	0,055	0,062	0,061	0,061	0,003	0,006	0,005	0,009	0,007									
I11	0,065	0,063	0,059	0,056	0,059	0,057	0,056	0,057	0,057	0,058	0,056	0,058	0,049	0,057	0,057	0,056	0,004	0,004	0,004	0,008	0,004	0,006								
I12	0,069	0,066	0,063	0,062	0,062	0,061	0,060	0,061	0,060	0,062	0,060	0,062	0,054	0,061	0,061	0,060	0,005	0,006	0,002	0,012	0,005	0,007	0,008							
I13	0,065	0,062	0,059	0,058	0,059	0,057	0,056	0,057	0,056	0,057	0,056	0,058	0,050	0,057	0,057	0,056	0,001	0,003	0,002	0,008	0,003	0,002	0,003	0,003						
I14	0,066	0,064	0,060	0,060	0,060	0,058	0,058	0,058	0,058	0,059	0,058	0,060	0,052	0,058	0,058	0,057	0,007	0,005	0,003	0,013	0,004	0,008	0,006	0,006	0,004					
I18	0,066	0,064	0,061	0,060	0,061	0,059	0,058	0,058	0,058	0,059	0,058	0,060	0,052	0,059	0,058	0,058	0,008	0,006	0,002	0,013	0,005	0,009	0,007	0,007	0,005	0,006				
I20	0,066	0,063	0,060	0,060	0,058	0,058	0,057	0,058	0,056	0,059	0,057	0,060	0,051	0,058	0,058	0,057	0,004	0,002	0,002	0,010	0,004	0,005	0,004	0,006	0,003	0,004	0,006			
I21	0,065	0,062	0,059	0,058	0,059	0,057	0,056	0,057	0,056	0,058	0,056	0,058	0,050	0,057	0,057	0,056	0,002	0,004	0,002	0,007	0,005	0,003	0,002	0,005	0,001	0,005	0,006	0,002		
I29	0,065	0,063	0,060	0,059	0,060	0,058	0,057	0,058	0,057	0,058	0,056	0,059	0,051	0,058	0,057	0,057	0,006	0,006	0,006	0,013	0,010	0,007	0,006	0,012	0,006	0,006	0,010	0,007	0,005	

4.2: Phylogenetic analysis

4.2.1.: 16S

42 Sequences with a length of 369 BP are in the 16S alignment, gaps included. 74 positions are variable and 42 are parsimony-uninformative. The Croatian population consists of 23 individuals and 5 unique haplotypes, the Italian population consist of 19 individuals and 4 unique haplotypes. In all the analysis, the calculated trees of ML, Bayesian analysis and Arlequin, are identical. As seen in Table 7 and 8, there are just few haplotypes available for the analysis and therefore only few haplotype groups remain. Figure 3 shows the unrooted Bayesian tree. It has high posterior probability (PP) values, the bootstrap (BS) values range from 99 to 100. In every analysis the "Italian Clade" is shown in red and it could not be properly resolved, and in the "Croatian Clade", shown in green, K13 was always between the two clades (Figure 3 and 4). The used substitution model was TrN+G. The TCS network shows the clades, but could not connect the different clades.

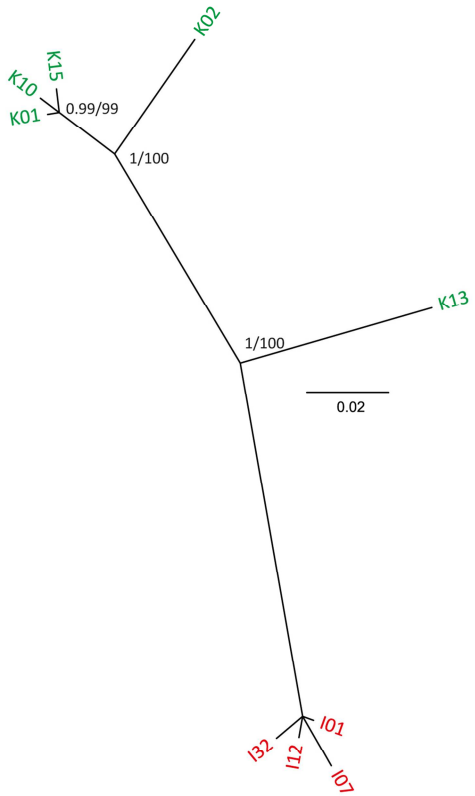

Figure 3: 16S Bayesian tree with PP /BS values, green is the Croatian clade, red the Italian clade.

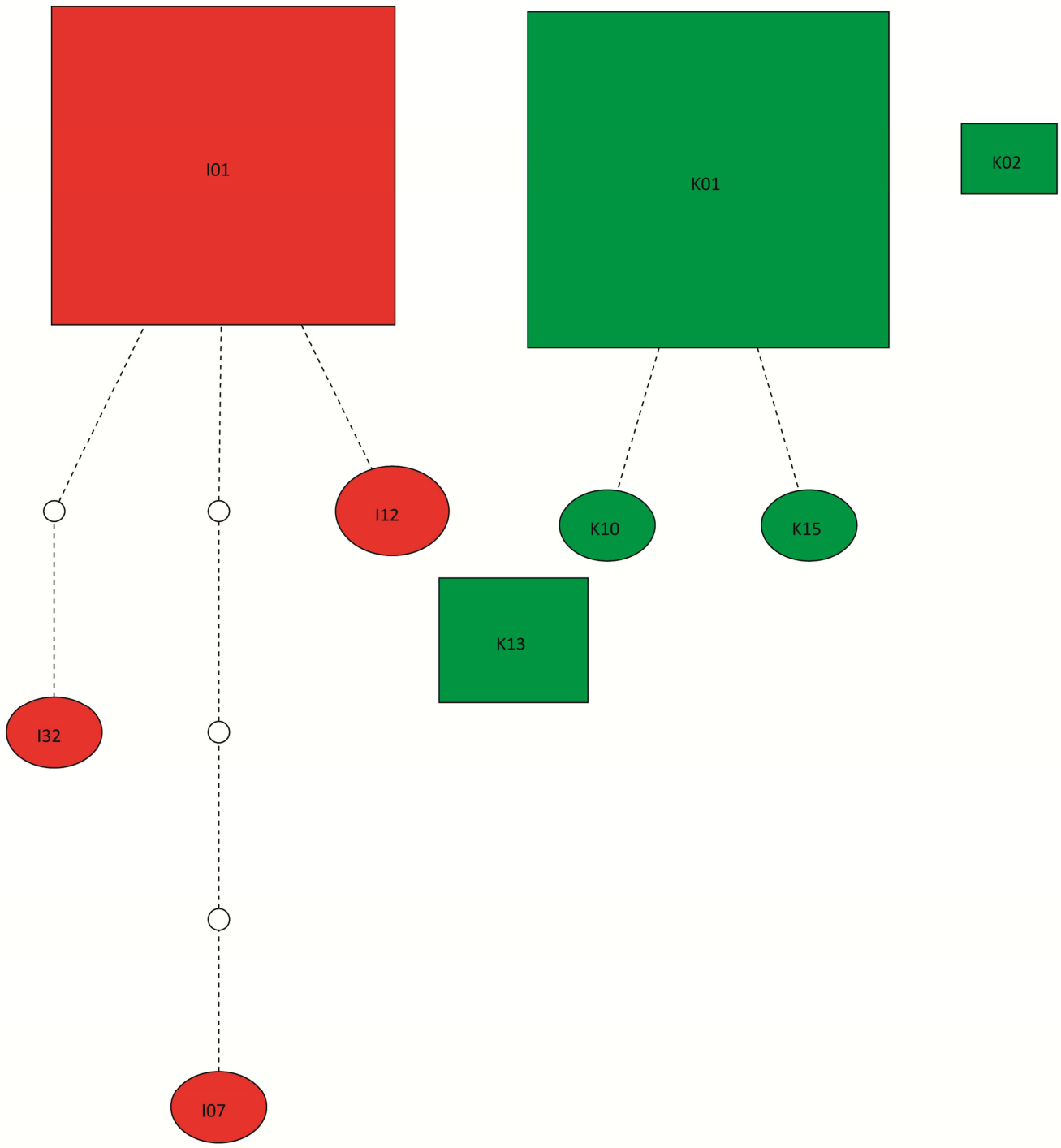

Figure 4 16S TCS network, red is the Italian clade, green the Croatian clade

4.2.2: ITS

The Italian Clade has two groups. The first clade consists of I01, I07, I10, I11, I13, I21 and I29, the second with the rest (Figure 5). In the TCS network (Figure 6) and the minimum spanning tree, the first group has I15 in it, but in all the PAUP* v.4.0b10 analysis it belongs to the second group. ITS alignment has 41 sequences and a length of 967 BP, 19, divided into 13 haplotypes, come from Croatia and 22 specimens come from the Italian west coast, consisting of 19 haplotypes. There are variable 107 positions and 50 parsimony-uninformative positions. The substitution model was HKY+I+G (Table 7-9). The bootstrap value for the "Italian Clade" is 100, PP is 1 and for the "Croatian Clade" the BS is 66, PP 0.88. In the TCS network the two clades could not be connected.

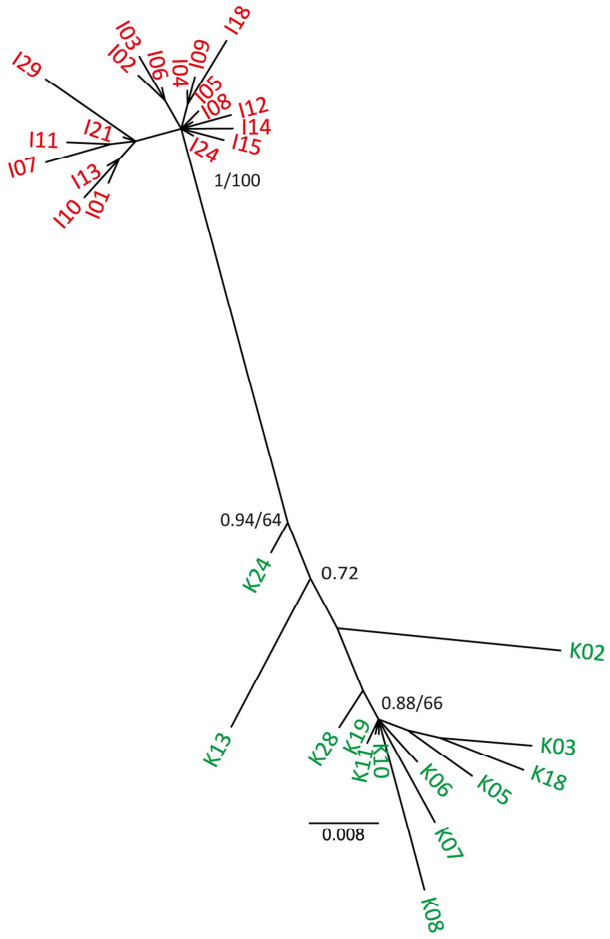

Figure 5: Bayesian tree from the ITS analysis with PP/BS values, green is the Croatian clade, red the Italian clade.

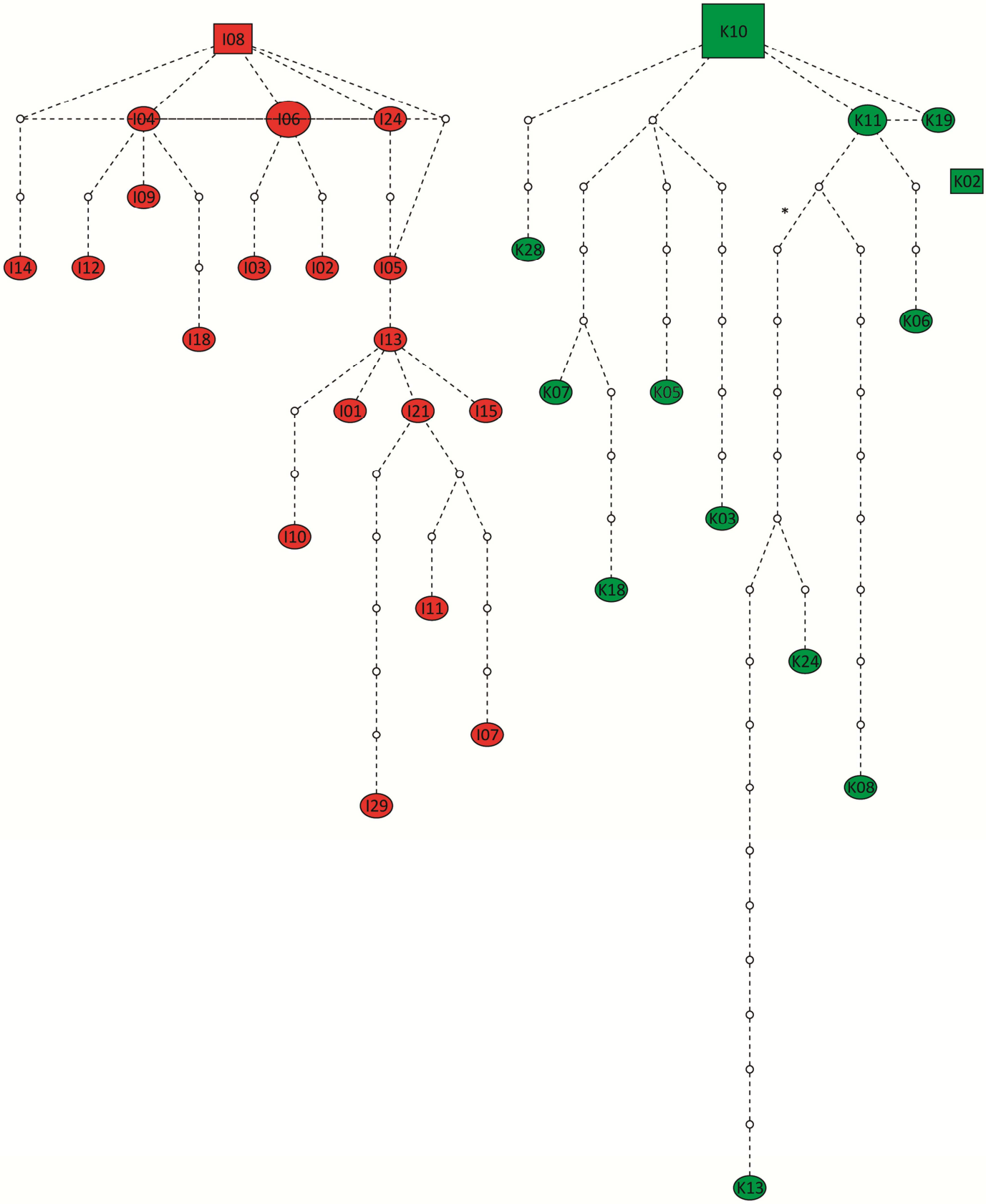

Figure 6: Network for ITS, green is the Croatian clade, red the Italian clade

4.2.3: Combined

The combined data-set consist of 30 sequences with a length of 1336 BP. 16 sequences are from Croatia, combined to 13 haplotypes, and 14 sequences are from Italy, all unique haplotypes There are variable 166 positions and 103 parsimony-uninformative positions The substitution model was TVM+I+G for 30 sequences (Tab. 7-9). The “Croatian & Italian Clades” are in the combined analysis similar to the ITS analysis, except for maximum likelihood, where I02 and I20 clusters into the first and not into the second group. The BS for Italy is 100, PP is 1 and the “Croatian Clade” has PP of 1 and BS of 100. The “Italian Clade” is connected in the TCS network where as the “Croatian clade” is not fully connected and splits into two networks.

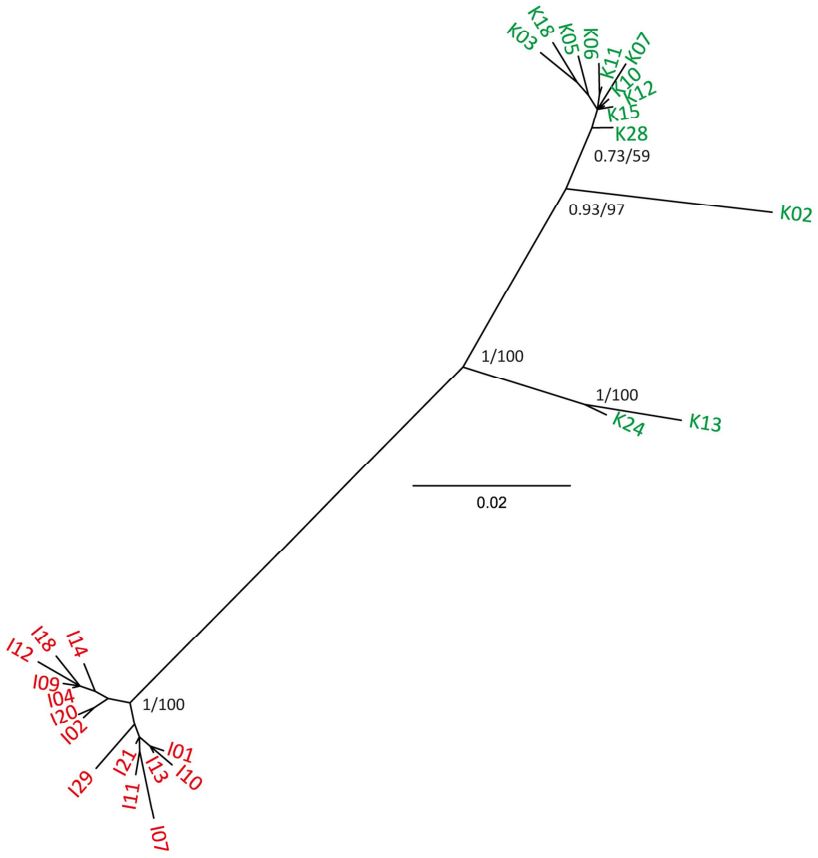

Figure 7: Bayesian combined tree with PP/BS values, green is the Croatian clade, red the Italian clade

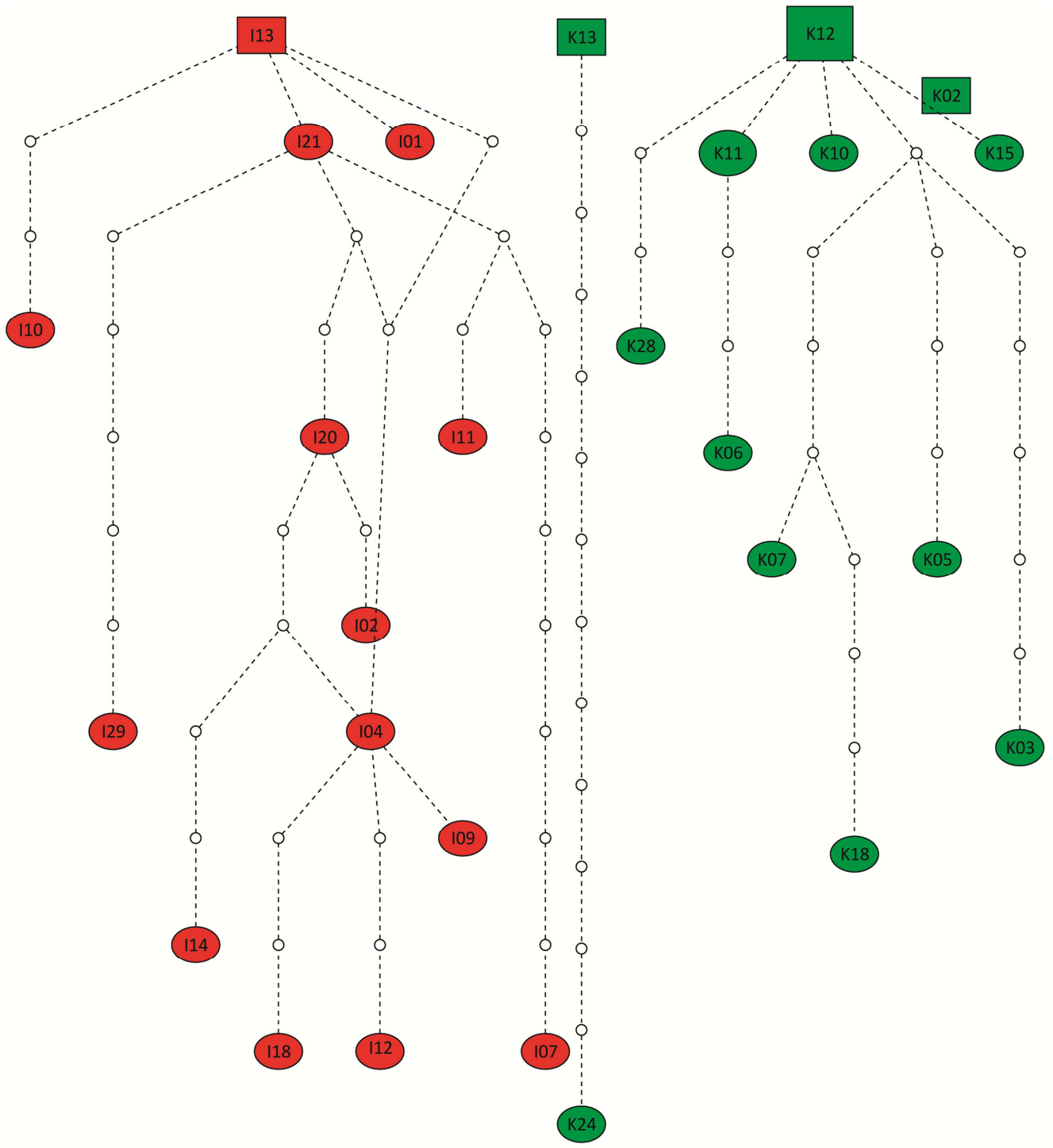

Figure 8: TCS combined network red is the Italian clade, green the Croatian clade

4.2.4: *Sphaerosyllis sp.* Relationship

To clarify if *Sphaerosyllis sp.* could be *S. hystrix*, because of similar appearance, 16S and 18S NJ analysis were made. The 16S analysis was made with all available sequences from this study and from NCBI (Fig.9). Unfortunately no sequences from *S. hystrix* were available and so it was tested if *Sphaerosyllis sp.* belongs to *Sphaerosyllis* or *Prosphaerosyllis*. It turned out, that the present *Sphaerosyllis sp.* clustered with *Sphaerosyllis sp*. from the study Aguado`s (et al., 2007 and 2012) and was collected in Port de la Selva, Spain. For *S. hystrix* only 18S and CO1 sequences were available and so an 18S data-set was made with 4 "Croatian Clade", 2 "Italian Clade" specimens, two *Sphaerosyllis hystrix*, the same *Sphaerosyllis sp.* from above and two other *Sphaerosyllis*. *Sphaerosyllis sp.* also clusters with *Sphaerosyllis sp.* from Aguado`s study and not with *S. hystrix.*

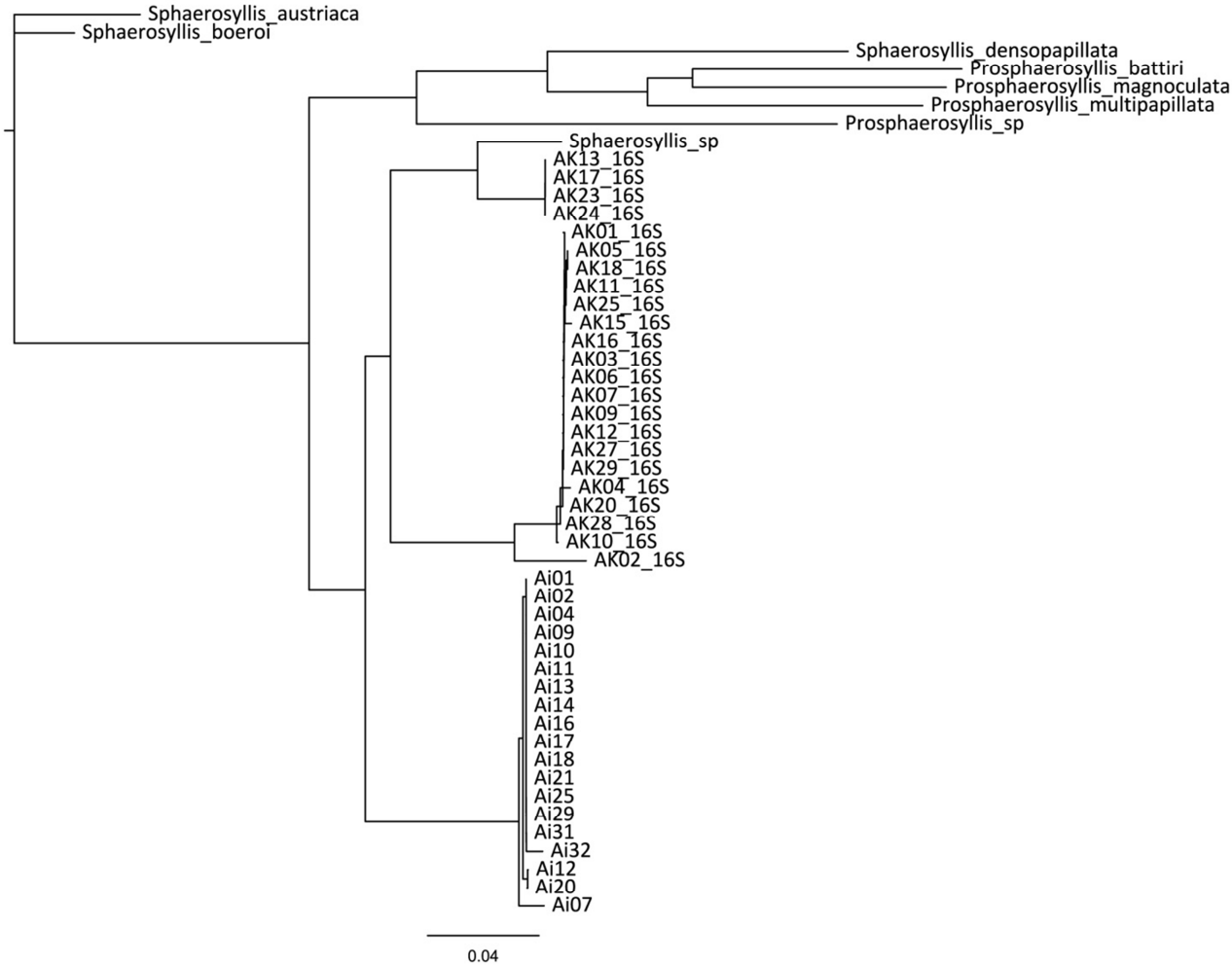

Figure 9: NJ tree for 16S data-set for different syllids and species from Aguado et al. (2007 & 2012) and the sequences obtained in this study.

4.3: Statistical analysis

The distance analysis data for all the three different data-set is shown in Figures 9 to 11 and in Tables 10 to 12. The Genetic distances within and among populations of the 16S data-set show the highest pairwise distances, ranging from 0% to 11.2% within populations, and among populations they range from 11% to 14.1% (Fig. 10).

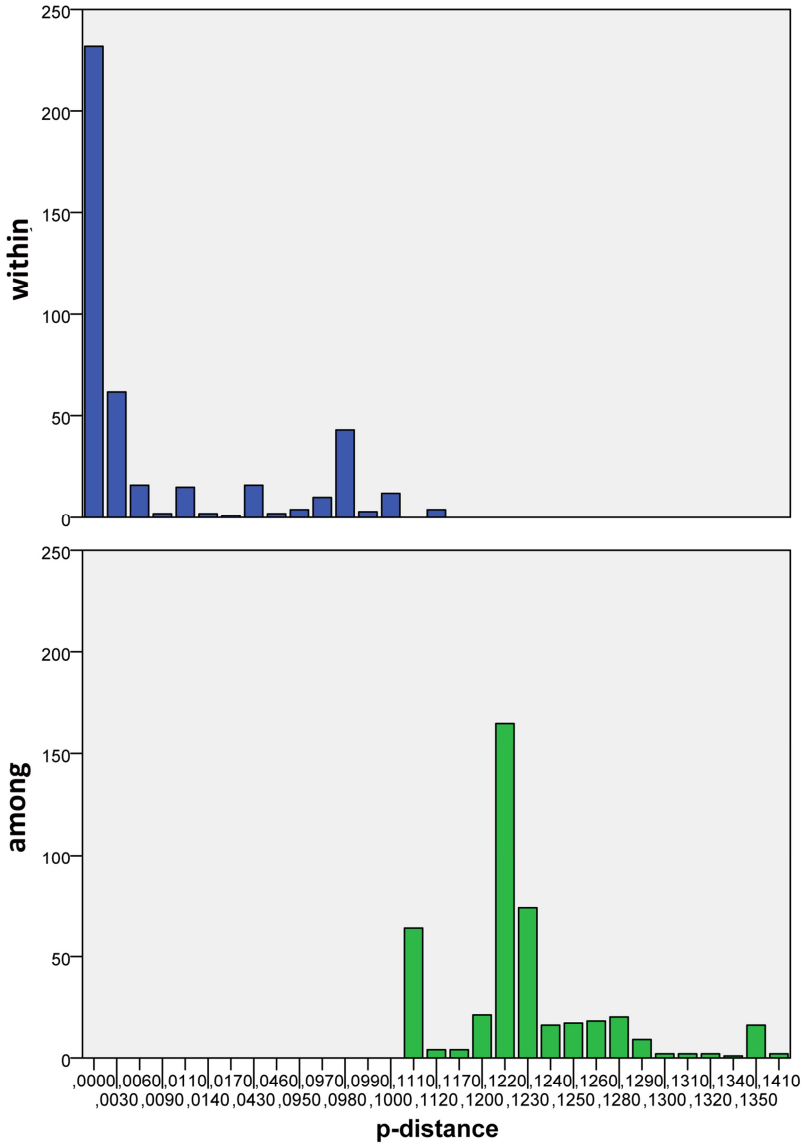

Figure 10: percentages of the uncorrected p-distances of the 16S data-set

For ITS the pairwise distances are much lower, the range from 0% to 2.8% within populations from 2.5% to 4.7% among populations.

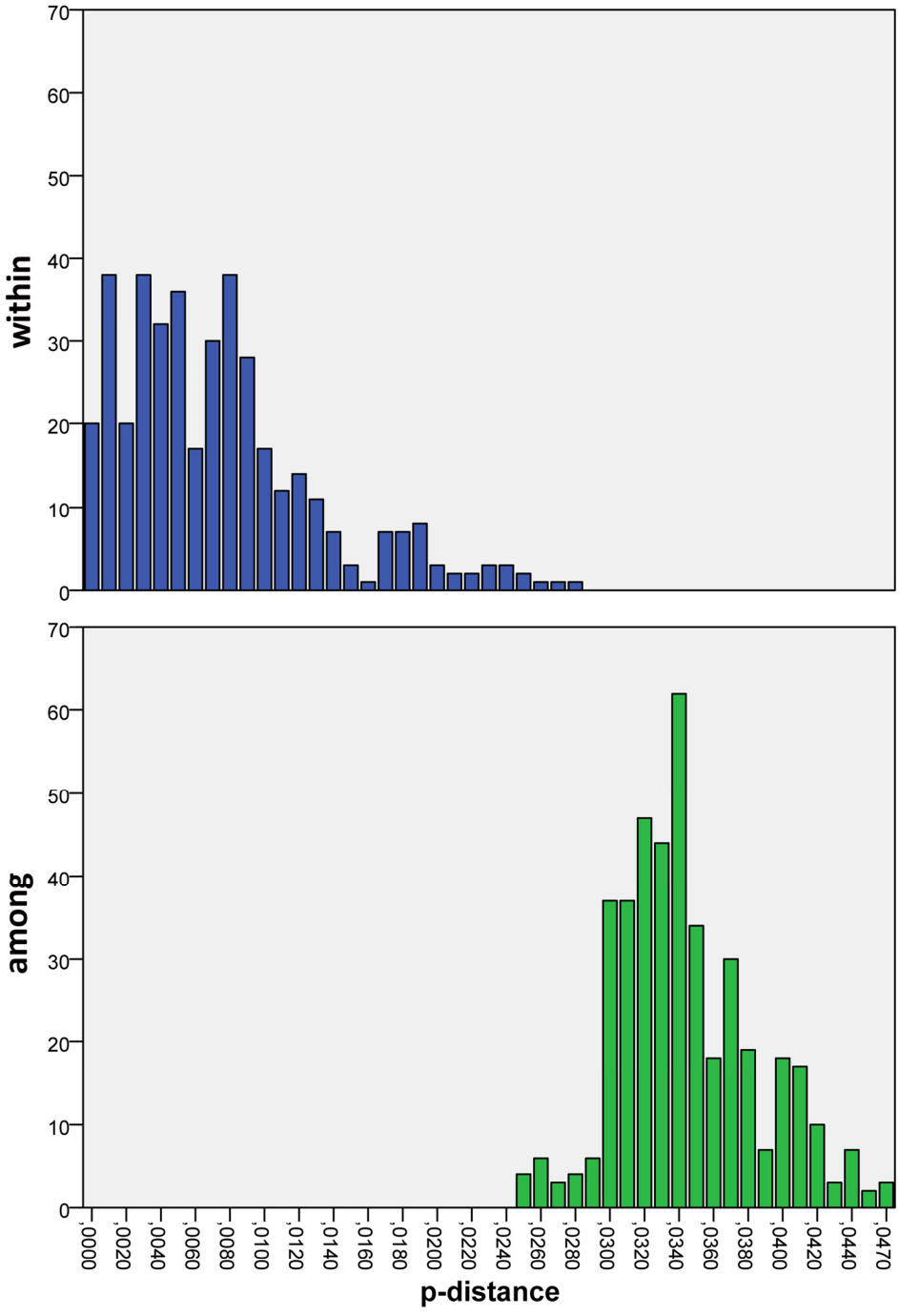

Figure 11: ITS uncorrected p-distances in percentage.

In the combined analysis the distances are a little bit higher than in the ITS alignment. Here they range from 0% to 4.8% within the populations and between the two populations from 4.8% to 7%. It's the only analysis where almost no overlap within and among populations. In 16S and ITS there was always an overlap between within and among populations. The explanation for this is, that the cause of the overlap is different in the 16S and the ITS analysis. In 16S K02 with K13, K17, K23 and K24 has the within population distance that causes the overlap, in ITS, K02 and K13 has the highest within distance with K03 and K08.

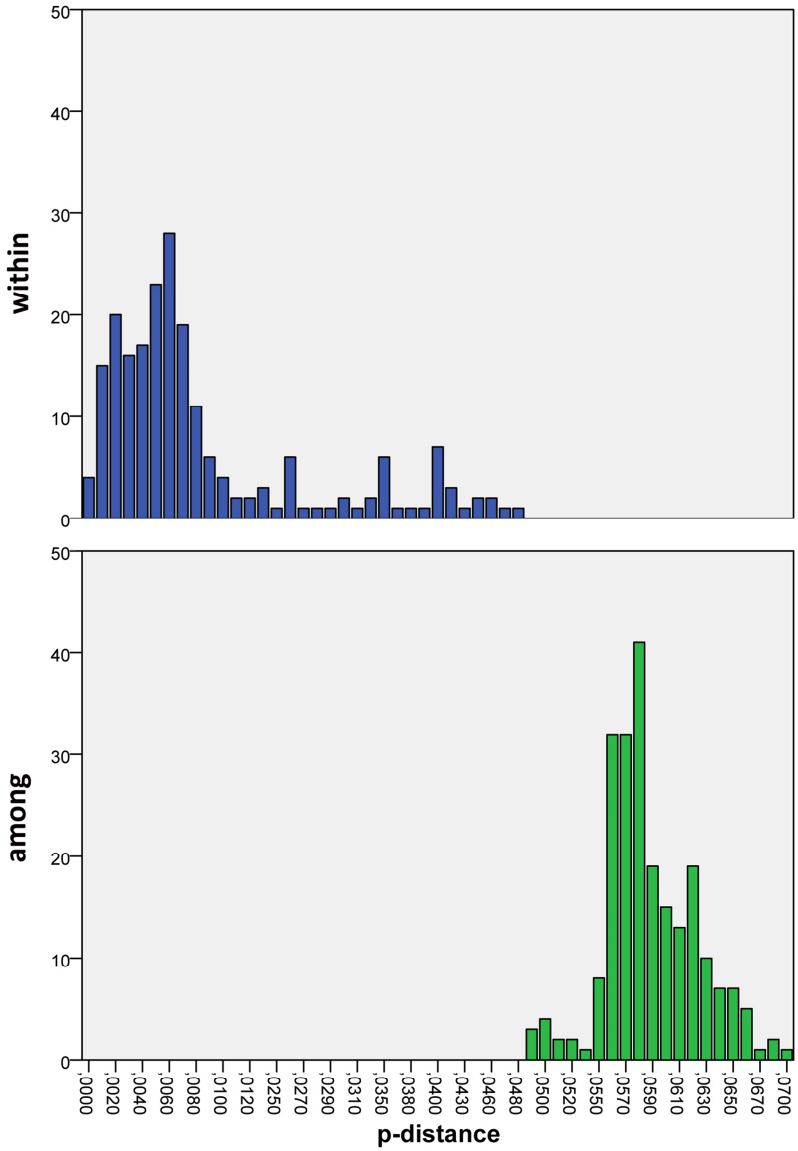

Figure 12: uncorrected p-distances of the combined data-set in percentage.

Table 13 shows the calculated F_{st} values from the Arlequin analysis. Migrants per generation were calculated using this formula $(Nm)_f=0.5*((1/F_{st})-1)$ (Wright, 1978). The resulting value was 0.4 migrants per generation, using an average F_{st} value of 0.83563.

Table 13: F_{st} values from Arlequin analysis

Data-set	average Fst	Croatia FST	Italy Fst
16S	0.92798	0.92546	0.93064
ITS	0.78308	0.78026	0.78552
Combined	0.79584	0.79133	0.80100

The variation among populations is high in contrast to the variation within populations (Table 14), which is 7.20% in 16S compared to 21.69% in ITS and 20.42% in the combined data-set.

Table 14: Sum of squares (SSD), variance components and percentage of variation within and among all data-sets

Data-set		SSD	Variance components	% of variation
16S	Among populations	585.573	29.92997 Va	92.80
	Within populations	85.939	2.32269 Vb	7.20
	Total	671.513	32.25266	
ITS	Among populations	885.490	42.84508 Va	78.31
	Within populations	462.876	11.86861 Vb	21.69
	Total	1.348.366	54.71369	
Combined	Among populations	1.051.412	69.21802 Va	79.58
	Within populations	497.188	17.75670 Vb	20.42
	Total	1.548.600	86.97472	

In Figure 13 and 14 the saturation of the ITS analysis are shown.

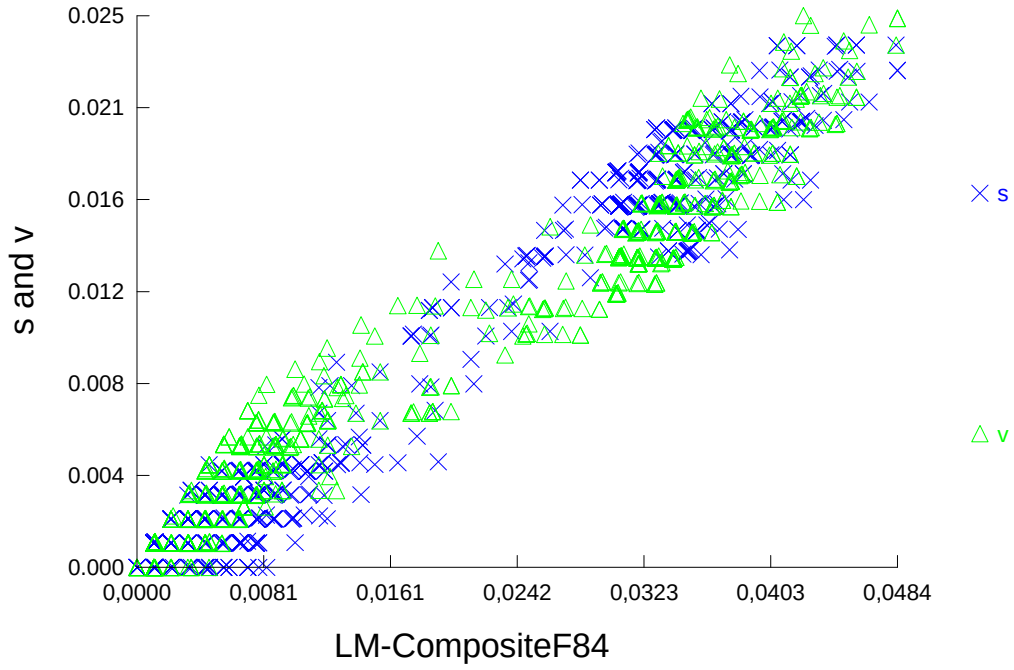

Figure 13: DAMBE plot of the transition (x) rate and transversion (Δ) rate against the *Felsenstein (F84)* distance for pairwise comparisons of the sequences

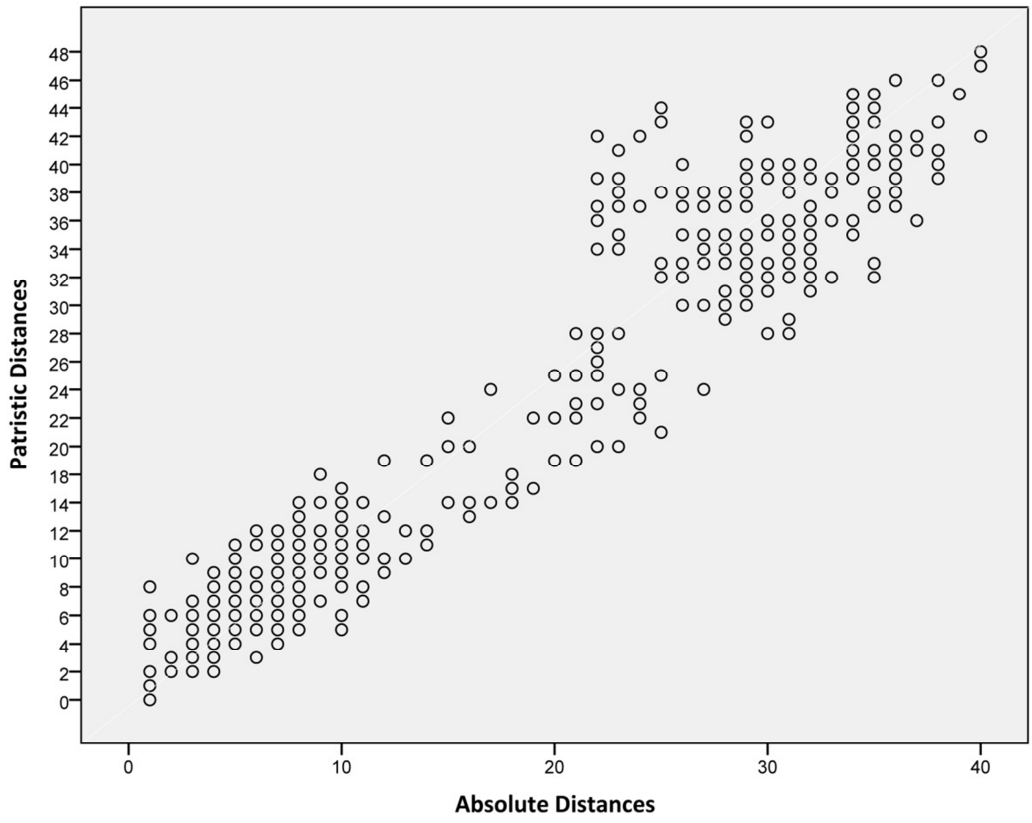

Figure 14: Patristic vs. absolute Distances computed in PAUP* v.4.0b10

Figure 13 shows the DAMBE plot, R^2 for transition is 0.959, for transversion 0.950, Figure 14 shows the results from the PAUP* v.4.0b10 analysis, visualized in SPSS v.21. Both have a linear progression and, therefore, substitutions are not saturated in this data-set and the BEAST analysis can be made.

The BEAST analysis is shown in Figure 15.

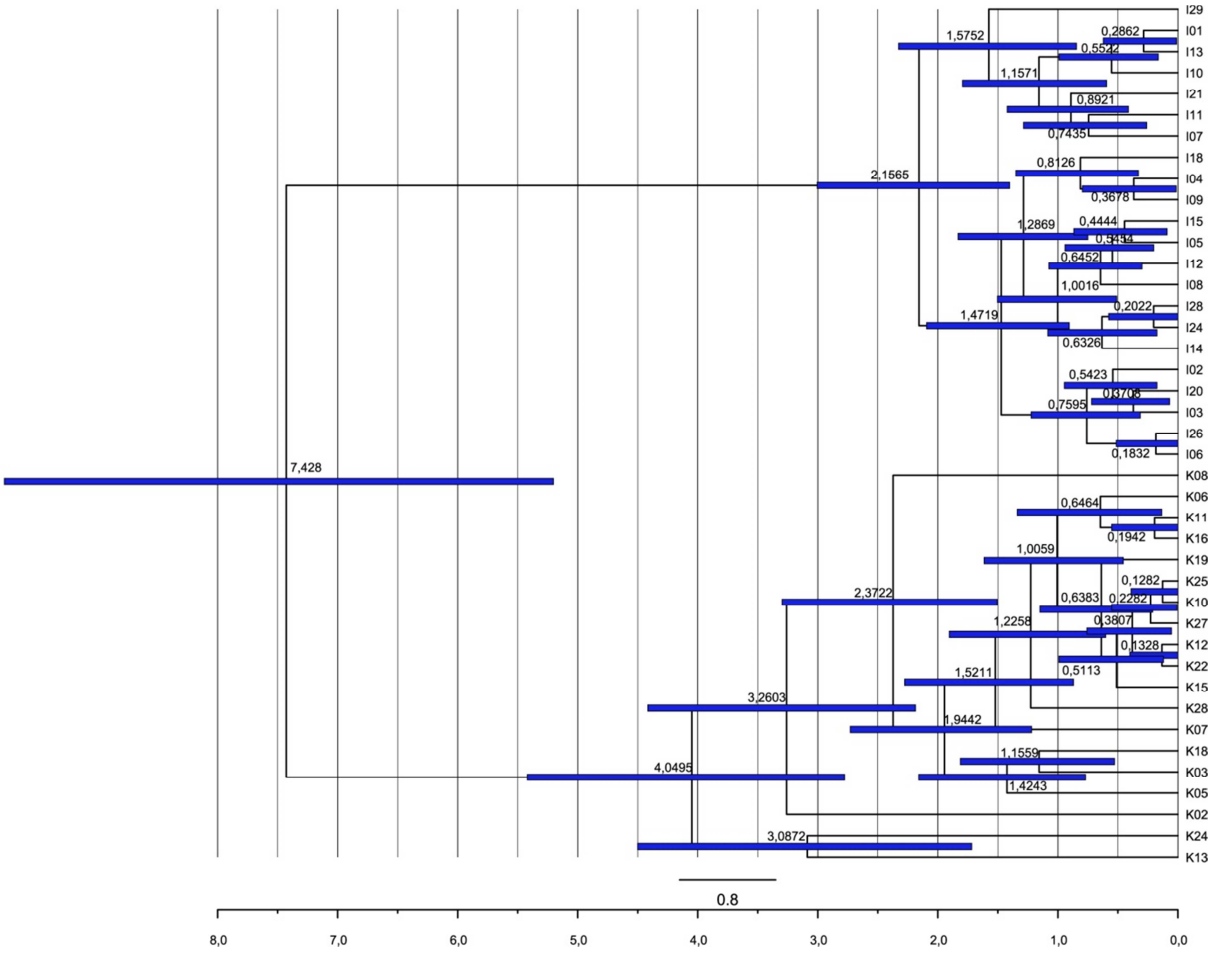

Figure 15: Tree from the BEAST analysis. Divergence time is 7.4 million years, the stat. error bar reaches from 5.2 to 9.7 million years

The divergence time of the Italian and the Croatian linage is 7.4 million years with an error bar from 9.7 to 5.2 million years. The tree is similar to the tree returned by Mr. Bayes.

5: Discussion

To clarify if *Sphaerosyllis sp*. is two separated species, as assumed, or conspecific, phylogenetic and statistical analysis were made. Also to verify the species identity of *Sphaerosyllis sp*., with was tested against *S. hystrix.*

5.1: Comparison of the data-sets

5.1.1: Phylogenetic analyses

All analyses of the three different data-sets, 16S, ITS and combined, show a clear geographical separation with a high support from the bootstrap (BS) and posterior probability (PP) values. Unfortunately only the reverse primer worked in 16S and therefore only 369 BP were useable and it seems that is a conservative region. This could suggest that they are separated species. In the 16S and 18S analysis with different *Sphaerosyllis* and *Prosphaerosyllis* specimens all the *Sphaerosyllis sp*., even the specimen from the Spanish coast, clustered together.

5.1.2: Statistical analyses

The F_{st} Indices have a range from 92.8% to 78.3% and would suggest a high level of differentiation (Wright, 1978). The computed migrants per generation also suggest this, but it was calculated using the F_{st} value. But the problem with F_{st} values is that they depend on high within-population diversity (Meiermans & Hendrick, 2011) and that is not the case with this data-set. The problem with the F_{st} value is well known and often gives a lower differentiation with completely separated subpopulations as with closely related ones, because F_{st} does not really measure differentiation (Jost, 2008). The distance analysis showed an overlap between within populations and among populations for the 16S and ITS data-set, in the combined analysis there is almost no overlap between within and among populations. ITS and the combined data-set have very same differences for within and among populations. In 16S the within differences are lower and the differences among populations is higher, but this could be a result of the short 16S. The F_{st} value would suggest cryptic species, but the p-values would indicate conspecific populations. Mayer (2013) had

similar ITS values for *Plakosyllis brevipes* (Syllidae) an also suggests, that *P. brevipes* is conspecific.

The BEAST analysis of the ITS data shows, that the Italian and Croatian haplotype lineages have split about 7 million years ago. The clock rates used are from hydrothermal vent vestimentiferans provided by Chevaldonné (et al., 2002) that were also applied by Nygren (et al., 2009). These rates may be too low for the syllid polychaetes in this study. The split of the haplotype lineages using these rates is calculated at 7 million years BP, thus dating it before the Messinian Salinity Crisis about 6 million years ago. Small populations can have a strong bottleneck leading to increased substitution rates and, thus, high genetic (and morphological) divergence. For the 16S analysis the split was 530 million years ago and that seems even more improbable than the ITS results.

5.2: Cryptic or conspecific species?

The F_{st} value, the Beast analysis and the separation of the populations into an “Italian and Croatian clade” would suggest cryptic species. But the distance analysis, the 16S and 18S analysis with other *Sphaerosyllis* specimens and the morphological similarity rather suggest conspecific species. As mentioned above, Mayer (2013) had similar result for her conspecific populations. Maybe with a larger sampling size and more sampling spots, like the coast of Albania, the results would be clearer. It seems that *Sphaerosyllis sp* .is conspecific rather than cryptic species, because the support is not sufficient to assume cryptic species.

5.3: Gene flow

If there are conspecific, how does gene flow occur over such great distance, 2.500 km, with such small specimens? They only have a small dispersal potential and lack a pelagic larval stage. There are different ways to explain this. One way to explain this is due passive drift. Breaking waves and tidal currents churn up sand and its attached inhabitants. Hagerman and Rieger (1981) estimated, that through erosion and passive drift an animal could travel 10 km per day. Anthropogenic transport of interstitial fauna plays an important role. Historical viewed specimens could be transported via wet ballast sand from sailing vessels (Gerlach, 1977). Today they could travel on the fouling from macrofauna on the ship’s hull, where polychaete larvae were found and should be a suitable substrate for interstitial animals (Carlton and Geller, 1993). If the habitat is continuous, gene hopping could take effect, meaning that the genes can spread steadily without migration of the individuals. During the

Last Glacial Maximum (LGM), 18.000 years ago, the sea level was about 120 meters lower (Bard et al., 1996). During this time only the south of the Adriatic Sea was flooded. Then the sea level rose again 11.000 years ago, and the north of the Adriatic Sea was flooded again and animals migrated northwards. The elapsed time could be not long enough to separate them.

5.4: Conclusion

The data-set points rather to a conspecific and widespread species in the Mediterranean Sea. The distances between Italy and Croatia for the ITS and combined data-set were small and even in the 16S and 18S *Sphaerosyllis* data-set, the distances between the three different geographical clades of *Sphaerosyllis sp.* were alike and smaller than to other specimens of *Sphaerosyllis*. The spreading potential for these animals is low and if and how gene flow is happening cannot be satisfactory explained. It would be of interest to have a larger sample stock and more sample spots, like the coast of Albania, do research if individuals from the south Adriatic Sea are more closely related to the “Italian Clade”. Maybe the North Adriatic individuals have adapted to colder habitats and that is a reason why they do not co-occur.

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