Diplomarbeit

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Molecular genetic, chemotaxonomic, and autecological investigations of European Sericostomatidae (Insecta: Trichoptera)

Molekulargenetische, chemotaxonomische und autökologische Untersuchungen europäischer Sericostomatidae (Insecta: Trichoptera)

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1. PREFACE

Several methods employed in this work are rather complex. Therefore in the ‘material and methods’ chapters basic principles of these methods are explained for better understanding of the results. Maps were created using the program PanMap (Diepenbroek et al. 2000). Illustrations (graphs, map design) were prepared with Photoshop 7.0 and Corel Draw 9.0. If not created by myself, the source of the graphics is mentioned in the text. Sequence data can be found in the appendix.

2. ZUSAMMENFASSUNG

Über den taxonomischen Status der zahlreichen beschriebenen westpaläarktischen Arten von *Sericostoma* Latereille, 1825 (Trichoptera: Sericostomatidae), sowie deren Verbreitung wird seit Mitte des 19. Jahrhunderts kontrovers diskutiert. Artunterscheidungen basieren zumeist ausschließlich auf der Morphologie der ♂ Genitalien, mit häufig nur geringen Unterschieden. Zur Überprüfung der zurzeit als gültig angesehenen Arten, speziell der beiden mitteleuropäischen Morphospezies *S. personatum* und *S. schneideri,* wurden autökologische, chemotaxonomische, sowie molekulargenetische Untersuchungsmethoden gewählt.

Autökologische Untersuchungen konzentrierten sich auf Unterschiede hinsichtlich der Schlupfdynamik, diurnalen Aktivität, sowie Quellung und temperaturabhängiger Entwicklung der Ei-Gelege. Der chemotaxonomische Ansatz beschränkte sich auf die Identifizierung von Pheromonen der beiden mitteleuropäischen *Sericostoma* ‚Arten'. Für die genetischen Untersuchungen wurden zwei mitochondriale Gene (16S rDNA, Cytochromoxidase I) und ein nukleäres Gen-Fragment (ITS-1, 5.8S rDNA, ITS-2) als molekulare Marker verwendet.

Die autökologischen Untersuchungen zeigten, dass Unterschiede in Schlupfdynamik und diurnaler Aktivität zwischen *S. personatum* und *S. schneideri* Populationen existieren.

Es wurden keine Unterschiede hinsichtlich des Quellungsverhaltens der Ei-Gelege festgestellt. Über die Temperaturabhängigkeit der Entwicklung konnten keine Angaben gemacht werden, da sämtliche Ei-Gelege, mit einer Ausnahme, unabhängig von der Inkubationstemperatur in Diapause gingen. Ein Einfluss von während des letzten Larven- oder des Puppenstadiums übermittelten maternalen Diapause-Hormonen in die Ei-Gelege wird vermutet.

Eine eindeutige Bestimmung von potenziellen Signalstoffen aus den Pheromondrüsen konnte nicht erreicht werden.

Es konnten keine genetischen Merkmale entdeckt werden, die eine eindeutige Trennung von Populationen von *S. schneideri* und *S. personatum* erlauben. Die mitochondrialen Haplotypen von *S. personatum* und *S. schneideri* ließen sich vielmehr in drei geographische Komplexe untergliedern: einen osteuropäischen (inklusive Finnland), einen mitteleuropäischen (inklusive Norwegen und Schweden) und einen alpinen Haplotypen-Komplex. Die als *S. flavicorne* SCHNEIDER, 1845 aus der Türkei, sowie die als *S. vittatum* RAMBUR, 1842 aus Spanien bezeichneten Tiere unterschieden sich signifikant von dem europäischen *S. personatum* / *schneideri* Komplex. Genetische Unterschiede lassen sich mit Einflüssen der Eiszeiten und den daraus resultierenden periodischen Vereisungen großer Teile Nordeuropas erklären. Die für die Bestimmung häufig verwendeten Merkmale sind eventuell lediglich Polymorphismen oder

oder das Resultat phänotypischer Plastizität und daher für eine systematische Einordnung nur eingeschränkt verwendbar. Die getroffene Unterteilung der zwei mitteleuropäischen *Sericostoma* ‚Arten' in *S. personatum* und *S. schneideri* erscheint in diesem Kontext nicht sinnvoll.

Alternativ könnte argumentiert werden, dass es sich bei *S. personatum* und *S. schneideri* tatsächlich um separate Arten handelt und dass beide Arten durch Introgression vor der Artaufspaltung dieselben Mitochondrien aus verschiedenen Ausgangspopulationen erhielten. Die unterschiedlichen Aktivitäten beider Populationen unterstützen dies.

Zusätzlich wurde eine Phylogeniehypothese der vier hauptsächlich in Europa vorkommenden Gattungen *Notidobia*, *Oecismus*, *Schizoplex* und *Sericostoma* basierend auf dem mitochondrialen 16S rDNA Gen konstruiert. Während manche Analysemethoden eine basale Stellung der Gattung *Notidobia* berechneten, wurden mit den meisten Analyseverfahren nur polytome Baumgraphen erhalten, was auf einen zu geringen Informationsgehalt der Sequenzabschnitte hinweist.

3. SUMMARY

The taxonomical status and distribution of the Westpalearctic *Sericostoma* species complex (Trichoptera: Sericostomatidae) is subject of controversial debate. Species descriptions are based in general on minor morphological differences of the male genitalia. In this work, species boundaries of these taxa, mainly the Central European 'species' couple *S. personatum* (SPENCE in Kirby & Spence, 1826) and *S. schneideri KOLENATI, 1948*, are reconsidered using autecological, chemotaxonomic, and molecular genetic methods.

Autecological analyses focused on differences concerning emergence period, diurnal activity as well as swelling of the egg masses and temperature dependence of embryogenesis. The chemotaxonomic approach focused on identification of compounds of the pheromone glands to detect differences concerning the communication system. For molecular genetic investigations two mitochondrial genes (16S rDNA, cytochrom oxidase I) and one nuclear gene fragment (ITS-1, 5.8S rDNA, ITS-2) were used. Members of all Westpalearctic sericostomatid genera were investigated, especially specimens of the *S. personatum* and *S. schneideri* 'group'.

Autecological investigations revealed differences concerning the emergence period and diurnal activity between the two populations. No differences concerning the swelling of egg masses were observed. All but one of the egg masses remained partially developed diapausing at all incubation temperatures thus limiting the interpretation. Maternal induction of diapause by means of diapause hormones due to unfavourable environmental conditions simulated in the laboratory during development of last instar larva and pupa seems most likely to explain this phenomenon

No components directly related to pheromones could be identified from male and female pheromone glands.

Molecular genetic investigations do not support a division of *S. personatum* and *S. schneideri* into two distinct groups or potentially sibling species. Mitochondrial haplotypes thus exhibited a unique geographical pattern with one group primarily found in Eastern European regions (including Finland), one in Central Europe (including Norway and Sweden), and one group predominantly located in the Alps. Specimens morphologically described as *S. flavicorne* SCHNEIDER, 1845 from Turkey and *S. vittatum* ROMBUR, 1842 from Spain were distinct from the European *S. personatum / schneideri* complex. The haplotype pattern can be explained with the Quaternary ice ages and the resulting glaciation of major parts of Europe.

Morphological characters frequently used for the identification of species may be polymorphisms or the result of phenotypic plasticity and were therefore considered as not useful to resolve systematical questions. The division of the Central European *Sericostoma* 'species' into *S. personatum* and *S. schneideri* seems to be questionable.

An alternative hypothesis supports the division of *S. personatum* and *S. schneideri* populations and explains overlapping haplotype data with introgression before speciation. In this case, both distinct species received the same mitochondria from different ancestral populations. This hypothesis is well supported by activity patterns.

From the available data, a phylogeny hypothesis of the four European sericostomatid genera *Notidobia*, *Oecismus*, *Schizoplex* and *Sericostoma* was deduced. While some methods resolved a basal position of the genera *Notidobia*, most methods resolved solely polytomic relationships.

"Seek simplicity, but mistrust it"
(A. N. Whitehead)

4. INTRODUCTION

The knowledge of species and their phylogenetic position offers important information for ecological, evolutionary, and conservation biological research. Correct identification of species is of major importance in both basic and applied biological sciences.
In zoology, morphological characters frequently do not provide sufficient information for correct classification due to undetectable or high variable morphological differences. Furthermore, populations of the same species may be separated into distinct species by mistake due to observed intraspecific phenotypic plasticity. In general, this seems to be the case within the caddisfly family Sericostomatidae Stephens, 1836 (Malicky 1983; Wood and Resh 1991; Jackson and Resh 1998) and in particular in its European genus *Sericostoma* Latereille, 1825. For a long time uncertainty prevailed concerning the species number, respectively the taxonomical status of European *Sericostoma* 'species', and this problem is subject of an intensive, controversial debate (e.g. Malicky 1999; Botosaneanu 2001).
The central point of the biological and the evolutionary species concept is the postulate that species represent reproductively isolated entities, maintaining separate evolving genetic lineages (Mayr 1942; Wiley 1978). In the present work, molecular genetic as well as autecological and chemotaxonomic approaches were chosen to search for evidence for the existence of reproductive barriers and the existence of separate evolving genetic lineages within in the genus *Sericostoma*.

4.1 An overview of the order 'Trichoptera'

It is scientific consensus that the Trichoptera Kirby, 1813 are the sister group of the Lepidoptera Linné, 1758 (*greek*: *lepidos = scale*) and that both together constitute the superorder Amphiesmenoptera (*greek: amphiesma = coat*) (Kristensen 1968; Friedländer 1983; Kobayashi and Ando 1988; Dathe 2003). The name 'Trichoptera' refers to the characteristically haired wings (*greek: trichos = hair; pteron = wing*) of the adults, one diagnostic character of that order. The English name 'caddisflies' and the German name 'Köcherfliegen' emphasize another character - the portable case many larvae bear for protection, camouflage, or respiratory

improvement (Williams et al. 1987). With about 10.000 extant species the caddisflies represent a relatively small order of holometabolous insects (Morse 1997b). With the exception of the Antarctic continent, the Trichoptera are distributed worldwide (Banarescu 1990), with biodiversity centres in the Oriental region (Schmid 1984). From Germany 314 extant species are described (Robert 2003).

Eggs, larvae and pupae are usually aquatic, whereas the adults are terrestrical. Caddisflies inhabit almost all freshwater habitats and constitute a major proportion of the benthic macroinvertebrate community in most natural freshwater systems (Botosaneanu and Malicky 1978). The major part of that ecologically highly diverse group (MacKay and Wiggins 1979) is intolerant of pollution, a fact that facilitates their use in freshwater biological monitoring programs (Rosenberg and Resh 1993; Stuijfzand et al. 1999). The determination of larvae to generic or even to species level (Waringer and Graf 1997) is of great advantage for this aim.

Phylogenetic relationships among the Trichoptera families and suborders have been investigated by several authors (Matrynov 1924; Milne and Milne 1939; Ross 1967; Friedländer 1992; Wiggins 1992; Weaver 1992; Frania and Wiggins 1997; Morse 1997b) using morphological characters. Especially before the 1970s, it was a problem that not explicitly homologous characters were used for the inference of the clades. Two important publications based on molecular data (Kjer et al. 2001; Kjer et al. 2002) and one morphological, computer-assisted review (Frania and Wiggins 1997) resolved a great deal of questions regarding the relationships of Trichoptera families and superfamilies (for an overview see Morse 1997b).

First publications dealing with population genetic topics of caddisflies appeared only recently (Jackson and Resh 1998; Guinand and Tachet 2000; Wilcock 2001; Plague et al. 2001; Myers et al. 2001; Myers and Sperling 2002). One deals with cryptic species within the Nearctic sericostomatid genus *Gumaga* McLachlan, 1871 (Jackson and Resh 1998). The usage of molecular markers has proven to be a powerful tool in this group. Nevertheless, molecular genetic investigations and experiences with the order Trichoptera are rare compared to other orders (see the small number of gene sequences available at the central database 'GenBank' of the NCBI[1]).

[1] NCBI = National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank/index.html)

4.2 The family Sericostomatidae Stephens, 1836

The family Sericostomatidae presently includes 115 extant and four fossil species (Morse 1997a) being distributed over all continents with the exception of Antarctica and Australia (Banarescu 1990). Adults are large (10 – 15 mm), heavy insects without ocelli and exhibit a characteristically sexual dimorphism in maxillary palps: in males, the palp consists of only two to three segments, the distal one is often conspiciously enlarged and adpressed to the head, forming a 'mask' (Botosaneanu 1992). In females, the palps are five-segmented without alteration of shape. Larvae of Sericostomatidae build up a smooth, slightly rounded case of sand with a rounded terminal opening. Adults bear two spurs on fore- and midlegs and four spurs on hindlegs (spur formula: 2 – 2 – 4) (Botosaneanu 1992).
In Europe, the family Sericostomatidae consists of five extant genera:

Tab. 1: Number of extant species within the European genera of the family Sericostomatidae (after Botosaneanu and Malicky 1978, Morse 2004, and Tobias and Tobias 1981).

genus	no. of species
Cerasma McLachlan, 1876	1 - 2
Notidobia Stephens, 1829	5 - 8
Oecismus McLachlan, 1876	2 - 4
Schizoplex McLachlan, 1876	5 - 7
Sericostoma Latereille, 1825	5 - 29 (?)

No information exists about the relationship between sericostomatid genera (Wagner, Kumanski, and Malicky, pers. comm.). Malicky (pers. comm.) even questions the correctness of the status of the genera with the exception of *Notidobia* that differs significantly on the morphological level. Information on *Cerasma* of which no species occurs in Central Europe is weak (see: Botosaneanu and Malicky 1978, Morse 2004).
According to most recent publications (Botosaneanu and Malicky 1978; Tobias and Tobias 1981; Pitsch 1993; Robert 2003), in Central Europe, particularly in Germany, only four sericostomatid 'species' in three genera exist:

- *Notidobia ciliaris (*Linné, 1761);
- *Oecismus monedula* Hagen, 1859;
- *Sericostoma personatum* (Spence in Kirby & Spence, 1826);
- *Sericostoma schneideri* Kolenati, 1848.

Notidobia ciliaris was abudant until the second half of the 20th centuary, but has become rare now (Tobias 1986). Water pollution is thought to be the main factor limiting the occurrence of *N. ciliaris,* as it inhabits lower stream regions that today are normally affected by pollution (Neu, pers. comm.; Tobias 1986). *Oecismus monedula* is locally common with abundance maxima in lower spring stream (Fischer et al. 1992).

4.3 The *Sericostoma* species complex

Information about species number and distribution of *Sericostoma* species in Europe varies to a high extent (Botosaneanu and Malicky 1978; Tobias and Tobias 1981; Morse 2004). This is due to (i) different attempts to synonymize some of the enormous number of *Sericostoma* 'species' described during the 19th century (see tab. 2) and (ii) due to recently described species (Botosaneanu and Malicky 1978; Tobias and Tobias 1981; Malicky 1999; Sipahiler 2000).

Tab. 2: Synonymous names for *S. schneideri* and *S. flavicorne* according to Morse (2004) and Tobias and Tobias (1981).

current name	synonymous names
S. personatum (Spence, in Kirby & Spence, 1826)	*S. analis* (Stephens, 1836)
	S. assimilis (Stephens, 1836)
	S. chrysocephalum (Zetterstedt, 1833)
	S. collare Pictet, 1834
	S. foyanum Navas, 1919
	S. hirta (Kawall, 1964)
	S. hyalinum (Stephens, 1936)
	S. latereillii Curtis, 1834
	S. multiguttatum Pictet, 1834
	S. spencii (Kirby & Spence, 1828)
	S. memorabile McLachlan, 1876
S. schneideri Kolenati, 1848	*S. pyrenaicum* Pictet, 1865
	S. turbatum McLachlan, 1876
	S. timidum Hagen, 1864

In general, *S. personatum* is referred to as the *Sericostoma* species with short, forked penis sheaths (lateral sclerites of the 10th abdominal segment) and smaller palps in males. The other

Sericostoma species in Germany, distinguished from *S. personatum* by bearing elongate and almost parallel lateral sclerites, is *S. schneideri* (see fig. 1).

Recent investigations by Botosaneanu (1992; 2001) revealed that the former name *S. flavicorne* Schneider, 1845 that was used until recently for Central European sericostomatids with long, parallel lateral sclerites (fig. 1) is invalid and must solely be applied to species in Oriental regions. Some taxonomists still doubt this (e.g. Malicky 1999 and pers. comm.).

In the following, European sericostomatids with long and parallel lateral sclerites are referred to as *S. schneideri* and S*ericostoma* species from Turkey with long and lateral sclerites as *S. flavicorne* for pragmatically reasons. Whether the status of a separate species is objectively legitimate will be discussed later.

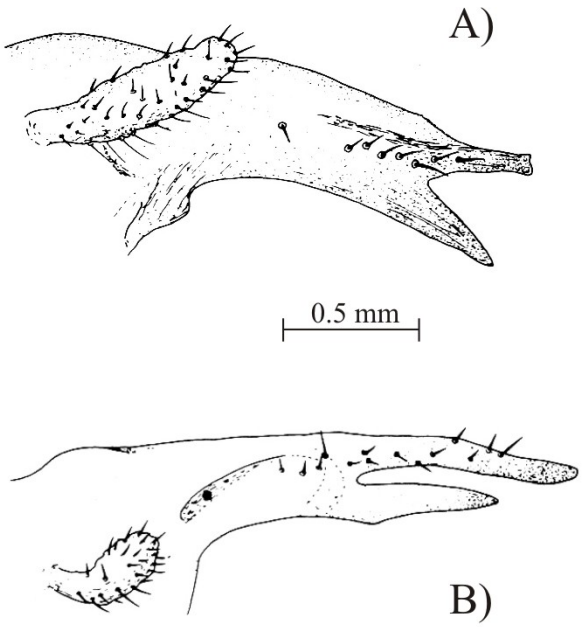

Fig. 1: Lateral sclerite of the 10th abdominal sclerite of A) *S. personatum* and B) *S. schneideri* (Tobias and Tobias 1981). Shape of sclerites varies between geographically separated populations (see Malicky 1983).

As mentioned above, few objective criteria allow the distinction of *S. personatum* and *S. schneideri.* Several authors question the division into two separate species (Wagner 1990; Tobias and Tobias 1981; Malicky and González, pers. comm.). During the last years, detailed studies (Neu 2002; Neu 2004) offered objective criteria for the distinction of Central European populations of *S. personatum* and *S. schneideri.* With these criteria, it is often even possible to distinguish final instar larvae by coloration of the anterior pronotum and females by the shape of the inner anatomy of genitalia. However, the problem whether this variation is due to (i) phenotypic or ecotypic plasticity or whether (ii) specimens exhibiting these characters are distinct species of simply differing populations remains unsolved. Additionally, in the

neighbouring countries of Germany populations were found that exhibit clearly intermediate morphological characters (Neu, pers. comm.; own observations).

It has been previously stated that intraspecific phenotypic variation among caddisflies and other aquatic insects obviously exists (e.g. Malicky 1983; Otto 1984; Hogue and Hawkins 1991; Sipahiler 2000; Klaithong 2003), therefore these taxonomical questions cannot be answered using solely typological approaches to define species boundaries (Doyen and Slobodchikoff 1974).

4.4 Aims of this study

Three different approaches were chosen to resolve the taxonomical problem of the *Sericostoma* species complex, because a thorough investigation involves not just an typlogical examination of morphological characteristics but must seek to analyse reproductive, ecological, and molecular genetic distance data as well (Doyen and Slobodchikoff 1974; Wood and Resh 1991). These three approaches are:

1. Autecological investigation
2. Chemotaxonomical investigation
3. Molecular genetic investigation

Results are compared with studies on the Nearctic sericostomatid species complex of the genus *Gumaga* McLachlan, 1871 (Wood et al. 1982; Wood and Resh 1984; Resh and Wood 1985; Resh and Wood 1987; Jackson and Resh 1991; Wood and Resh 1991; Jackson and Resh 1998). Both situations are comparable, since both genera belong to the same family, exhibit no prominent morphological characters for distinction and show a distribution of larvae in two favourite stream regions – the upper and lower parts of the stream (Wood and Resh 1991).

The molecular genetic investigation was expanded to the other three frequent sericostomatid genera *Notidobia*, *Oecismus*, *Schizoplex.* Their phylogenetic relationship was investigated by means of mitochondrial DNA (16S rDNA).

5. AUTECOLOGICAL INVESTIGATIONS

5.1 Introduction

Ecological research can be subdivided into at least three different fields of study: autecological, demecological and synecological approaches (Schwerdtfeger 1975). While demecology focuses on populations and synecology deals with biocoenoses, the relations of individual organisms to their biotic and abiotic environment are of primary importance for the field of autecology. Results obtained from autecological investigations may explain observed distribution patterns and provide the basis for further investigations at population and biocoenose level.

The competitive exclusion principle (Begon et al. 1998) states that a stable coexistence of two species is only possible, when intraspecific competition is greater than interspecific competition of both species. This can only be observed, when species differ in the usage of resources available in their habitat, a phenomenon described as 'resource partitioning' (Begon et al. 1998). This fact has led to the postulation of the ecological species concept (Van Valen 1976). Yet, resource partitioning can be observed between members of the same species as well (see Wägele 2001) and consequently it is not possible to define species solely by their ecological differences. Together with further information about species, like morphological and genetical data, ecological results might seriously underpin a species postulation.

5.1.1 Ecological research among the *Sericostoma* species

Many ecological studies on *S. personatum* have been performed (Ellliot 1969; Iversen 1973; Iversen 1980; Rennerich and Schuhmacher 1984; Wagner 1990; Kiss 2002) with main focus on lifecycle and nutrial aspects. The results were contradictory (for an overview Iversen 1973, Wagner 1990), in particular the duration of the aquatic development (one to five years) and the number of larval instars varied considerably. Wagner (1990) assumed that contradictory ecological results might be due to the existence of different subspecies or geographical races of a *S. personatum* 'superspecies'.

Few ecological investigations dealt with *S. schneideri* and only one recent publication investigated lifecycle aspects; however a comparison of the results with the lifecycle of *S. personatum* revealed no significant differences (Komzák and Sedlák 2002).

By comparing the main niche dimensions (Schwertfeger 1975) space, time and nutrients, the following differences between *S. personatum* und *S. schneideri* are described:

- Circum European distribution (figs. 18 and 19) of *S. personatum* includes Great Britain, Ireland and the Scandinavian countries, whereas *S. schneideri* is located more to the South, not found above N 54° (Botosaneanu and Malicky 1978; Reusch 1988).
- Local distribution differs to a certain amount (Botosaneanu and Malicky 1978; Pitsch 1993) with aquatic states of *S. personatum* found in springs and spring streams and *S. schneideri* inhabiting lower stream regions, nevertheless sympatry occurs.
- *S. personatum* is said to emerge early in the summer with adult abundance maxima from May to July, whereas *S. schneideri* emerges mainly in July and August (Komzák and Sedlák 2002).

Differences in diurnal activity remain unknown. Larvae of *S. personatum* are definitively night active (Ellliot 1969; Wagner 1990) and spend the day buried in the sand (Rennerich and Schuhmacher 1984), beneath stones (Ellliot 1969), or attached to organic matters (Wagner, pers. comm.; own observations). According to Elliot (1969), adults are active during the day; Nielsen (1942) states that adults are active during the night.

S. personatum and *S. schneideri* both seem to prefer sandy sediments with high amounts of coarse particular organic matter (CPOM).

Space and aspects of reproduction

Egg masses

Female Trichoptera, like many other insects, possess accessory glands, secreting a gelatinous substance in which individual eggs are embedded (Dathe 2003). Some *Sericostoma* species belong to the group of Trichoptera in which females submerge into the water in order to deposit egg masses beneath stones. Egg masses of *S. personatum* were typologically described several times (Ellliot 1969; Bjarnov and Thorup 1970; Wagner 1990). They are blackish, gel like, approximately 3 – 4 mm in diameter in water and differ in shape. The shape was described as ‘wedge-shaped’, ‘pyramidal’, or ‘spherical’ (Nielsen 1942; Ellliot 1969; Wagner 1990). Elliott (1969) observed ovipositing females of *S. personatum* crawling into the water, using a partially submerged stone for hold, depositing their egg masses beneath stones. At least one of the females reemerged after deposition. Often females die after egg deposition, as own observations similar to the ones of Nielsen (1942) revealed. As oviposition only occurs at locations with large, stable, nd partially submerged stones or wood – suitable sites near the source – *S. schneideri* probably oviposits egg masses on the water surface or may use roots of

the alder (*Alnus glutinosa*), as in the lower stream regions partially submerged stones are rare. No further information exist. It remains unknown whether more than one egg mass is produced per female within *Sericostoma*.

The spring and springstream regions *S. personatum* inhabits are characterized by intermittent discharge (fig. 2). Consequently, in dry summers brooks are endangered by dehydration. Therefore, egg masses of species living in these areas must be resistant to dry conditions. Differences in desiccation resistance among trichopteran species ovipositing egg masses in temporary dry ponds or above the water on the one hand and species ovipositing in water on the other were described (Wiggins 1973; Middleton 1977). Middleton (1977) found that the amount of the matrix-stabilizing molecule glucuronic acid was significantly increased in the egg matrix of species inhabiting habitats endangered by desiccation. As a consequence, viscosity of those egg masses is high in contrast to egg masses of species depositing submerged, which take up more water but lose it in dry conditions just as quickly. Water is retained by the high complex three-dimensional matrix network that forms a viscous superficial cortex (Berté and Pritchard 1983). The strong black gelatinous matrix observed in *S. personatum* egg masses may provide several advantages (Wiggins 1973). Most important for *S. personatum* might be the protection against dehydration and attacks of fungi, bacteria and copepods.

As desiccation events are least likely to occur in the lower stream parts, substantial differences concerning the composition of the egg matrix might exist between spring stream (*S. personatum*) and lower stream (*S. schneideri*) inhabiting populations. Comparative egg masses analysis of morphologically cryptic species of the Nearctic sericostomatid genus *Gumaga* showed that species inhabiting lower stream regions produce egg masses differing in shape from species inhabiting the springstream regions (Wood and Resh 1991, Jackson and Resh 1998).

Morphology and swelling dynamics of egg masses were compared, since swelling depends on the composition of the egg matrix (Berté and Pritchard 1983).

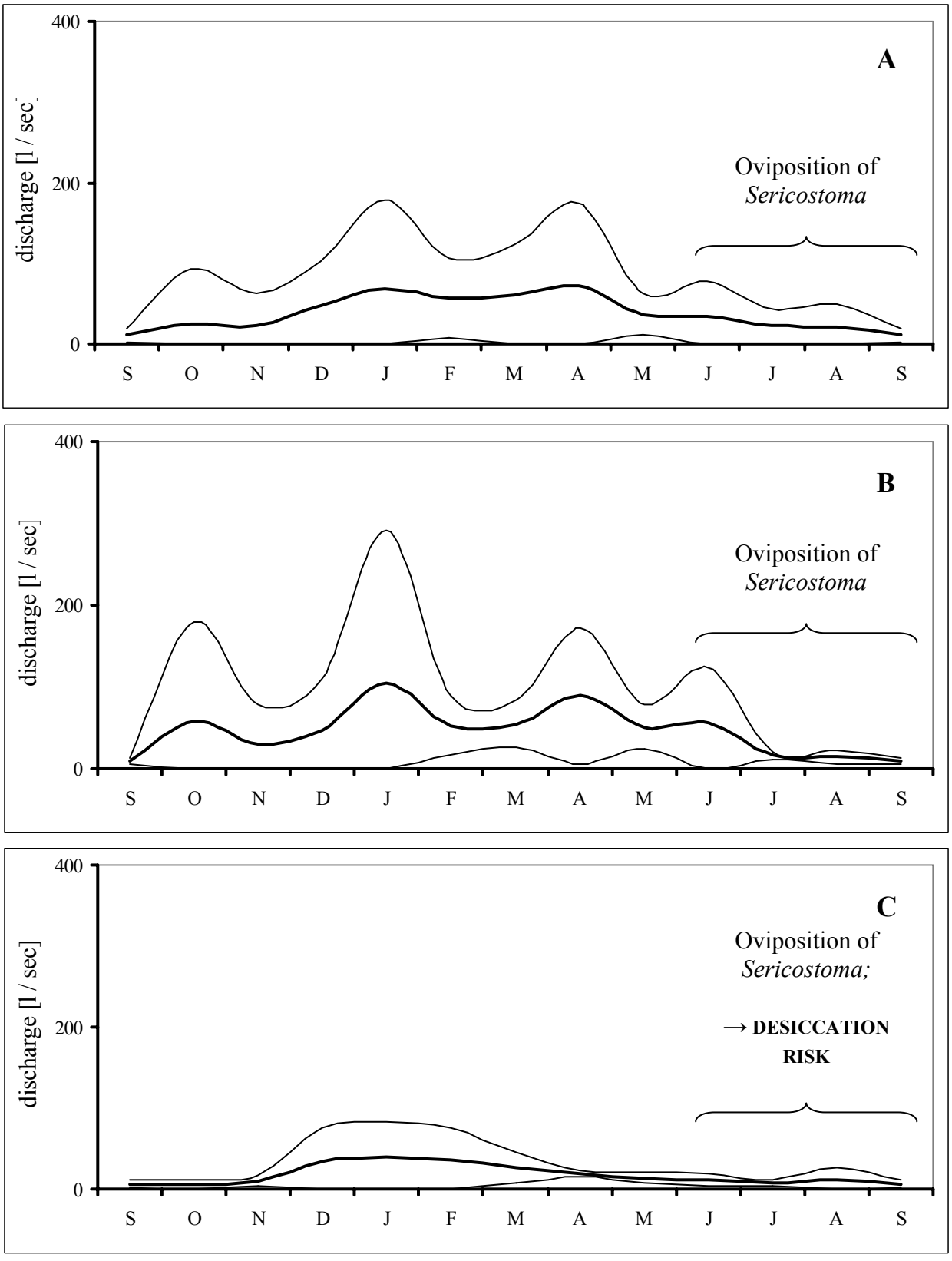

Fig. 2 : Different annual discharge patterns (mean ± standard deviation) of the Breitenbach (Hesse). A) Mean maximal monthly discharge (1969 – 2000), B) includes all years between 1969 and 2000 with non-seasonal floods, and C) presents all years with low precipitation and low discharge all over the year (after Wagner and Schmidt, unpublished data).

Niche dimension 'time'

Temperature dependance of embryonic development

Lifecycle studies investigating the development of freshwater animals in dependence of temperature offer data that may explain observed contemporary distribution patterns of a species. Sternberg and Wagner (in prep.) revealed that several caddisflies inhabiting upper stream regions differ in embryonic development from Trichoptera inhabiting the lower parts of the stream as well. In particular the duration of embryonic development at low constant temperatures and the maximal temperature for successful development differed. The duration of embryonic development at cold constant temperatures was almost linearly or slightly exponentially increased in species inhabiting spring and spring stream (*Apatania fimbriata, Crunoecia irrorata, Drusus annulatus*). In species inhabiting the lower stream regions (*Chaetopteryx villosa, Rhyacophila fasciata*) the duration of embryonic development increased exponentially at low temperatures. Additionally, embryonic development of spring and spring stream species was often not successful at constant temperatures above 18°C, whereas other species developed successfully up to 24°C. Embryonic development of *S. personatum* was observed by Wagner (1990) and it did not differ from other spring and spring stream inhabiting species: embryonic development was relatively fast at low temperatures and not temperature dependent at constant temperatures between 12 – 18°C.

In general, fast development at low temperatures might be of major importance for species inhabiting spring regions that exhibit constantly mild climates with temperatures fluctuating only slightly over the year. In contrast, the yearly temperature fluctuations are stronger in the stream regions *S. schneideri* inhabits. No need for fast development at low temperatures must be considered.

Diurnal activity and mating

Conclusions about the taxonomic relationship of two species differing in diurnal activities can only be drawn, if circadian activity patterns are genetically determinated. Evidence for this was first obtained over 30 years ago (Konopka and Benzer 1971). Today many genes and molecular mechanisms of circadian clocks are described (Giebultowicz 2000), although these activities often seem to be evolutionary highly flexible.

In the order Trichoptera, no genes controlling diurnal activites have been described yet. Nevertheless, differences in the circadian rhythmic of Trichoptera triggered by no detectable exogenous stimulus have frequently been described, for example based on data obtained from research on the two closely related Nearctic sericostomatid species *Gumaga nigicula* and

Gumaga griseola. Both exhibited significant different diurnal activity patterns concerning emergence, mate attraction and flight activity (Jackson and Resh 1991). These differences might act as ethological barriers, reducing gene flow and thus leading to the establishment of different evolving lineages.

In field and laboratory trials Elliot (1969) showed that adults of *S. personatum* hatched chiefly during day and only very few at night. Mating couples of *S. personatum* were frequently observed in the early morning hours by Wagner (Wagner, pers. comm.). In contrast to the activity of adults, larval activity was found to be highest in the dark and lowest in the light, but activity was solely triggered by changes in light quantity (Ellliot 1969; Wagner 1990). Little is known about any endogenous diurnal activity of *S. schneideri* adults., therefore emergence patterns and circadian activity of both *S. personatum* and *S. schneideri* were observed for possible differences.

5.1.2 Aims of autecological investigations

All experimental procedures aimed at the collection of information to verify or falsify the hypothesis that *S. personatum* and *S. schneideri* maintain separate evolving lineages with respect to the niche dimensions time and aspects of reproduction.

5.2 Material and methods

Studies have been conducted at the Max Planck Institute for Limnology, Limnological River Station Schlitz (Hesse).

5.2.1 Collection sites of specimens

Pupae and final instar larvae of *S. personatum* were collected in May 2003 along the Breitenbach (BTB) (N50°39' E9°37') and at one site of the Richthofbach (RB) (N50°45' E9°35'). Animals were collected by hand or with a sieve (2.0 mm mesh size). Larvae and pupae of *S. schneideri* were collected in May 2003 along the river Fulda (Hesse) next to the village Lütter (N50°28' E9°45') (see fig. 3). Animals were brought to the laboratory in plastic tanks with small amounts of water. At the institute, the larvae were transferred into artificial recirculating laboratory streams ('labstreams') filled with BTB water.

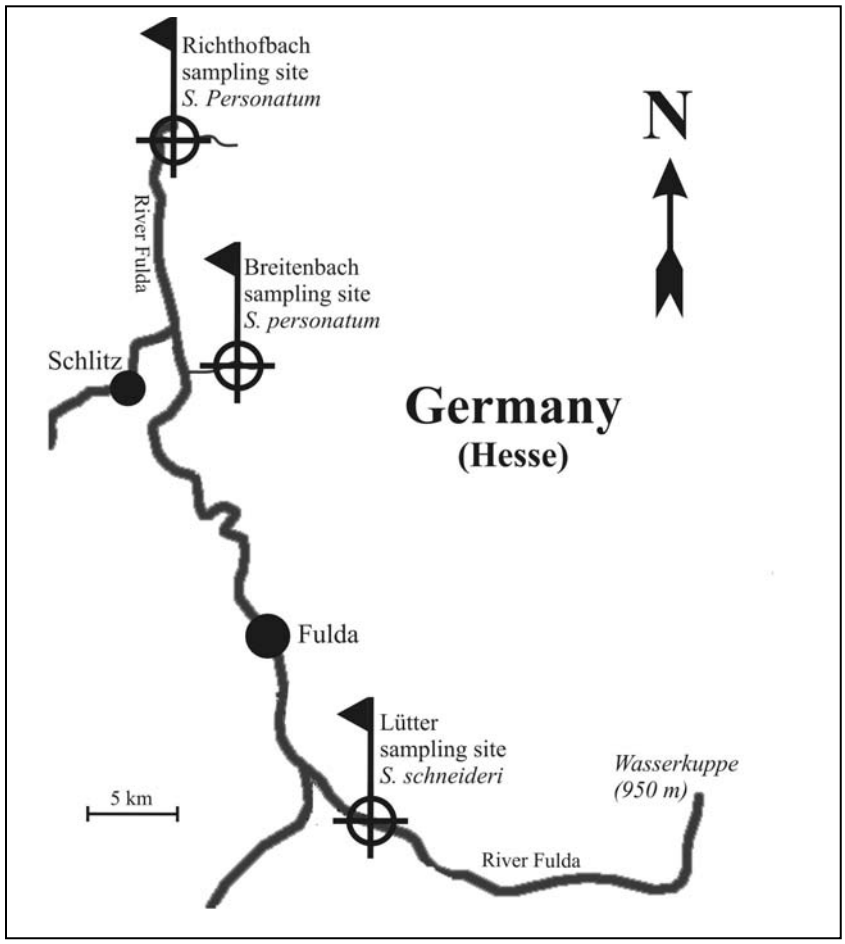

Fig. 3: Hessian sampling sites of specimens for ecological investigations: *S. personatum* from the Breitenbach and the Richthofbach; *S. schneideri* from the river Fulda near Lütter. Brooks are excluded from graphics.

5.2.2 Recirculating laboratory streams

Various types of recirculation laboratory streams have been constructed for ecological experiments. Their common purpose is to move water around in a circular way. The main advantage of labstreams is obviously the possibility to rear aquatic organisms in the laboratory under most natural conditions. Particularly for this purpose, special labstreams were planned at the River Station. They are made of translucent plexiglass and are rectangular (length, width, height: 1.6 x 1.0. x 0.4 m). The water is moved by an oversized water wheel with many paddles. This guarantees high flow velocity with simultaneous low turbulence. The walls opposing the waterflow in a 90° angle (see fig. 4) act as breakwaters and thus generate natural flow patterns. The sediment in the labstream is sorted out due to the same physical principles, mainly drag forces, as in the field.

One of the most important factors of ecological science is the controlled alteration of natural parameters influencing the experiment. These labstreams allow a highly specific alteration of several abiotic parameters, in particular temperature, waterfilling, flow velocity, oxygen content, and pH-number by a computer interface:

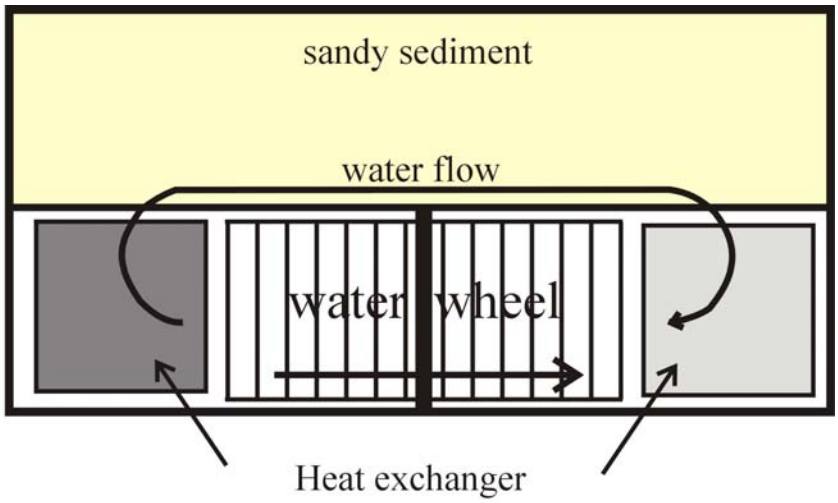

Fig. 4: Modelled view of a recirculating laboratory stream (mod. after Schmidt, http://www.mpil-schlitz.mpg.de)

Sandy sediments and water from the BTB were brought into the laboratory streams, flow velocity was set to 0.2 m / s, and temperature (see 'rearing of larvae and pupae') was adjusted.

5.2.3 Rearing of larvae and pupae

Larvae and pupae of *S. personatum* were transferred to one labstream with a constant temperature of 16°C, those of *S. schneideri* to another labstream with constant 18°C. Light re-

gime was light (L) : dark (D) 12:12 h (± 0.5 h due to manual treatment). The entire labstreams were covered with gauze (1.0 mm mesh size) to collect all emerging adults. Larvae were fed with dead leaves from the BTB and freshly collected alder leaves (*Alnus glutinosa*). Emergence was registered at least twice a day. Emerged adults were used for mating experiments or pheromone analysis.

5.2.4 Mating experiments

Hatched adults were separated immediately. One day after hatching, conspecific couples were placed into small glass vials with a wet grass or plantain (*Plantago lanceolatum*) leave. Lids of the vials were perforated and the perforations closed with gauze for adequate oxygen supply. Crossmating was tested among individuals of *S. personatum* and *S. schneideri* (five couples). The couples were kept at room temperature, avoiding direct insolation, in an office at the Max Planck Institute. Adults were fed every second day with one to three drops of water, occasionally enriched with saccharose. At least twice a day, glass vials were checked for egg masses. Dead animals were transferred into 70% ethanol for conservation.

5.2.5 Thermostat-controlled water-bath system

Investigating temperature dependence of the duration of embryonic development, growth rates, and hatching-success requires different test temperatures with high temperature stability for comparison. This was achieved by using the thermostat-controlled water-bath system developed by Marten (1990). The water-bath system utilizes the heat conductivity of water for the temperature regulation of several interconnected water baths. It consists of an elongate silicone glued glass-tank (190 x 50 x 40 cm), which is divided crosswise by glass panes (5.0 mm thick) and filled with tap water. The complete system is almost isolated from environmental temperature fluctuations by styrofoam (40 mm). No light source was placed above or in the experimental construction. Temperature of the first chamber (Aq1) was 4°C, using a cooler (Huber TC 40 E) and the last chamber (Aq8) was heated up to 18°C by a conventional aquarium heater (25 W). Temperature was controlled (± 0.1 °C) three times a day by a digital and a conventional rod thermometer at the beginning of the experiment, after the establishment of a constant temperature regime once or twice a week due to low temperature fluctuations. Temperature stratification was prevented by aeration of each chamber by a conventional aquarium aeration system.

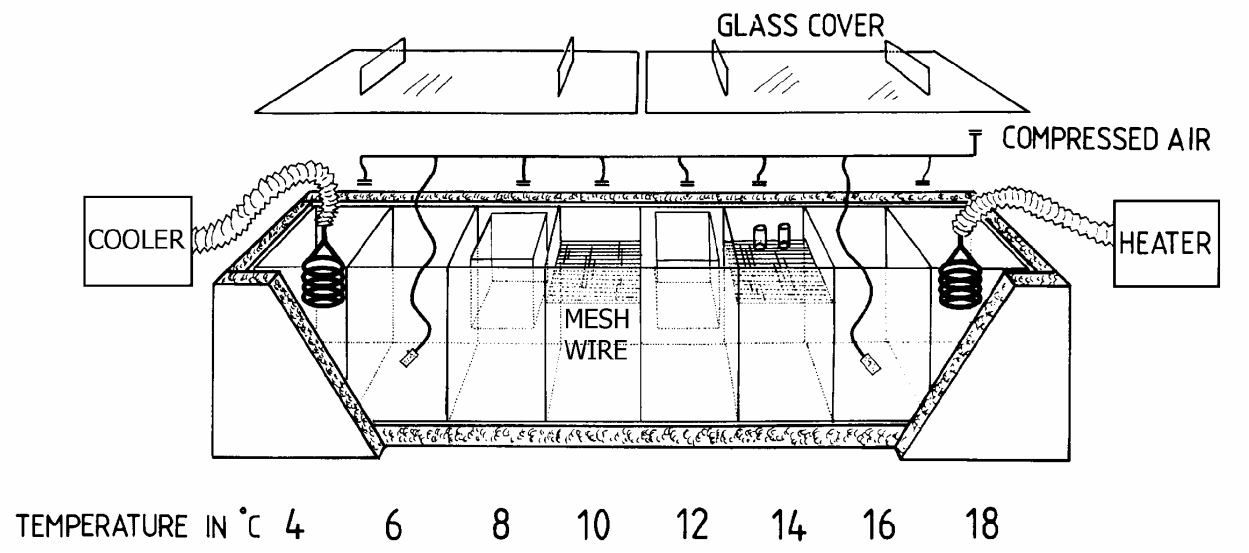

Fig. 5: Schematic view of the water-bath system (mod. after Marten 1990).

The first chamber, containing the rod cooler, had to be aerated by an additional aeration system (placed in Aq1 on the 18.06.2003) to allow adequate circulation of the water within the bath, as ice formation quickly occurred at the cooler and heat exchange between Aq1 and Aq2 was suboptimal. The chambers between the 4°C and the 18°C chamber build up a constant gradient that differed from the expected 2°C intervals (4°C – 6°C – 8°C – 10°C – 12°C – 14°C – 16°C – 18°C), because the second chamber had to be fixed with an additional glass pane. This led to a reduced heat exchange between chamber one and two, explaining the temperature patterns observed among the different chambers. Nevertheless, temperatures in the chambers were almost constant (see fig. 6). For the deposition of glass vials containing samples, wire mesh was fixed in each chamber two to three centimetres below the water surface.

Tab. 3: Mean temperature (M) and standard deviation (± SD) in °C of the eight chambers (Aq1 – Aq8) of the water-bath system (data from 11.06.2003 to 20.12.2003).

	Aq1	**Aq2**	**Aq3**	**Aq4**	**Aq5**	**Aq6**	**Aq7**	**Aq8**
M ± SD	3,9 ± 0,1	6,7 ± 0,5	9,5 ± 0,7	11,5 ± 0,6	13,4 ± 0,5	15,1 ± 0,4	16,6 ± 0,4	18,0 ± 0,3

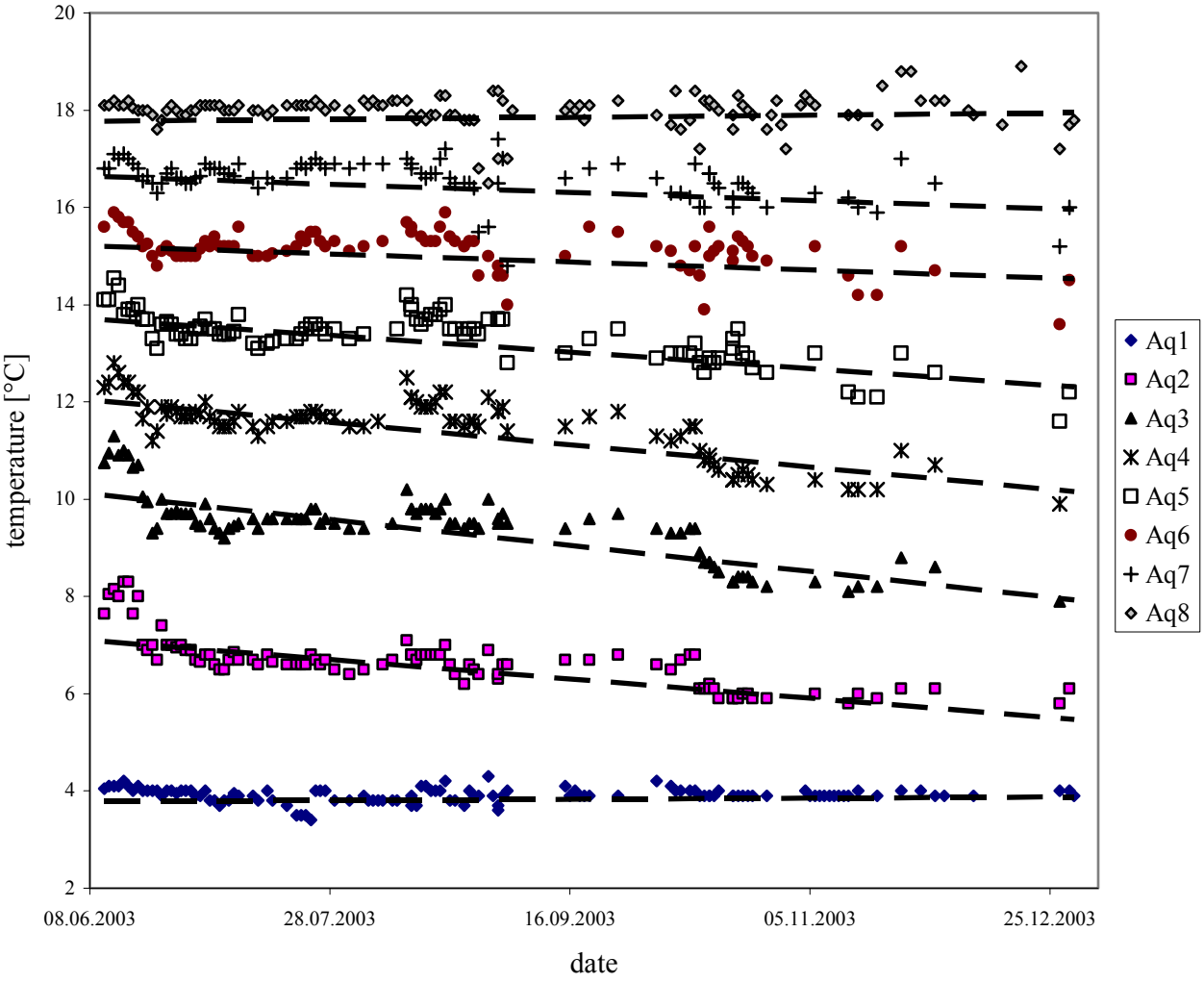

Fig. 6: Temperature of the eight chambers (Aq1 – Aq8) of the water-bath system between 08.06.2003 and 25.12.2004. Interrupted lines represent linear trend lines.

5.2.6 Egg mass swelling and embryonic development

Volume of selected egg masses was determined directly after deposition and again seven days later after swelling in the water. Therefore, two randomly chosen edges of the tetrahederal egg masses were measured. Measurements were made under a calibrated stereo microscope. Since all edges of the selected egg masses were almost equal, the lengths of two randomly chosen edges were measured under the stereo microscope. The arithmetic mean was calculated and with that information the volume calculated according to the formula:

$$V = \frac{\sqrt{2}}{12} a^3$$

(a = arithmetic mean of two edges of the tetrahedral shaped egg mass)

After determination of the volume, the egg masses were placed in clean glass vials half filled with water from the source of the BTB. To allow adequate supply with oxygen, the lid was perforated (5.0 mm diameter) and the hole covered by fine gauze (300 µm mesh size). The vials were individually labelled, using a glass cutter. Immediately after transferring the egg masses into the vials, they were placed onto the wire mesh in the chambers of the water-bath system. Thus, egg masses were not in direct contact with chemical compounds of the tap water in the aquarium but took up the temperature from the surrounding tap water. Date, chamber number and temperature were registered for each egg mass. Hatching of larvae was controlled daily and the number of hatched larvae registered.

5.3 Results

5.3.1 Emergence

Adults of both *S. personatum* populations emerged from end of May until the end of July, with the maximum increase in the first half of June. Emergence patterns between both *S. personatum* populations were almost similar, but differed from the patterns observed in the field (BTB). Increase in number of the emerging adults was not as steep as in the labstreams (fig. 6). *S. schneideri* emerged from begin of June until end of July with a maximum increase in number at the end of June. The emergence pattern of *S. schneideri* in labstreams was almost similar to the emergence patterns of *S. personatum* in the field (BTB). 50% of the emergence of both *S. personatum* populations in labstreams was 10 to 15 days earlier than *S. schneideri* and *S. personatum* (field).

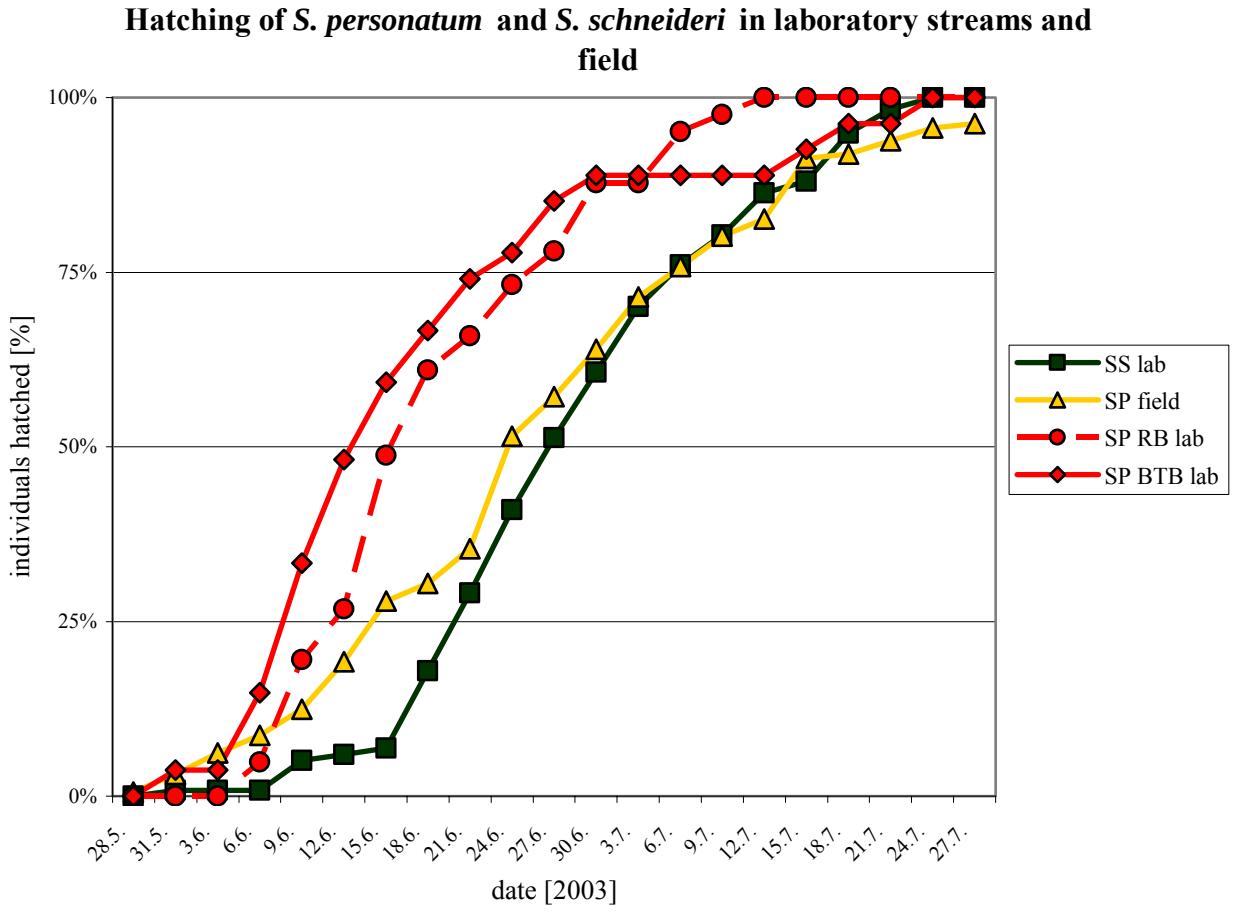

Fig. 6: Emergence patterns (cumulative percent) of *S. personatum* and *S. schneideri* reared in recirculating laboratory streams and of *S. personatum* hatched at the BTB (SP field). Data points represent 3-day-means.

5.3.2 Diurnal activity

Hatching of both *S. personatum* populations did not follow a conspicuous diurnal pattern. In contrast, *S. schneideri* emerged exclusively during the 12 h darkness. Mating couples of both *S. personatum* populations were observed from 6:00 a.m. to 10:00 a.m. (n = 7 couples). In contrast, mating couples of *S. schneideri* always occurred between 4:00 p.m. and 8:00 p.m. (n = 9 couples). Only one mating couple was observed at 1:00 p.m. on a cloudy day.

Mating was very stereotypic with the partners sitting lengthwise on the leave interconnected by the abdominal tips. The wings of the often bigger female were laid above the wings of the male. Couples remained in that position 1.5 to 3 hours. These observations are consistent with the field observations by Wagner (1990).

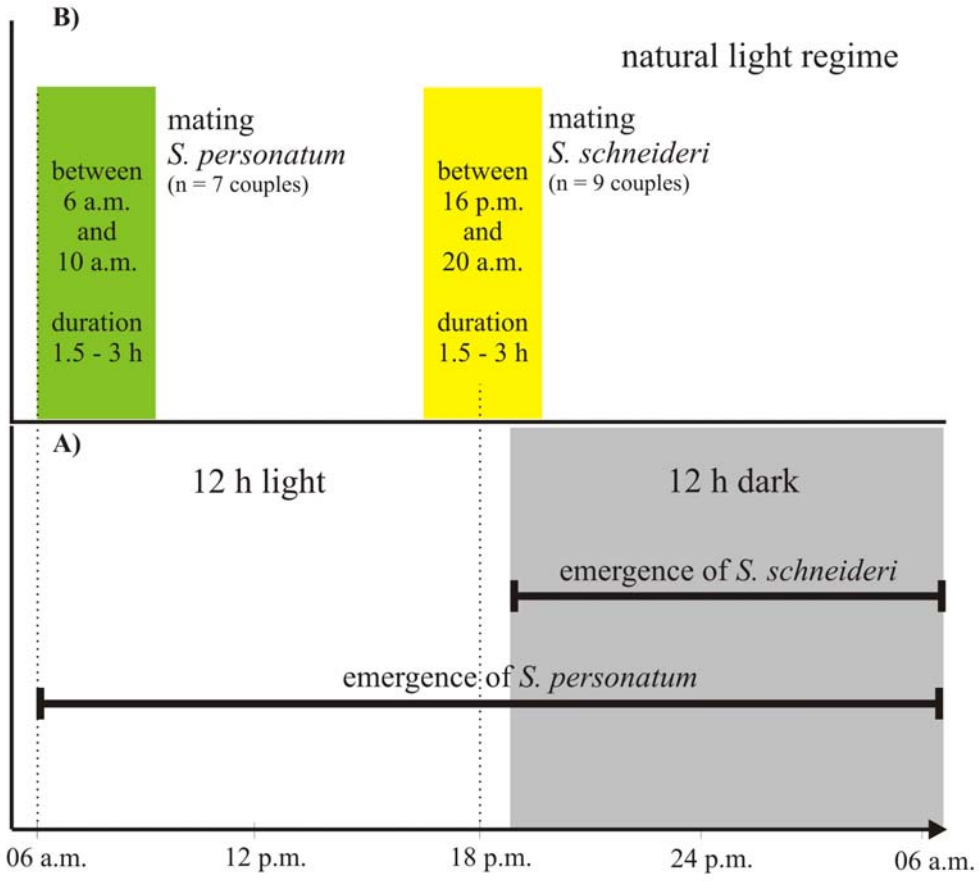

Fig. 7: Diurnal activity of *S. personatum* and *S. schneideri* observed in the laboratory; A) emergence of adults reared in the recirculating laboratory streams, B) mating observed in *S. personatum* and *S. schneideri*

One female was observed that died during mating, nevertheless the male stayed connected to the female carrying it around for about half an hour before loosening the connection. Individuals of both species lived two to ten days (see tab. 4).

Tab. 4: Lifespan (in days) of both sexes of *S. personatum* and *S. schneideri*; n = number of specimen, M = arithmetic mean, SD = standard deviation.

	S. personatum ♂	***S. personatum*** ♀	***S. schneideri*** ♂	***S. schneideri*** ♀
n	18	22	37	40
M ± SD	6.2 ± 2.3	4.5 ± 2.2	5.0 ± 2.3	5.5 ± 2.7

5.3.3 Aspects of reproduction

The general appearance of the egg masses did not differ significantly between *S. personatum* and *S. schneideri*. Both were gelatinous, shaped bluntly tetrahedrally to spherically and covered by a black matrix. They were similar in shape and construction to the egg masses of the Nearctic sericostomatid *Agarodes libialis* (see Wood et al. 1982).

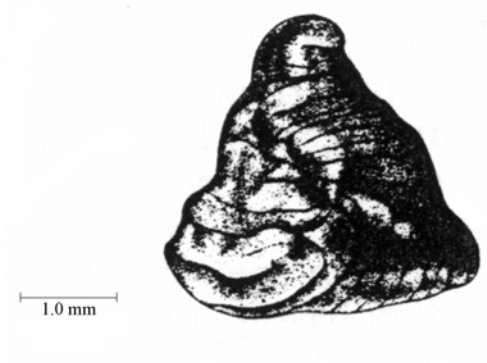

Fig. 8: Egg mass of *Agarodes libialis* (mod. after Wood et al. 1982)

When put into water, caddisfly egg masses swell due to the uptake of water. Egg masses of *S. personatum* and *S. schneideri* did not differ significantly, neither in shape nor in swelling (see tab. 5, fig. 10). Dry egg masses had an average edge length of 1.64 ± 0.31 mm (M ± SD), swollen egg masses 3.9 ± 0.55 mm. Volume increased by a factor of 7.9 to 28.9 due to the uptake of water (see fig. 10). High variation was observed in particular among small egg masses. Egg masses from *S. personatum* and *S. schneideri* did not differ significantly.

55.3% of *S. schneideri* (n = 21) females produced no egg masses, 26.3% (n = 10) produced one egg mass, 13.2% (n = 5) produced two egg masses, and three and four egg masses were produced each once (2.6 %). The latter were significantly smaller. In *S. personatum* 52.2% (n = 12) of the females deposited no egg masses. 34.8% (n = 8) produced one and 13.0% (n = 3) produced two egg masses.

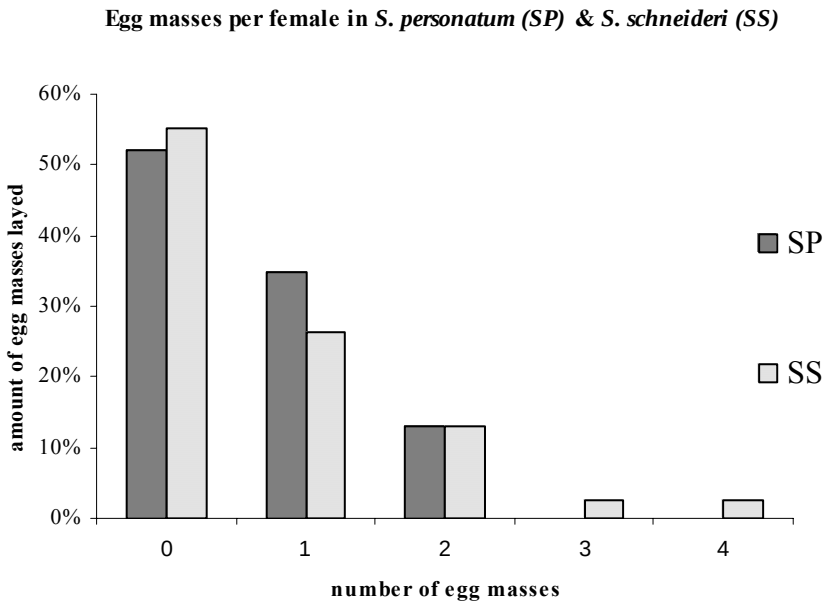

Fig. 9: Amount of female *S. personatum* and *S. schneideri* specimens producing no, one, two, three, or four egg masses.

Tab. 5: Swelling of egg masses. Edgelength (a) and volume (V) of dry and swollen egg masses, factor of volume increase (F), means (M) and standard deviations (±SD).

Egg mass	species	a dry [mm]	a swell [mm]	V dry [mm^3]	V swell [mm^3]	F
L	*S. personatum BTB*	0.89	2.72	0.08	2.36	28.89
XXIXd	*S. schneideri*	1.09	2.57	0.15	2.00	13.17
XXVIIb	*S. schneideri*	1.32	3.71	0.27	5.99	21.94
XXXIIc	*S. schneideri*	1.37	2.72	0.30	2.36	7.87
XXXIIIb	*S. schneideri*	1.39	4.15	0.32	8.42	26.52
XXIXc	*S. schneideri*	1.44	3.26	0.35	4.08	11.56
LIII	*S. schneideri*	1.47	3.85	0.37	6.74	18.11
XXXIIb	*S. schneideri*	1.59	3.95	0.48	7.27	15.24
XXXVII	*S. schneideri*	1.64	4.05	0.52	7.83	14.95
XXIXb	*S. schneideri*	1.70	3.80	0.57	6.49	11.30
XLb	*S. schneideri*	1.80	4.05	0.68	7.83	11.47
IIL	*S. schneideri*	1.82	4.15	0.71	8.42	11.82
IILb	*S. schneideri*	1.82	4.15	0.71	8.42	11.82
XL	*S. schneideri*	1.90	3.95	0.81	7.27	9.03
XXXII	*S. schneideri*	1.90	4.30	0.81	9.36	11.62
XLIII	*S. personatum RB*	1.90	4.54	0.81	11.06	13.74
XXXIII	*S. schneideri*	1.92	4.30	0.84	9.36	11.17
XLII	*S. schneideri*	1.95	4.20	0.87	8.73	10.01
XXVb	*S. schneideri*	1.97	4.25	0.91	9.04	9.98
LV	*S. personatum BTB*	2.02	4.10	0.98	8.12	8.31
M ± SD	all above	1.64 ± 0.31	3.84 ± 0.56	0.58 ± 0.26	7.06 ± 2.46	13.93 ± 5.62

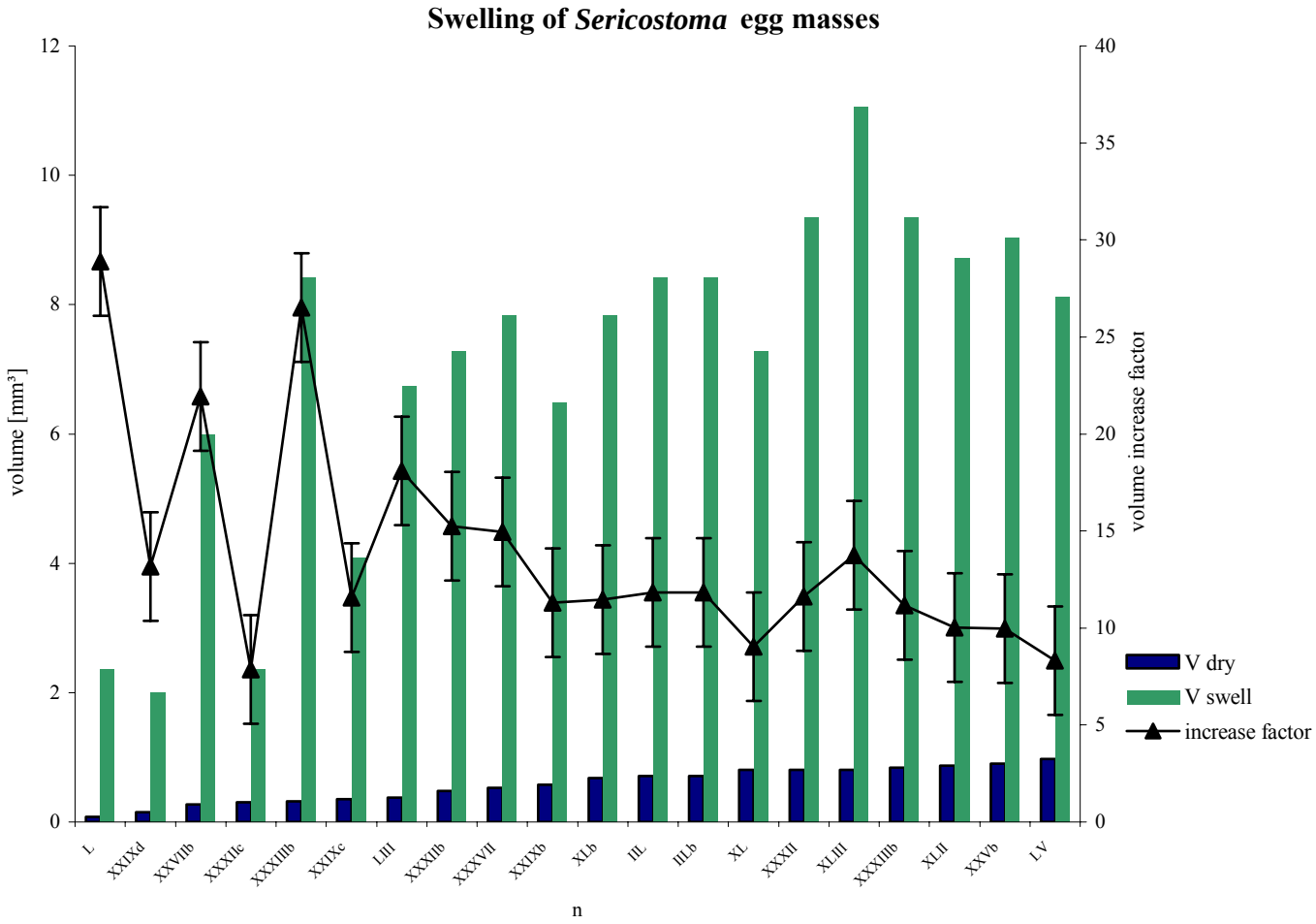

Fig. 10: Volume increase of 20 randomly selected egg masses of *S. personatum* (n = 3: LV, L, and XLIII) and *S. schneideri* (n = 17). Volume of dry egg masses was measured shortly after deposition and of swollen egg masses after seven days in the water-bath system. Volume increase factor with standard deviation (±) was calculated (secondary axis).

Only one egg mass deposited by a *S. personatum* female from the RB hatched successfully (fig. 11). It took 38 days for the first larvae to hatch and the last larvae hatched after 67 days. No larvae of *S. schneideri* hatched. Hatching number was almost constant until 75% of the larvae had hatched. Then, for one week, hatching was interrupted. The last almost 25% hatched in a short period after this week.

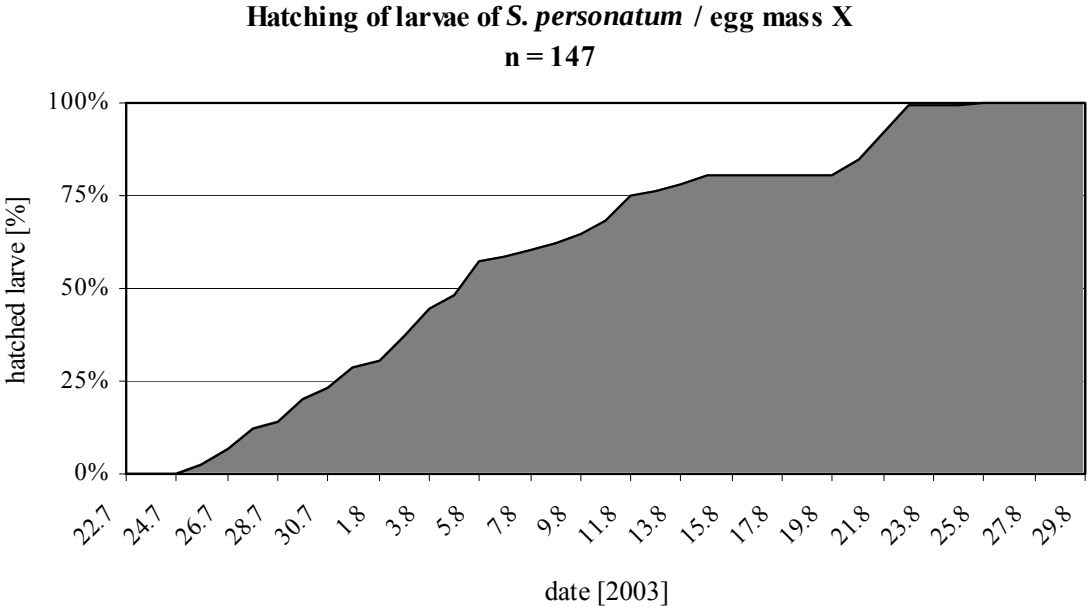

Fig. 11: Number of larvae (cumulative percent) hatched of the *S. personatum* egg mass (label: X); 3-day-means.

To investigate whether the constant temperatures were inhibiting development, on October 15th, some vials of *S. personatum* and *S. schneideri* were transferred from the thermo-constant water-bath system to the labstream with sinuously diurnal variation between minimum and maximum temperature in 24 h (see tab. 6). Nonetheless, up to now no hatching occurred.

Tab. 6: Rearing conditions of egg masses within the small glass vials in the recirculating laboratory streams 1 and 2.

labstream	min / max temp. [°C]	light condition
1	6 / 14	Darkness
2	8 / 12	Darkness

Until now, no more egg masses hatched, neither in the water-bath system nor in the laboratory streams exhibiting diurnally fluctuating temperatures. Some egg masses were already destroyed due to fungal infections or were eaten up by harpacticoids. The other egg masses still remain diapausing in the thermo-constant aquariums as in the daily fluctuating channels.

5.4. Discussion

5.4.1 Emergence

Since live span of single specimens is short and emergence only occurs in a short period, the differences in emergence time between *S. personatum* and *S. schneideri* might constitute a premating isolation mechanism leading to reduced gene flow in sympatric, potentially interbreeding populations. Additionally, it has to be noted that final instar larvae and pupae of *S. schneideri* were reared at constant 18°C in contrast to *S. personatum*, being reared at 16°C. Since development (in a certain range) usually increases exponentially with linearly increasing temperature (Begon et al. 1998), it can be assumed that development of *S. schneideri* would have been delayed even further at 16°C. The niche separation concerning the dimension 'time' would consequently be more prominent in nature than the observed delay of about two weeks (fig. 6).

Emergence pattern of *S. personatum* from the RB and BTB reared in labstreams did not differ significantly. It differed significantly from the emergence pattern of *S. personatum* in the field leading to the conclusion that constant temperatures modify the mode of development. This observation is congruent with the investigations of Wagner on the trichopteran species *Chaetopteryx villosa* (Wagner 1986 and unpublished results).

5.4.2 Diurnal activity

Emergence and mating at different times of specimens of both *Sericostoma* taxa indicates that they might represent separate groups. Wagner (1990) always observed mating couples of *S. personatum* in the early morning hours (~ 7 – 8 a.m.) in the field. This is congruent with the present observations. Crossmating trials might have been without success due to these differing activity patterns. Since crossmating was conducted only with five couples, this statement has to be taken with caution. Mating success of conspecific *Sericostoma* couples was also low (less than every second couple produced egg masses, see fig. 9). For the cryptic *Gumaga* species, Jackson and Resh (1991) proved that differences in diurnal activity were one major important factor maintaining premating isolation in sympatry between the separate lineages.

5.4.3 Aspects of reproduction

The fact that only one egg mass of *S. personatum* hatched in the laboratory was unexpected. At least 18% of the egg masses hatched in incubation experiments Wagner (1990) performed, using the same water-bath system and specimens from the same brook. In all brooks observed in the field (BTB and RB), first instar larvae were found in late summer and no egg masses were detected indicating that hatching of first instar larvae in the field occurred soon after the deposition of egg masses without diapause in contrast to the laboratory. A further comparison of the temperature dependence of *S. personatum* and *S. schneideri* was therefore impossible.

To search for reasons for this phenomenon, eggs were at first investigated under a stereo microscope to confirm partial development of the egg masses since the lack of hatching egg masses might otherwise be explained with the absence of fertilization. Partial development was observed and documented with photographs. Consequently, it must be assumed that egg masses in the laboratory underwent a dormant state, obviously a kind of diapause.

In a second step, rearing conditions of the experiment conducted here were compared with those of Wagner (1990) to search for differences that might explain the dissimilar outcomes. At least one was found: Wagner (1990) collected mating couples in the field, whereas in the present study final instar larvae and pupae were collected in the field and reared in the laboratory under artificial conditions. The conditions within the labstreams that differed most from natural conditions were the usage of constant temperatures (16°C for *S. personatum* and 18°C for *S. schneideri*) and a light regime (LD 12:12 h). Compared to the natural light regime in early summer this is relatively short. Temperature nevertheless was considerably higher than in the natural habitat at this time. These two parameters could possibly have initiated diapausing of *Sericostoma* egg masses. Examples for diapausing in other insect orders exist. It is a widespread phenomenon in life history of insects (Dathe 2003). Its adaptive value is (i) to survive unfavourably environmental conditions (Behrens 1984) and (ii) to synchronize the life cycle of insects, as Schmidt (1984) had shown for the aquatic Ephemeroptera *Baetis vernus*. Numerous different diapause types have been described (e.g. Mansingh 1971). Some authors still divide dormant states into diapause and quiescence. While the former is described as a genetically programmed and obligate dormant state, the latter is seen as a reaction to unfavourable environmental conditions and is nonobligtate. In reality, a distinction is often difficult and no general classification can be given (e.g. Beck 1980). Consequently, here diapause is referred to as a dormant state that is accompanied by markedly decreased metabolic rates (Behrens 1984). Diapause results from the exposure of sensitive stages to distinct stimuli and may occur in every stage of the life cycle, however, within one species it is generally confined

to a single and well-defined period proving the genetic basis. Among closely related species, this period can vary, indicating that diapause has evolved independently and that selective pressure is little affected by the stage in which it occurs (Behrens 1984). The quantity of the stimulus inducing diapause can vary between local populations inhabiting different habitats (e.g. Holzapfel and Bradshaw 1981). Initiation and termination of diapause are normally triggered by a particular set of environmental stimuli, most often temperature and photoperiod. The environmental signals are transduced into endogenous chemical messengers, neurohormones and hormones, in the endocrine organs. Neurohormones, often dopamine derivates (Noguchi and Hayakawa 2001), bring about the phase change from development to diapause or vice versa through a metabolic shift in the target organs or cells (Yamashita and Hasegawa 1985). In some species, e.g. *Bombyx mori* (Lepidoptera), the physiological state of the parents is known to affect the incidence of egg diapause (Behrens 1984; Xu et al. 1995).

In the present study direct development occured neither at high, medium, nor at low temperatures (except one egg mass incubated at chamber 4). Thus it must be assumed, that the stimulus triggering diapause has been induced earlier, probably by means of diapausing hormones (DH) produced by the female. Considerably high rearing temperatures in the labstreams might be associated with the production of DH in pupae or final instar larvae. High temperatures have proved to initiate diapause in B*ombyx mori* eggs through DH (Xu et al. 1995) and might be responsible here too.

An influence of the light regime remains uncertain. Often, long day conditions (quantity as well as quality) have proven to act as diapausing inductors in several insect taxa and only few taxa start diapause as reaction to short day conditions (Behrens 1984). Since adults were reared under almost natural light conditions, it is unlikely that this led to the production of DH in adults. Larvae pupae might be sensitive for short day conditions producing DH.

Egg mass number and swelling

Elliott (1969) observed that females might reemerge after deposition of egg mass and supposes that more than one egg mass might be produced and deposited. The present results support this assumption, since individual females even produced four egg masses, though smaller in size.

High variability of the volume increase factor was observed among smaller egg masses. This can be explained most likely with less precise measurements due to the smaller size rather than with different composition, since no different trend of swelling between small and big egg masses exists except the differences in variability. Thus it must be considered, that desic-

cation resistance does not differ significantly between both *Sericostoma* groups (*S. personatum* and *S. schneideri*). Further investigations concerning the composition of the egg matrix would be useful to prove similarity.

5.4.4 Conclusions

Differences in emergence patterns and diurnal activity support the hypothesis that *S. personatum* and *S. schneideri* represent distinct evolving groups. Nevertheless, niche overlap cannot be denied. Since only few animals were investigated, further studies must be conducted for confirmation.

Ecotypic and phenotypic plasticity concerns a high number of animal taxa (see Via et al. 1995) including the order Trichoptera (e.g. Hogue and Hawkins 1991; Klaithong 2003). To exclude that observed differences are results of phenotypic plasticity it would be useful to conduct reciprocal egg transfer experiments, as Wood and Resh (1991) did with the *Gumaga* species. Egg masses from *S. schneideri* inhabiting the lower stream regions should be transferred to spring-stream regions where usually *S. personatum* occurs (and vice versa). If morphology, emergence pattern and diurnal activity of adults hatched from the transferred egg masses were not altered, this would confirm the existence of a genetic basis of the differing phenotypes and support the postulation of two distinct species.

6. CHEMOTAXONOMIC INVESTIGATIONS

6.1 Introduction

The term 'pheromones' was introduced in 1959 to scientific discourse by Karlson and Luscher and refers to various volatile compounds, which act as intraspecific chemical messengers. Pheromones are secreted into the environment by one individual of a species and perceived by another individual of the same species, triggering a behavioural response or a physiological change. Reactions on pheromones range from the more obvious mate, food, and habitat location over alarm behaviour to the essential cohesion, coordination, and caste determination of social insect colonies (Birch and Haynes 1982). While pheromones are usually specific, some are common to several species, e.g. the lepidopteran pheromone component 7-Dodecenylacetate that is produced as sex pheromone by female elephants as well.

6.1.1 Pheromones in Trichoptera

From the 19th century onwards, 'scent' production in Trichoptera was reported (Müller 1888). Intensive studies of the compounds started only about 25 years ago (Duffield et al. 1977; Dettner 1979; Duffield 1981; Ansteeg and Dettner 1991). The major task of pheromones was believed to be a defensive one (Wagner et al. 1990). First proof that pheromones play a decisive role for mating systems[2] within Trichoptera was demonstrated by Wood and Resh (1984). Since then, attraction of conspecific males by a chemical extractable from females has been documented in six trichopteran families including the Sericostomatidae (Wood and Resh 1984; Resh and Wood 1985; Jackson and Resh 1991). In all investigations, the site of pheromone production was shown to be located around the fifth abdominal segment. Experimental procedures aiming at a more precise investigation of volatile pheromones were further developed, and in 1994, the first trichopteran sex pheromone was chemically described by Löfstedt et al. (1994). Most pheromones are biosynthetically derived from the lipid metabolism, modified by a special subset of enzymes (Bergmann 2002; Blomquist and Vogt 2003). In the order Trichoptera the production of short-chain secondary alcohols and ketones (C_6 – C_9) seems to be quite common (Duffield 1981; Löfstedt et al. 1994; Bergmann et al. 2002; Bergmann 2002). This proved to be the case in primitive Lepidoptera as well, whereas more aberrant lepidopteran taxa commonly use long-chain primary alcohols, aldehydes or acetates (Roelofs 1969; Bergmann 2002) (Fig. 12).

[2] According to Emlen and Oring (1977): General behavioural strategies employed in obtaining mates.

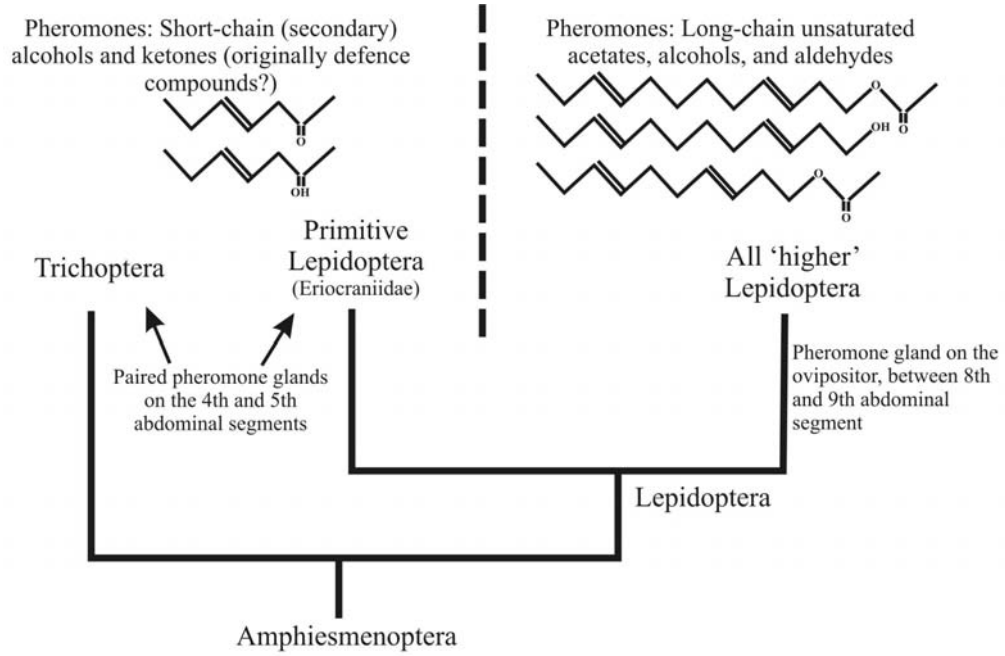

Fig. 12: Phylogenetic relationship between Trichoptera, primitive and more aberrant Lepidoptera, derived by chemical structure of sex pheromones used (drawn according to Löfstedt, http://www.pheromone.ekol.lu.se/detTrichoptera.html).

The potential influence of pheromones on speciation has been demonstrated for lepidopteran and trichopteran taxa: distinct pheromone communication between competitive, closely related species, provided premating isolation mechanisms that may prevent hybridization (Roelofs 1969; Klun et al. 1973; Cardé et al. 1977; Wood and Resh 1984; Resh and Wood 1985; Jackson and Resh 1991; Wood and Resh 1991). For the order Trichoptera, Houghton (2002) even described differences in pheromonal communication between geographically separated populations of one species, a fact he described as 'pheromonal dialects' (Houghton 2002). Bergmann (2002) discovered the existence of chemically different pheromones used in closely related species, but did not test its effect in field trials. In the USA, a similar situation occurs for two closely related species of the Nearctic sericostomatid genus *Gumaga*. Wood and Resh (1991) revealed that individuals of spring and stream populations were almost separated in sympatry by means of chemical communication. Dettner (1979) was the first to use the chemical differences in pheromonal communication for taxonomical purpose.

6.1.2 Aim of chemotaxonomic investigations

The aim of this study was to investigate whether *S. personatum* and *S. schneideri* can be successfully distinguished by the quantitative and qualitative composition of their pheromones

that might constitute a premating isolation mechanism. The morphology of the glandular system was superficially compared as well.

6.2 Material and methods

6.2.1 Material

66 specimens of two populations of *S. personatum* (BTB and RB, both Hesse), one population of *S. schneideri* from the river Fulda near Lütter (Hesse), and one population of *O. monedula* from the Engelbach (Hesse) provided the basis for research. The exact location of sampling sites and rearing of specimens is described in the 'material and methods: ecology' and 'material and methods: molecular genetics' chapters. After emergence, adults were kept separated in small glass vials. One to three days after emerging, living specimens were (i) transferred to n-hexane or dichloromethane filled vials to obtain body extracts (three to six specimens per vial, incubated over one night), or (ii) dissected under a stereomicroscope to obtain filled pheromone glands, which were placed in n-hexane or dichlormethane. Male and female specimens were separately used for analysis.

Unfortunately, only ten extracts were finally in the condition for investigation at the Institute of Organic Chemistry, University of Hamburg, by the working group of Prof. Dr. Dr. W. Francke. The other samples evaporated due to an extremely long postal transport to Hamburg.

6.2.2 Methods

In a first step, the extracts were concentrated (see tab. 7, 8) and afterwards analysed by means of coupled gas chromatography and mass spectrometry (GC/MC).

Tab. 7: 'Species', sample type, concentration, and abbreviation of dichloromethane samples investigated by FFAP:

species / sex	body / glands	n	concentration	abbreviation
S. personatum ♂	b	3	20 µl → 1 µl	P5
S. personatum ♀	b	6	20 µl → 1 µl	P6
S. schneideri ♂	b	3	20 µl → 1 µl	S3

Tab. 8: 'Species', sample type (whole body or single glands), concentration and abbreviation of dichlormethane and n-hexane conserved samples investigated by DB5MS:

species / sex	body / gland	n	carrier	concentration	abbreviation
S. personatum ♂	b	3	dichloromethane	15 µl → 1 µl	P5
S. personatum ♀	b	6	dichloromethane	20 µl → 1 µl	P6
S. personatum ♀	g	2	hexane	30 µl → 1 µl	P9
S. personatum ♀	g	2	hexane	30 µl → 1 µl	P10
S. schneideri ♂	b	3	dichloromethane	15 µl → 1 µl	S3
S. schneideri ♀	g	4	hexane	30 µl → 1 µl	S11
S. schneideri ♂	g	4	hexane	30 µl → 1 µl	S12
S. schneideri ♀	g	4	hexane	30 µl → 1 µl	S13
O. monedula ♀	g	2	hexane	30 µl → 1 µl	O2
O. monedula ♂	g	2	hexane	30 µl → 1 µl	O3

Principle of coupled gas chromatography and mass spectrometry

GC/MC is one of the most effective and popular methods to separate, identify, and quantify compounds in mixtures. The GC/MS procedure consists of two different methods used in combination: (i) GC performs the separation of the sample and characterizes components by their retention time and (ii) MS allows chemical identification by mass number, since every compound has a specific molecular weight and charge. These features allow separation and identification of almost all compounds.

1. GC: The concentrated sample, including all potential pheromones, is heated to transfer it from liquid to gaseous state. The gas then flows through a long, silica capillary column with a polar (FFAP) or a non-polar (DB5MS) stationary phase allowing the separation of chemically different compounds on the gas chromatograph. Each distinct chemical of the sample comes off the column at a known time interval and is collected. Once the metabolites of the samples are collected, they are moved to the mass spectrometer.

2. MS: The mass spectrometer determines compound identification by molecular weight and charge. The molecules are first ionized by bombardment with electrons. The charged molecule is then accelerated by an electric field and passes through a slit, which allows only certain particles to pass. Afterwards, the sample passes through a magnetic field of known strength. Sample and magnetic field have opposite charges, which causes a repulsion or deflection of the compounds. The compounds are made to pass through another slit into a collector. Because the strength of the electric and magnetic field is selectively controlled, the

mass of the charged molecule can be determined with great accuracy. A database containing information about mass number of ten thousands of molecules exists for comparison.

Equipment

A gas chromatograph (Hewlett-Packard 5890) was linked to a mass spectrometer (VG 70/250 SE) operated at 70 electron volt (eV). Two different separation columns were used for identification of low and high volatile components. Using helium as carrier gas, separations were achieved using a 50 m (length) x 0.25 mm (inner diameter), 0.25 µm film FFAP fused silica capillary column under the following conditions: 3 min 60°C, then programmed to 220°C at a rate of 3°C per minute. For high volatile components, a 30 m x 0.25 mm, 0.25 µm film DB5-MS fused silica column was used under the following conditions: 2 min 60°C, then programmed to 160°C at a rate of 10°C per minute and finally programmed to 280°C at a rate of 3°C per minute. 1 µl extract was injected splittlessly. With a pressure of 100 kPa (FFAP) and 70 kPa (DB5MS) the flow rate of the sample was 30 cm per second. Results are offered by a chromatogram including information about mass number.

6.3 Results

6.3.1 Morphology

Pheromone glands of the fifth abdominal segment exhibited different shapes according to their filling state. No differences between S. *personatum* and *S. schneideri* specimens were observed. In general, the glandular system consisted of a pair of ampulliform glandular reservoirs, surrounded by glandular tissue, connected to an expel channel. The porus of the channel ended at the dorso-lateral part of the abdominal sternite next to the membranous connection to sternite IV (Ivanov and Melnitsky 2002).

6.3.2 Qualitative and quantitative composition phermononal glands

GC/MS using FFAP columns (fig. 13)

GC/MS revealed short-chain volatile compounds in P5 (peak 121, 139) and S3 (peak 121,139) after a retention time of seven minutes. Relatively high amounts of 2-chlorocyclohexanol (peaks 1652 – 1655) were detected in S3, P4, and P5 after a retention time of approximately 32 minutes. Different amounts of 1,2-cyclohexanediol, 1-hexadecanol (peaks 3107 – 3110), 1-octodecanol (peak 3543 – 3546) and other long-chain hydrocarbons (peaks 3295 – 3298, 3378 – 3380) were detected in all three samples. In addition, only S3 showed long chain hydrocarbons in the range 35 – 45 minutes retention time (peaks 1853, 1948, 2002, 2146, and 2287). Extracts from female *S. personatum* (P6) revealed high amounts of isopropylic palmitate (peak 2841), while less hydrocarbons were present, and 1-eicosylacetate and 1-eicosanol (peaks 3886, and 4218) in males (P5).

Tab. 9: GC/MS analyses on FFAP columns; components identified by mass spectra (n.i. = not identified).

samples	peak	component	no. C atoms
S3, P4, P5	1652 - 1655	2-chlorocyclohexanol	C_6
S3, P4, P5	2372 - 2374	1,2-cyclohexandiol	C_6
S3, P4, P5	3107 - 3110	1-hexadecanol	C_{16}
S3, P4, P5	3295 - 3298	n.i.	$C_{17\text{-}21}$
S3, P4, P5	3378 - 3380	n.i.	$C_{17\text{-}21}$
S3, P4, P5	3543 - 3546	1-octadecanol	C_{18}
S3	1696 - 2287	div n.i.	$C_{17\text{-}21}$
S3	3229	3,4-methylendecanacid	C_{12}
P5	3886	1-eicosylacetate	C_{20}
P5	4218	1-eicosanol	C_{20}
P6	2841	isopropylpalmitate	C_{19}

GC/MS using DB5MS columns

Analyses on DB5MS confirm the compounds identified by FFAP separation (fig. 14). In addition, several volatile alkanes and alkenes, chemically located between tricosaen (peaks 1675 and 1676 in P5 and P6) and pentatriacontadien (peak 3508 in S3), were registered. S13 exhibited high amounts of long-chain fatty acids and corresponding ethylic esters (peaks 1209, 1483, and 1515) and lacked volatile components. Samples O2, O3, P9, P10, S11 and S12 appear to have only very few components in a detectable degree. All these components were found after eight to 12 minutes retention time (fig. 15). Scan 2570 is an electrical artefact.

Tab. 10: GC/MS analyses on DB5-MS columns; components identified by mass spectra.

samples	**peak (M+)**	**component**	**no. C atoms**
O2, O3, P9, P10, S11, S12	294 – 295	undecan	C_{11}
O2, O3, P6, P9, P10, S11, S12, S13	384 – 387	dodecan	C_{12}
O2, O3, P9, P10, S11, S12, S13	468 – 470	tridecan	C_{13}
P5, P6	1675	tricosen	C_{23}
S3	3508	pentatriacontadien	C_{2X}
S3	2784	cholesterol	C_{27}
S13	1173, 1209	palmitoleic and palmitic acid	C_{16}
S13	1483	stearic acid	C_{18}
P5	1512	ethylic ester of stearic acid	C_{20}

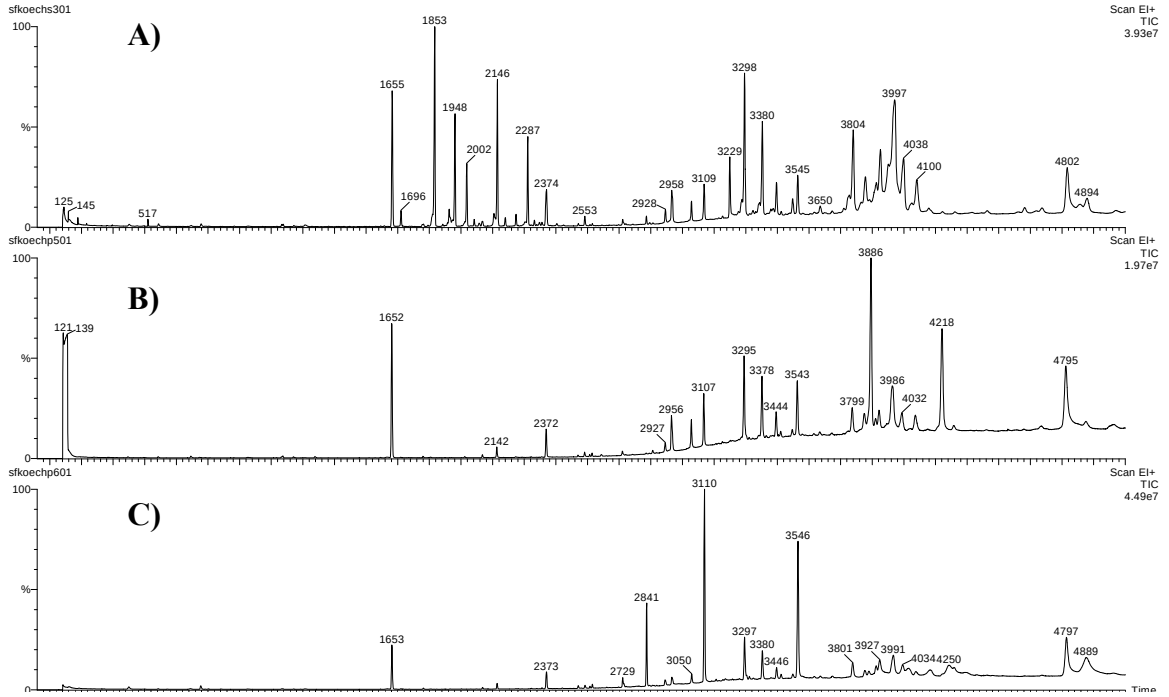

Fig. 13: GC/MS: Gas chromatograms and 70 eV mass spectra of dichloromethane extracts of whole male specimens of A) *S. schneideri* (S3), B) *S. personatum* (P5) and C) female *S. personatum* (P6) obtained with polar FFAP columns. Peak numbers represent mass number.

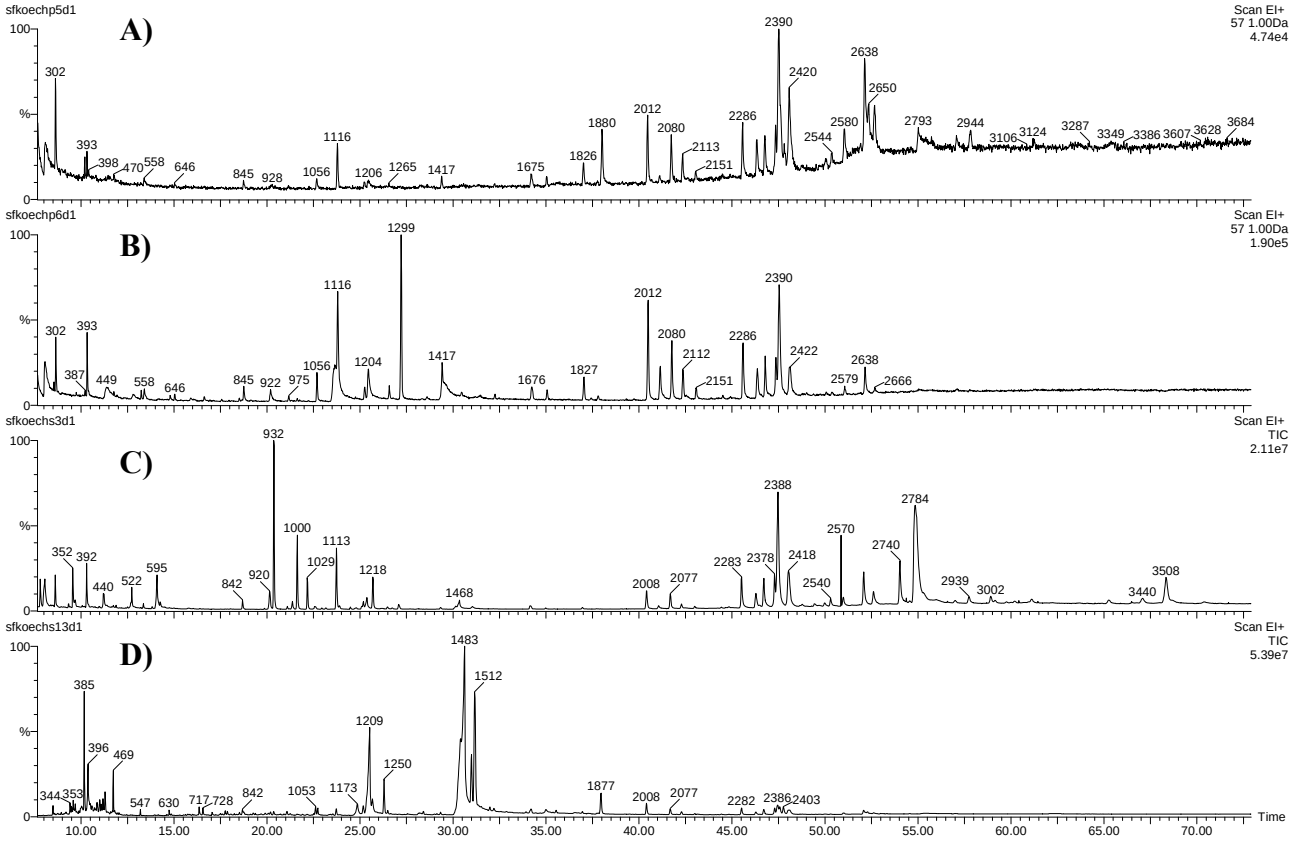

Fig. 14: GC/MS: Gas chromatograms and 70 eV mass spectra of dichloromethane extracts of complete male A) and female B) specimens of *S. personatum* (P5 and P6), C) male *S. schneideri* (S3), and D) of glandular hexane extracts female *S. schneideri* (13) obtained with non-polar DB5MS columns. Peak numbers represent mass number.

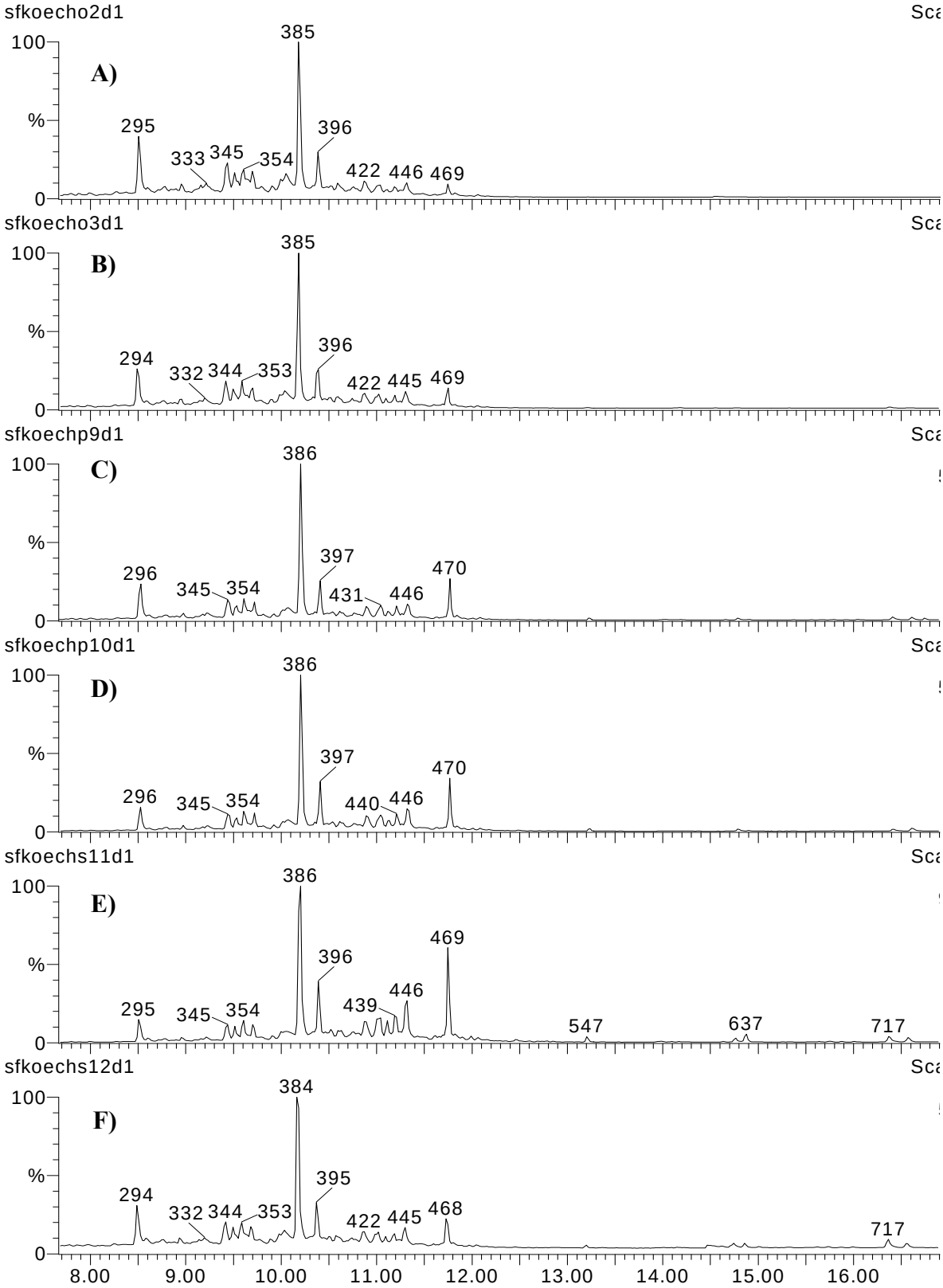

Fig. 15: GC/MS: Gas chromatograms and 70 eV mass spectra of hexane glandular extracts of A) male and B) female specimens of *Oecismus monedula* (O3 and O2), C) / D) female *S. personatum* (P9 and P10) and E) / F) male and female *S. schneideri* (S12 and S11) obtained with non-polar DB5-MS columns. Peak numbers represent mass number.

6.4 Discussion

No components that could be directly associated with pheromone-like chemicals were found within the samples containing only glandular extracts (O2, O3, S11, S12, S13, P9, and P10). This could be due to the low concentration (one to two pairs of glands per vial), to transport conditions in summer or to six-month storage until investigation. Body extract samples (S3, P5, and P6) revealed high amounts of long-chain hydrocarbons (> C_{17}). In the order Trichoptera sex pheromones usually appeared to consist of six to eight carbon atoms (Bergmann 2002), therefore it is uncertain, whether the detected components were pheromones, wax like metabolites that are commonly found on the chitinuous exoskeleton of insects (Dathe 2003), or simply other contaminants. Some components actually could act as pheromones (for example 1-hexadecanol, 3,4-methylendecanacid, 1,2-cyclohexandiol, tricosen, and 1-octadecanol), but since the few available samples differed significantly in composition, more samples have to be investigated for a representative result. In S13, many long-chain fatty acids (stearic acid, palmiate acid, cholesterol) were registered. This might be due to dissection conditions, for the glands were embedded in the surrounding tissue, which obviously was moved to the sample vials as well.

As shown for lepidopteran and dipteran taxa (e.g. Hamilton et al. 1999), long-chain hydrocarbons might play a major role in communication, despite their low volatile conditions. Usually the melting point of long-chain hydrocarbons is above 40°C. Since environmental temperatures in moderate climates seldom reach these high levels, it must be questioned, how or whether these components are held liquid or transferred to gaseous state. One possible answer might be that other short-chain hydrocarbons within the glands act as organic solvents (Veith, Wagner, pers. comm.).

Further investigations should focus on the analysis of glandular components, to ensure that components derived from the exoskeleton are almost excluded. For each extract, almost five to ten glands from species of one location should be used to guarantee high concentrations of the volatile components. Additionally, I would seriously recommend to secure fast and cold transport conditions and rapid analyses of the samples, otherwise the extracts are endangered by evaporation and the amount of the components detected is not representative anymore.

7. MOLECULAR GENETIC INVESTIGATIONS

7.1 Introduction

DNA sequence data can be used as molecular markers to resolve phylogenetic questions. Since the rate of sequence evolution varies extensively among different genes or DNA segments, it is possible to investigate evolutionary relationships on all levels of classification of organisms (kingdoms, phyla, classes, orders, families, genera, species, and intraspecific populations) (Nei 1996). DNA markers from a variety of different gene loci are used to trace phylogenetic signals. For most animal taxa mitochondrial DNA (mtDNA) markers have become commonly used for determining close phylogenetic relationships, since the generally maternal inheritance (Dawid and Blackler 1972), simple structure, small size, rapid evolution (Avise 1986), and the ease of isolation make mtDNA preferable to the nuclear DNA (Wolstenholme 1992). Many phylogenetic investigations using mtDNA as phylogenetic markers have been performed among insect orders (Pashley et al. 1993; Avise 1994) as well as on generic, species and population level (Avise 1986; Sperling 1993; Simon et al. 1994; Vogler and DeSalle 1994; Lunt et al. 1998; Brown et al. 1999; Nice and Shapiro 1999; Misof et al. 2000; Artiss et al. 2001; Wilcock 2001; Plague et al. 2001; Myers et al. 2001; Ribera et al. 2003). Nonetheless, as with any single cluster of tightly linked genes, phylogenies inferred with mtDNA may not correspond to the average gene phylogeny of the organisms involved. It remains important to establish the effectiveness of mtDNA as phylogenetic marker and to investigate conditions under which variation in the molecule may fail to indicate species boundaries (Sperling 1993). In order to delimit species boundaries, it is essential to prove the absence of gene flow. This cannot be achieved by the use of mtDNA and consequently the investigation of a second, nuclear locus is always useful.

In particular the fact that molecular markers proved to be a powerful tool for the detection of morphological cryptic evolving lines (Scholl and Pedroli-Christen 1996; Jackson and Resh 1998; Landry et al. 1999; Morrow et al. 2000; Muller 2000; Scheffer 2000; Morehead et al. 2001; Pfenninger et al. 2003) led to the investigation of the *Sericostoma* species complex with these methods.

Molecular genetic investigations in this work are subdivided into two major parts:

(i) Analysis of the *Sericostoma* species complex with particular focus concerning the two Central European forms *S. personatum* and *S. schneideri*

(ii) Investigation of the phylogenetic relationship between European Sericostomatid genera.

7.1.1 The *Sericostoma* species complex

The primary aim of molecular genetic investigations was to unravel the genetic structure of the Central European *Sericostoma* complex. Schmidtke (1994) already tried to resolve the taxonomic status of the problematic European species *S. personatum* and *S. schneideri* (former *S. flavicorne*) using allozyme technique. Unfortunately, the outcome of this work lacked clear results. Comparing the available allozyme data with other arthropod distance data, Schmidtke (1994) carefully stated that *S. personatum* and *S. schneideri* exhibit genetic distances usually observed between subspecies. Thus, results were not very expressive, since only two populations of *S. personatum* were investigated. A more detailed molecular genetic study dealt with the cryptic nearctic *Gumaga* complex (Sericostomatidae) (Jackson and Resh 1998). Jackson and Resh (1998) discovered that almost six separately evolving lines, lacking morphological differences, exist in Californian freshwater.

For this work, fragments of two mitochondrial (COI and 16S rDNA) and one nuclear gene (ITS-1) were chosen for investigation. All three, especially COI and ITS-1, have previously proven to exhibit adequate variability in genera and species level phylogenies (Vogler and DeSalle 1994; Schlötterer et al. 1994; Chang et al. 1997; Smith and Kambhampati 1999; Brown et al. 1999; Landry et al. 1999; Scheffer 2000; Wahlberg and Zimmermann 2000; Misof et al. 2000; Artiss et al. 2001; Damgaard and Sperling 2001; Myers and Sperling 2002; Bychek and Müller 2003).

Actually, it is undeniable that particularly in Central Europe the distinction between spring and spring-stream on the one side *(S. personatum*) and lower stream regions inhabiting populations on the other side (*S. schneideri*) is often possible (see fig. 1) (Pitsch 1993; Neu 2002). Neither genetic studies nor reciprocal rearing trials have been conducted in order to find out, whether these obvious phenotypic differentiations between *S. personatum*, *S. schneideri* as well as the other *Sericostoma* species (*S. vittatum*, *pyrenaicum*, *flavicorne*, *galeatum*, *herakles)* are correlated to significant amounts of genetic differentiation.

With the molecular genetic approach, several competing hypotheses are tested:

1. Do *S. persoantum* and *S. schneideri* represent genetically distinct evolving groups as assumed by most scientists (Botosaneanu and Malicky 1978; Pitsch 1993; Schmidtke 1994; Neu 2002; Robert 2003)?

2. Does S. *personatum* consist of several detectable geographically separated 'races' (Wagner 1990)?

3. Do *S. schneideri* and *S. flavicorne* represent separate evolving groups as proposed by Botosaneanu (2001)?

4. Do *S. schneideri* and *S. pyrenaicum* represent one entity, as proposed by Botosaneanu and Malicky 1978?

7.1.2. Phylogenetic relationship between European sericostomatid genera

A second aim of the molecular genetic investigation was the inference of the phylogeny of the family Sericostomatidae Stephens, 1836. Until know, no phylogeny hypothesis has been proposed, neither based on morphological nor on molecular data (Kumanski, pers. comm.). Since only larvae of certain taxa were available, comparative morphological investigation was excluded here. Molecular markers of species of all European genera except *Cerasma*[3] were investigated and phylogeny was estimated by several contemporary methods.

[3] Only one species is described from the Caucasus and one from Turkey.

7.2 Material and Methods

7.2.1 Material and collection sites (tab. 11)

For genetic analyses, freshly collected specimens were transferred into 70 – 80% ethanol and stored at 4°C to minimize enzymatic breakdown of the DNA. Specimens from several regions of Europe were kindly provided by several scientists and international museums. Unfortunately, the material was often older than 10 years and stored in alcohol of low quality. For that reason, it was often impossible to isolate an adequate amount of DNA.

Tab. 11: List of specimens (adults / larvae) and sampling sites used in this study, their abbreviations and geographical position. Individual label code: Country_collector_morphospecies + number (e.g. D_FL_SP01 = Germany (D), collected by F. Leese, *S. personatum* 01,).

genus* / *species	**individuals**	**larvae (L) Imago (I)**	**country**	**description sample site**	**site**	**coordinates**
Oecismus						
O. monedula	D_FL_OM01 D_FL_OM02	L L	Germany	Hesse, Biedenkopf, Engelbach	O1	N 50°55' E 8° 36'
O. monedula	D_PN_OM01	L	Germany	Rhineland Palatinate, Seffern, Balesf. Bach	O2	N 50°5' E 6° 29'
O. monedula	BG_SP_OM01	I	Bulgaria	Cerni Iskar River, Vezhen	O3	N 42°8' E 23°35'
Schizoplex						
Sch. festiva	E_MG_SchF03 E_MG_SchF04	I I	Spain	Galicia, Zamora, Rio tera	SchF1	N 41°50' E 6°0'
Notidobia						
N. ciliaris	D_BR_NC01 D_BR_NC02	L L	Germany	North Rhine-Westfalia, Bad Lippspringe	N1	N 51°47' E 8° 44'
Sericostoma						
S. personatum	D_FL_SP01 D_FL_SP02	L L	Germany	Hesse, Schlitz, Breitenbach	SP1	N 50°39' E 9°37'
S. personatum	D_FL_SP03	L	Germany	Hesse, Rhön, Wasser-kuppe	SP2	N 50°30' E 9°56'
S. personatum	D_FL_SP10	L	Germany	Hesse, Rimbach Weihersbrunnen,	SP3	N 50°43' E 9°33'
S. personatum	D_FL_SP20 D_FL_SP21	L L	Germany	Hesse, Rodenbach Unter-Wegfurth	SP4	N 50°45' E 9°33'
S. personatum	D_PN_SP01 D_PN_SP02	L L	Germany	Baden-Württemberg, Hinterzarten	SP5	N 47°57' E 8° 5'
S. personatum	D_PN_SP11	L	Germany	Rhineland Palatinate, Bauler, Gaybach	SP6	N 50°20' E 6° 51'
S. personatum	D_HR_SP01 D_HR_SP02	I I	Germany	Lower Saxony, Seeve	SP7	N 53°19' E 9° 57'
S. personatum	D_BR_SP01 D_BR_SP02	I I	Germany	North Rhine-Westfalia, Brünen, Hamminkeln	SP8	N 51°43' E 6° 40'
S. personatum	D_BR_SP08	I	Germany	North Rhine-Westfalia, Lichtenau	SP9	N 51°36' E 8° 54'

genus / species	individuals	larvae adults	country	description sample site	site	coordinates
S. personatum	D_PZ_SP01	L	Germany	Lower Saxony, River Zorge	SP10	N 51°39' E 10°38'
S. personatum	CH_VL_SP01 CH_VL_SP02	I I	Switzer-land	Pilatus, Mattalp	SP11	N 46°59' E 8°15'
S. personatum	CH_JG_SP01	I	Switzer-land	Waalensee, Weesen	SP12	N 47°10' E 9°5'
S. personatum	CZ_PK_SP01 CZ_PK_SP02	L L	Czech Republic	Near BRNO, Mokrá Hora	SP13	N 49°15' E 16°35'
S. personatum	CZ_PK_SP10	L L	Czech Republic	Jihlava, Moraviann Highlands, Smrčesnký	SP14	N 49°25' E 15°34'
S. personatum	BY_SC_SP1 BY_SC_SP2 BY_SC_SP3	L L	Belarus	Ruczei, Kemeliszki	SP15	N 54°49' E 25°49'
S. personatum	F_HM_SP01	I	France	Col du lautaret, hautes alpes	SP16	N 45°01' E 6°24'
S. personatum	IRL_JC_SP1 IRL_JC_SP2	I I	Ireland	Lough Inchiquin, Corofin,LAKE	SP17	N 52°58' W 9°3'
S. personatum	FIN_JI_SP1	L	Finland	Vihiti, Myllypuro Nuuksion kp.	SP18	N 60°15' E 24°30'
S. personatum	N_JS_SP01 N_JS_SP02	L L	Norway	River Dalaa, near Trondheim	SP19	N 63°26' E 11°26'
S. personatum	S_BM_SP01	L	Sweden	Västerbotten, Kulla-bächen, Umeå	SP20	N 63°52' E 20°11'
S. schneideri	D_FL_SS01 D_FL_SS02 D_FL_SS03	L L L	Germany	Hesse, River Fulda, near Lütter	SS1	N 50°28' E 9°45'
S. schneideri	D_HR_SS02	I	Germany	Schleswig Holstein, River Bille	SS2	N 53°37' E 10°25'
S. schneideri	D_PN_SS07	L	Germany	RP, Bauler, Gaybach	SS3	N 50°20' E 6°51'
S. schneideri	D_BR_SS02	I	Germany	NRW, Atteln, Lichte-nau, Altenau	SS4	N 51°35' E 8°47'
S. schneideri	A_JW_SS06	L	Austria	Lunz, Lunzer Seebach	SS5	N 47°51' E 15°04'
S. schneideri	A_HM_SS01	I	Austria	Lunz, Kothbergbach	SS6	N 47°53' E 15°2'
S. pyrenaicum	E_MG_SPy3 E_MG_SPy4	I I	Spain	Lugo, Valdomir, Rio Lor	SS7	N 43°2' W 7°33'
S. flavicorne	TR_FS_SF01 TR_FS_SF02	I I	Turkey	Fethie, Gelemis Seki Gay	SF1	N 36°29' E 29°17'
S. vittatum	E_MG_SV01 E_MG_SV02	I I	Spain	Galicia, Rio Ortigal, Os cabamnos,	SV1	N 49°15' W16°35'
Potamophylax						
P. cingulatus	D_RW_PC01 D_RW_PC01	L L	Germany	Hesse, Schlitz, Brei-tenbach	SP1	N 50°39' E 9°37'
P. luctuosus	D_RW_PL01 D_RW_PL02	L L	Germany	Hesse, Schlitz, Brei-tenbach	SP1	N 50°39' E 9°37'

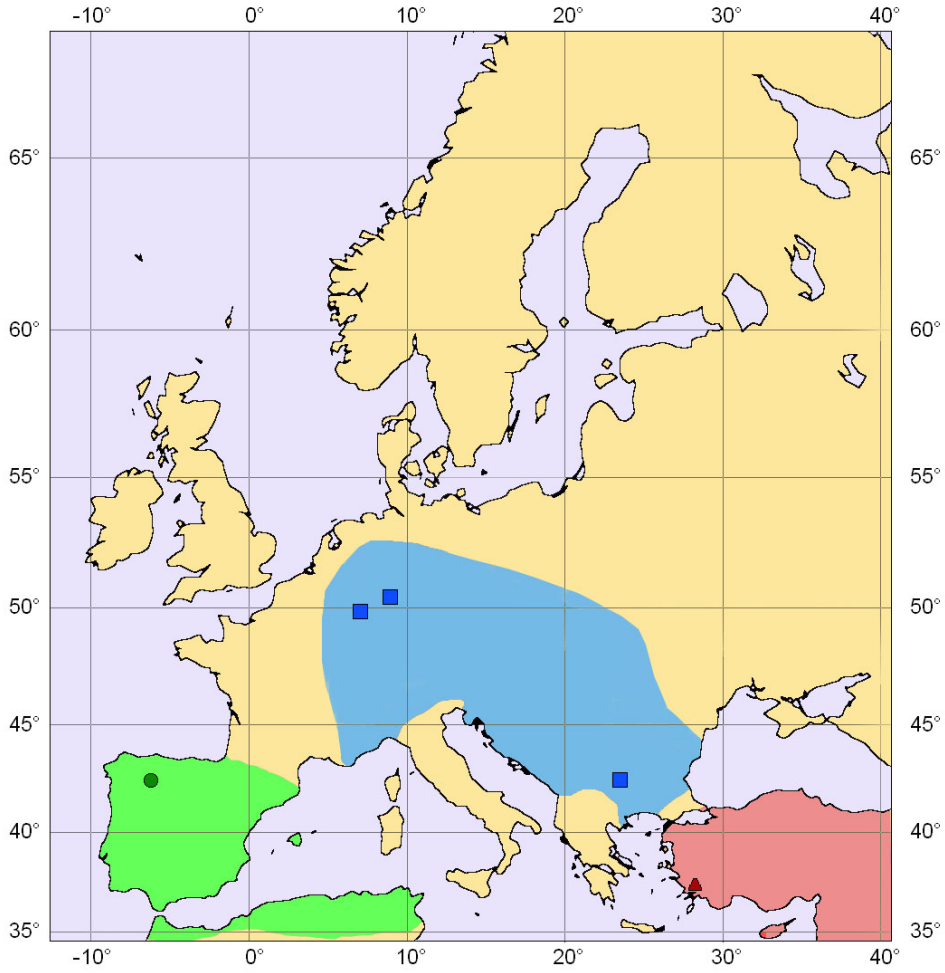

Fig. 16: Distribution and sampling sites of *Schizoplex festiva* (green circle), *Oecismus monedula* (blue squares) and *Sericostoma flavicorne* (red triangle) (after Botosaneanu and Malicky 1978; Tobias and Tobias 1981).

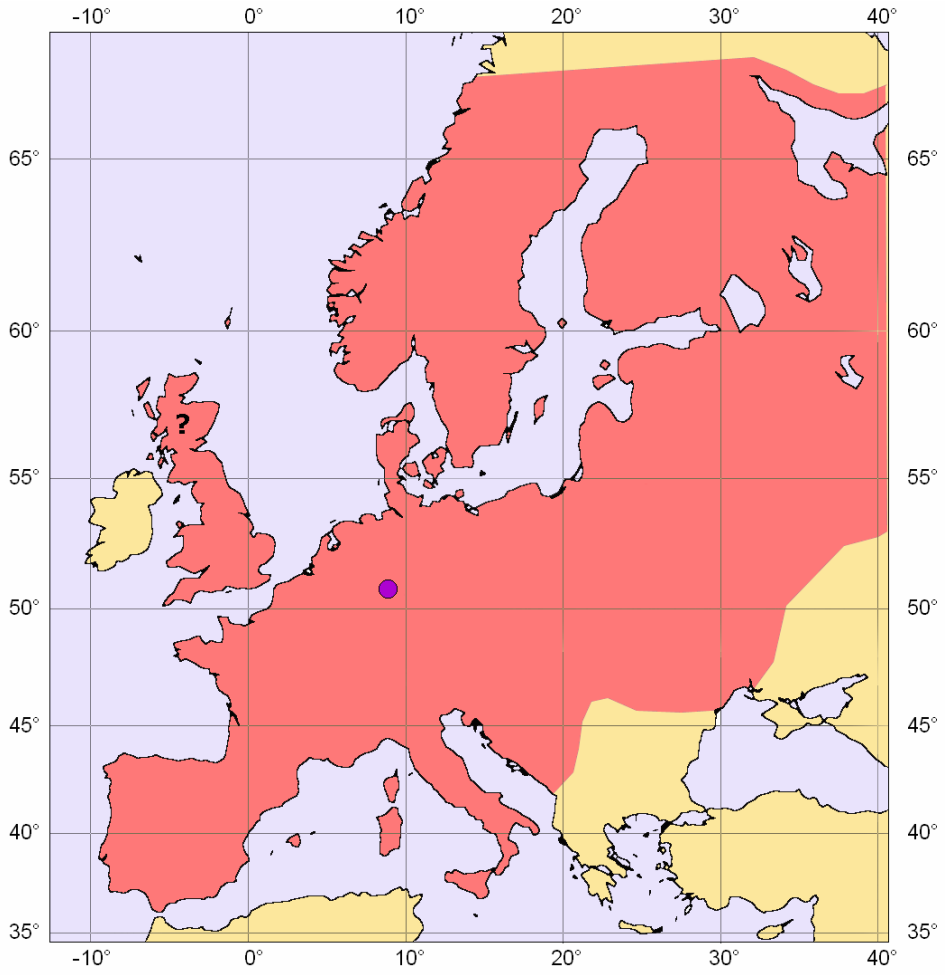

Fig. 17: Distribution and sampling site of *Notidobia ciliaris* (purple circle) (after Botosaneanu & Malicky 1978; Tobias and Tobias 1981).

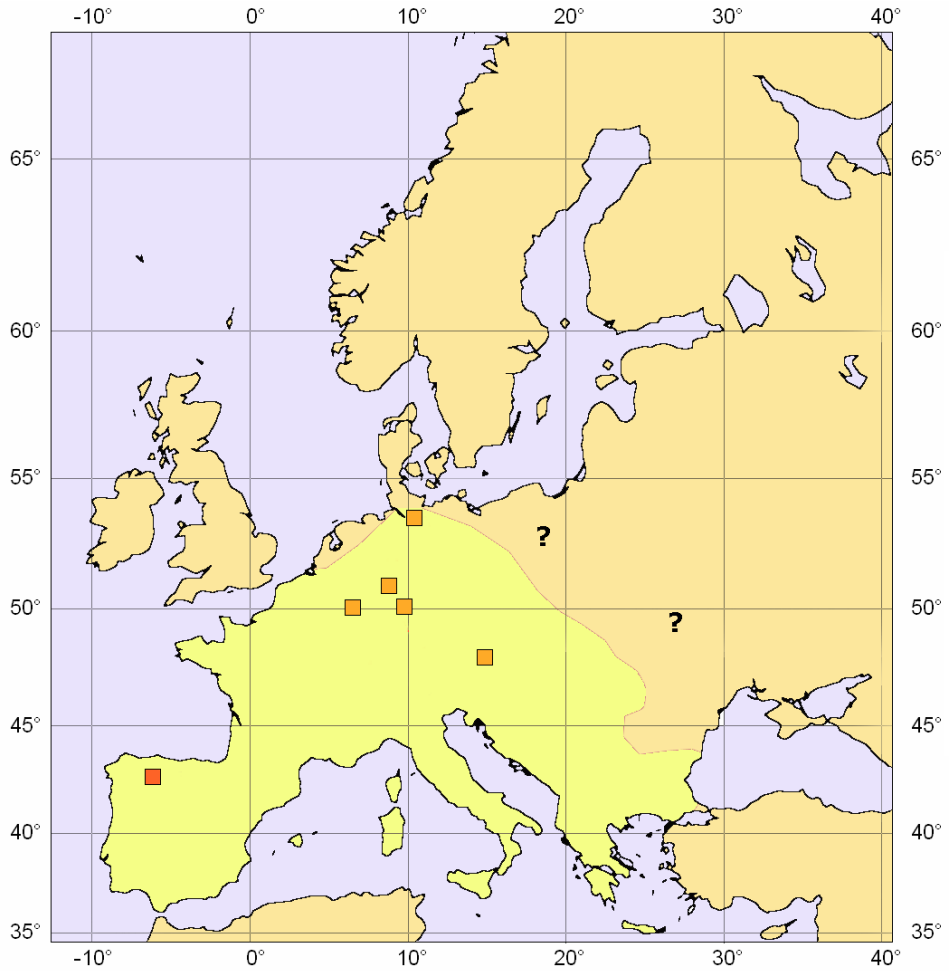

Fig. 18: Distribution (after Botosaneanu & Malicky 1978; Tobias and Tobias 1981) and sampling sites of *S. schneideri* (orange squares), and and *S.* cf. *pyrenaicum* (red square). According to the authors, both species are said to be synonymous. Question marks indicate regions where distribution is uncertain.

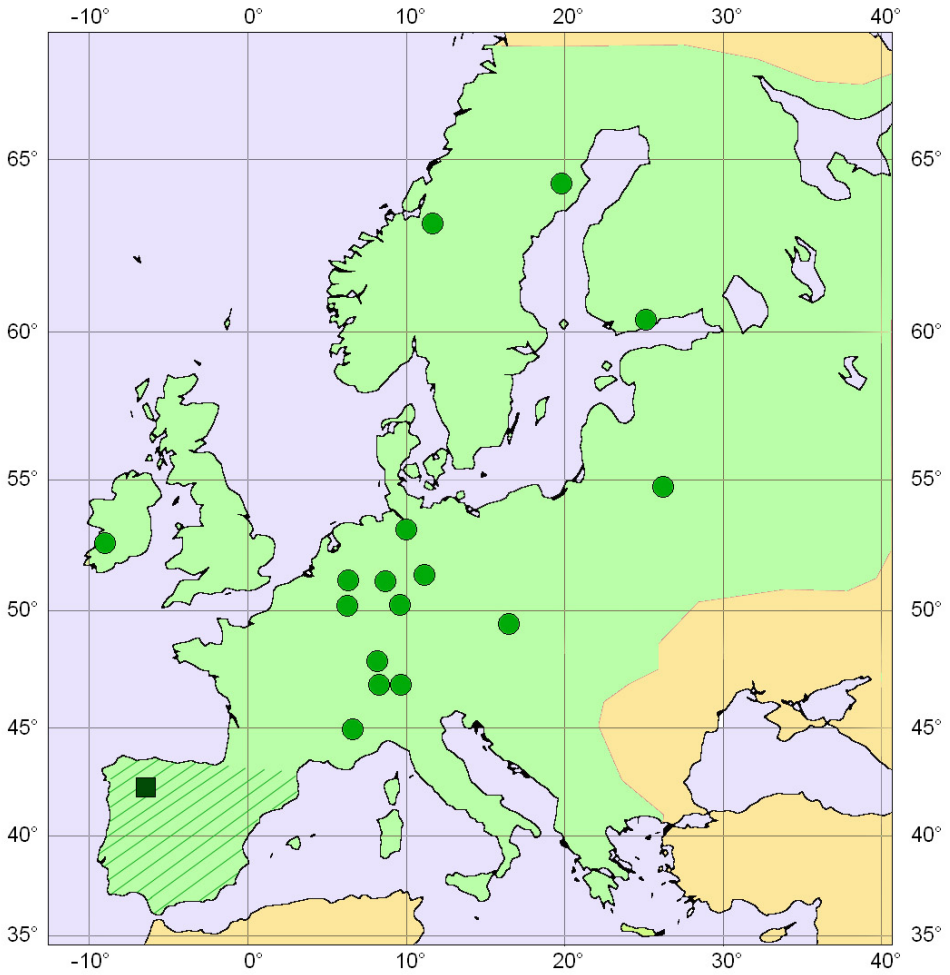

Fig. 19: Distribution (after Botosaneanu & Malicky 1978; Tobias and Tobias 1981) and sampling sites of *S. personatum* (green circles) and *S. vittatum* (green square). Distribution of *S. vittatum* is restricted to the Iberian Peninsula (lined parts of the map)

7.2.2 Laboratory methods

All laboratory work was carried out under sterile conditions using sterile tubes and pipette tips to minimize the risk of contamination. For all solutions, sterile distilled water was used. Forceps and scalpels used for dissection of specimens were meticulously cleaned and flamed using 70% ethanol.

DNA Isolation

The first step in order to get sequence data from the specimens collected is the isolation of nucleic acids, in our case DNA. In metazoans, DNA is found within the cell nucleus and in mitochondria.

At first, either thoracic muscle tissue as well as abdominal tissue was used. As the results did not differ, for subsequent DNA isolation tissue from the abdominal segments I – VIII was used. Depending on the size of the alcohol-stored larvae, one to eight abdominal segments (~ 3 mm^3) were dissected. The gut was carefully removed to reduce the risk of contamination and the surrounding tissue was used for DNA isolation. In case of the imagines the tissue of the abdominal segments was used without removing gut contents as in the order Trichoptera the gastrointestinal system is generally reduced and therefore the risk of contamination as well.

Isolation of nuclear and mitochondrial DNA was performed using the 'QIAamp® DNA Mini Kit' (Qiagen) according to the manufacturers 'Tissue Protocol' (02/2003) and the 'NucleoSpin® Tissue Kit' (Macherey & Nagel) according to the 'Standard protocol for human or animal tissue' (04/2000).

Methodology of both kits is similar: after breaking down cell layers and proteins by incubating tissue at 56 °C with proteinase K, the nucleic acids are released from their organelles (mitochondria, nucleus). In presence of chaotropic salts[4] nucleic acids are bound at a patented silicia membrane whereas all other cellular components and DNA fragments shorter than 100 bp are let through. The selectively bound DNA is released from the silica membrane after two three washing steps using a Tris-HCl (5 mmol, pH 8.5) buffer. In contrast to the recommended 200 µl elution buffer (AE, resp. BE-Buffer) only 100 µl were used to achieve higher

[4] Chaotropic salts disrupt the regular hydrogen bond structures in water. They denature proteins due to their ability to disrupt hydrophobic interactions. Nevertheless, they do not denature DNA and RNA. Their function during DNA isolation procedure is to denature cellular proteins (such as DNase and RNase). The high concentration of salts also neutralizes the negative charge of the silicia membrane and charges it positively – a fact that facilitates binding of the nucleic acids to the silica membrane in the column (see Mülhardt, http://www.biotech-europe.de/rubric/methoden/ methoden/v26.html).

concentrations of the isolated DNA. For short terms, DNA was stored at 4 °C, for longer terms at -20 °C. Attempts to isolate DNA from material stored in alcohol of low concentrations failed with the extraction kits as well as following the Chelex® isolation procedure (Singer-Sam et al. 1989; Walsh et al. 1991).

Chelex® isolation was performed placing approximately 1 mm^3 tissue into 300 µl of 5% Chelex® suspension. This sample was incubated at 98°C for 5 minutes. Afterwards the sample was centrifuged at 13.000 rpm (max. speed) for 5 minutes. During this process, divalent metal ions (chiefly Mg^{2+}) that activate enzymes (e.g. DNases) are bound and the denatured DNA is released into the medium where it can generally be found in adequate amounts within the supernatant. From old material, no reasonable amount of DNA was detected within the supernatant.

Genitalia and thorax with head were stored in well-labelled plastic vials. Success of isolation was controlled by gel electrophoresis.

Gel Electrophoresis

One of the most widespread methods to study nucleic acids is gel electrophoresis (Sambrook et al. 1989). Electrophoresis is a procedure to separate charged biomolecules, here DNA, in electric fields. Migration rate is determined by molecule size and electric charge. In this study, electrophoresis methods were used for control of DNA isolation, amplification and purification on the one hand and for sequencing of fluorescent-labelled DNA fragments on the other hand.

For the former, horizontal gel electrophoresis was used. The DNA sample was first mixed with bromphenole-blue for visual control to weight down the sample. Second, the sample was applied onto a horizontal agarose gel that represents a complex network of fibrils. Pore size of the gel can be varied with the amount of agarose. As a matter of size, smaller DNA molecules migrate faster than large molecules through the gel towards the positively charged anode. After a defined migration time, molecules in the gel are assessed by staining with ethidiumbromide. Ethidiumbromide binds with high affinity to the double-stranded DNA molecule. If the DNA is controlled under an ultraviolet light source, it fluoresces after the staining procedure. To characterize the length of the DNA fragment, a mix of DNA molecules of defined length (‘ladder mix’) is applied onto the gel with the sample DNA. The observed bands from the samples are compared to the bands of the ladder mix to distinguish the lenght of the DNA molecule. For sequencing, a vertical polyacrylamide gel was used (see below).

To roughly estimate quality and quantity of the isolated and amplified DNA, 3 µl of the isolated DNA were mixed with 2 µl loading dye and applied onto a 0.8%, respectively 1% horizontal agarose gel. Conditions for electrophoresis were 100 V for 30 minutes. Afterwards the gel was stained for two minutes with the ethidiumbromide. To remove surplus ethidiumbromide, the gel was kept for two to 30 minutes in distilled water and finally photographed under uv light.

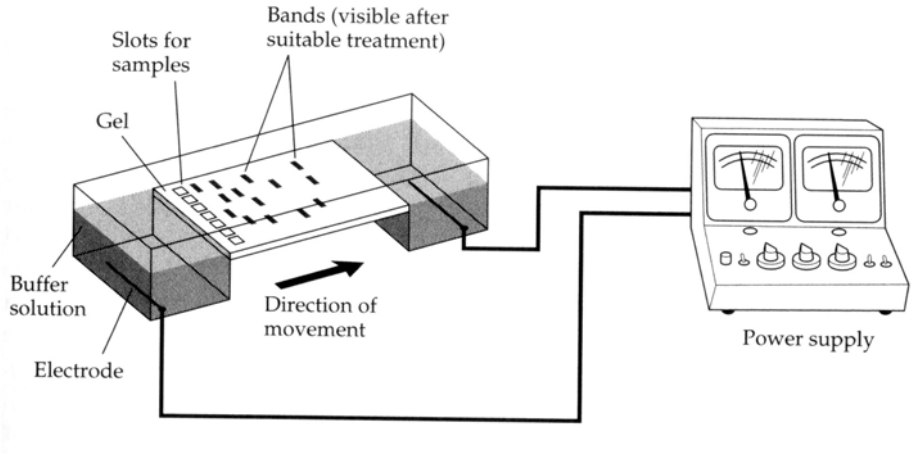

Fig. 20: Principle of horizontal agarose gel electrophoresis (after Hartl and Clarke 1997).

Investigated Genes

For the molecular systematic study, two mitochondrial and one nuclear gene (ITS-1) were chosen as markers:

Tab. 12: Locus and length of the gene fragment chosen for investigation.

gene	locus	length
16 S ribosomal DNA	mitochondrial DNA	~ 500 bp
cytochrome oxidase I	mitochondrial DNA	~ 450 bp
internal transcribed spacer 1, 2, and 5.8S rDNA	nuclear DNA	~ 1.000 bp

16S rDNA encodes for a single strand of rRNA that is later folded to pair with itself forming a secondary structure composed of helical ‘stems’ connected by unpaired ‘loops’ (see fig. 23). The 16S rRNA is an important structural element of the large subunit of the mitochondrial ribosomes. The cytochrome oxidase I is an enzyme of the complex within the respiratory chain that transfers electrons from cytochrome c to molecular oxygen, which is reduced to

water by accepting two protons (Babcock and Wickström 1992). Both mitochondrial genes were investigated separately and together.

DNA Amplification

For successful sequencing, DNA molecules of research interest have to be available in high quantity. Their selective amplification is achieved by a procedure called PCR (Polymerase Chain Reaction) (Saiki et al. 1986; Mullis and Fallona 1987; Saiki et al. 1988). Millions of copies from a single DNA template molecule may be produced with this technique[5].

PCR is an *in vitro* method to synthesize defined DNA sequences in high amounts. The reaction uses a surplus of two specific, approximately 17 – 25 bp long oligonucleotides, called primers. After the DNA double-strand is divided into single strands at high temperatures (DENATURATION), temperature is decreased to allow hybridization of specific primers to the target DNA sequence that is to be amplified (ANNEALING). For elongation of primers, a heat-stable DNA polymerase I with a temperature optimum at 72 °C is used. As the polymerase was originally isolated from the bacterium *Thermophilus aquaticus* that inhabits hot springs, it is called *Taq*-polymerase. *Taq*-polymerase attaches nucleotides at the 3'-OH-ends of the primers during ELONGATION and synthesizes the complementary strand of the template. Elongation time depends on the length of the amplified gene. After the elongation step the newly formed double-strands are again divided during the next denaturation step. A repetitive series of cycles, involving template denaturation, primer annealing, and elongation of the annealed primers by the *Taq*-polymerase, results in the exponential accumulation of the specific DNA fragment (see fig. 21).

Amplifications were conducted in a 25-µl total volume. *Taq*-polymerase and buffers of the companies Qiagen and Eppendorf were used according to the manufacturer's information (see tab. 13). To achieve good results a hot-start method was used placing the PCR-tubes in the thermocycler at the time it reached the denaturation temperature.

After the PCR-procedure 3 µl of the solution were mixed with 2 µl loading dye and controlled by gel electrophoresis. One negative control (without DNA) and one positive control (DNA that had proved successful amplification) were always investigated at each amplification step.

[5] PCR has changed the course of molecular biology and the scientists developing the technique were honoured 1993 with the Nobel Prize.

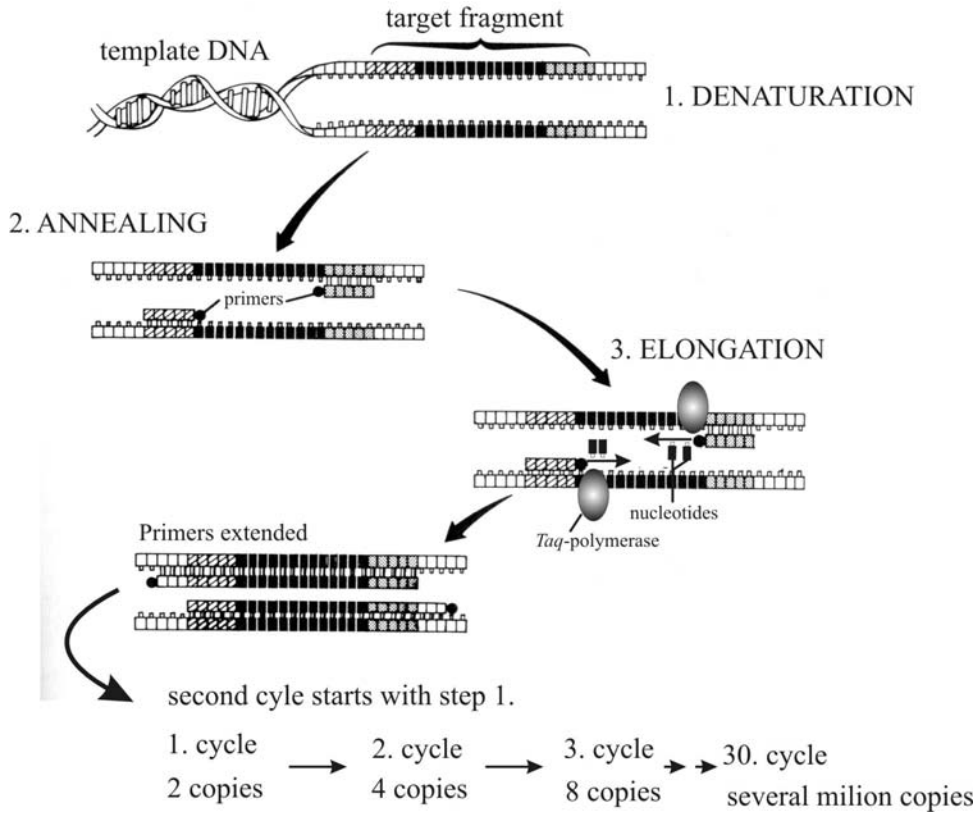

Fig. 21: Principle of the Polymerase Chain Reaction (mod. after Kirby 1990).

Tab. 13: PCR mixes used for amplification of gene fragments.

ingredients	**mix A** (amplif. of 16S / COI / ITS)	**mix B** (amplif. of 16S / COI)
10 x Puffer	2,5 µl	2,5 µl
dNTPs [2,5 mM]	2,0 µl	2,0 µl
Q-Solution / MgAC-Solution	-	5 µl
Primer 1 [100 mM]	0,25 µl	0,25 µl
Primer 2 [100 mM]	0,25 µl	0,25 µl
Taq-Polymerase	0,2 µl	0,2 µl
DNA	1 - 3 µl	1 – 3 µl
H_2O	filled up to 25 µl	filled up to 25 µl

Tab. 14: PCR reaction profiles for the genes investigated; function of the different thermal reaction steps.

gene	temperature	time	no. of cycles	function
16S rDNA	94°C	5 minutes	1	Inital denaturation
	94°C	45 sec	38	Denaturation
	52°C	45 sec		Annealing
	72°C	120 sec		Extension
	72°C	7 min	1	Final extension
COI	94°C	4 min	1	Inital denaturation
	94°C	45 sec	36 / 38	Denaturation
	42°C	45 sec		Annealing
	72°C	120 sec		Extension
	72°C		1	Final extension
COI	94°C	5 min	1	Inital denaturation
	94°C	45 sec	36 / 38	Denaturation
	52°C	45 sec		Annealing
	72°C	80 sec		Extension
	72°C	7min	1	Final extension
ITS1	94°C	5 min	1	Inital denaturation
	94°C	30 sec	35	Denaturation
	52.5°C	50 sec		Annealing
	72°C	200 sec		Extension
	72°C	7 min	1	Final extension

PCR Primer

Success of PCR depends to a high degree on the presence of primers that hybridize firmly to the ends of the DNA fragment of research interest. Therefore, knowledge of short conserved sequences within the gene fragment is essential for selective construction of primers. For the 16S rDNA universal primers already employed successfully for study of several arthropod classes were used (Simon et al. 1994). For the COI fragment primers were constructed. Since the complete mitochondrial genomes of several insect species (see GenBank of the NCBI) had been sequenced, it was possible to align homologous COI sequences of several arthropod species using the program Clustal X (Thompson et al. 1997). Conserved regions were identified using the program BioEdit (Hall 1999). Primers for these conserved sites were constructed considering the problems arising by hairpin-forming and dimer-forming as described by Palumbi (in: Hillis et al. 1996) using the program 'Fast PCR' (Kalender 2003) and the online java-applet ‘netprimer’ (http://www.premierbiosoft.com/net-primer/index.html).

Primers 1800F and 18SRC1 were constructed at the highly conserved terminal regions of the 28S and the 18S genes and included the ITS-1, 5.8S rDNA, and ITS-2 fragment (~ 1.0 kb).

Tab. 15: Primers used for DNA amplification. ‘1’ symbols forward and ‘2’ reverse primers.

primer	name	sequence	gene	source
1	**16a**	5’- CGC CTG TTT ATC AAA AAC AT – 3’	16 S	Simon et al. 1994
2	**16b**	5’- CCG GTC TGA ACT CAG ATC ACG T – 3’	16 S	Simon et al. 1994
1	**LCO_mod**	5'- TTC TAC AAA TCA TAA AGA TAT TGG AAC -3'	COI	mod. after Folmer 1994
2	**FL_rück1**	5'- TAA GCT CGG GTA TCA ACG TCT AT -3'	COI	Leese
2	**Pat_modified**	5’- TCC ATT ACA CTA ATC TGC CAT ATT – 3’	COI	mod. after Simon 1994
1	**1800F**	5’-GTA AAA GTC GTA ACA AGG TTT CCG TA-3’	ITS-1	Vonnemann
2	**28SRC1**	5’-ATA TGC TTA AAT TCA GCG GGT – 3’	ITS-1	Vonnemann

DNA Purification

Purification of the PCR generated template was performed to improve sequencing results since remaining primers, polymerase, nucleotides and salts might negatively influence the sequencing reaction (Hillis et al. 1996). The PCR-purification kits of Qiagen and Macherey & Nagel were used according to the manufacturer’s instructions. To achieve higher concentrations of purified DNA only 30 µl elution buffer were used. The patented procedure is similar to the washing steps at the silicia membrane during DNA isolation. After purifying, 3 µl of the DNA were mixed with 2 µl loading dye and again controlled by gel electrophoresis.

DNA Sequencing

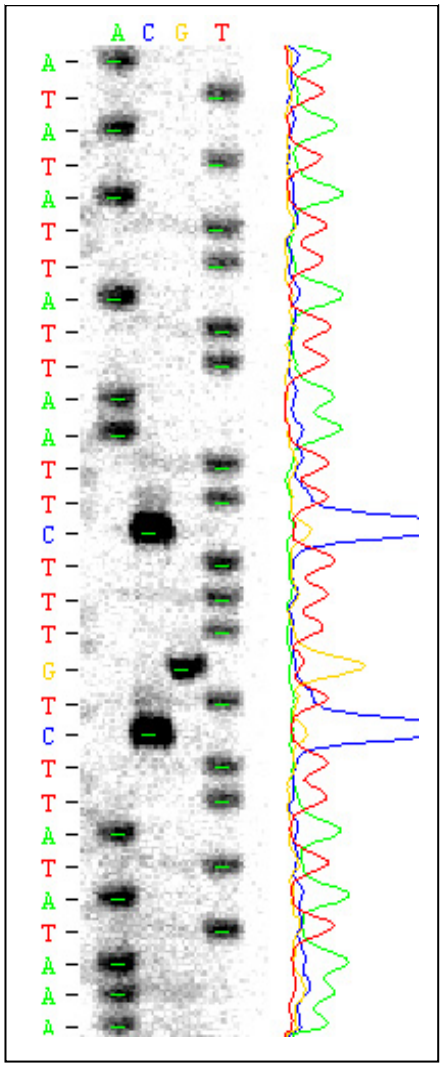

Fig. 22: Detail picture of a sequence gel. Each band column represents one nucleotide type: A, C, G and T (from left to right).

The aim of all laboratory work was to determine the nucleotide sequence of the particular genes of specimens. For this, the *Thermal Cycle-Sequencing* procedure was chosen. Cycle sequencing (Sambrook et al. 1989) is a coupling of two technologies: dideoxy-chain termination sequencing (Sanger et al. 1977) and the thermal cycling methodology as used in the PCR technique. The method can be used to obtain sequence information from a small (even femtomolar) amount of template. The sequencing method of Sanger uses so called didesoxyribonucleoside triphosphates (ddNTPs) as specific terminators of DNA chain elongation. In contrast to the common nucleotides (dNTPs) used in PCR, these ddNTPs lack the 3'-hydroxyl group necessary for incorporation of further dNTPs to the growing DNA chain. Consequently, the growing chain is terminated whenever a ddNTP molecule is incorporated.

Four separate primer extension reactions are performed for each sequence, using each of the specific ddNTP analogues (ddATP, ddCTP, ddGTP, and ddTTP). The polymerase reaction is carried out under conditions such that the incorporation of ddNTPs is rare and random. Each reaction produces a series of products of varying lengths, terminating in the relevant nucleotide (A, C, G, resp. T). These are then resolved by vertical electrophoresis on a polyacrylamide gel providing four alternating sequence ladders per sample, which are compared to provide the sequence of the template. In principle, cycle sequencing is exactly the same as Sanger (1977) sequencing, but the reaction proceeds in a series of cycles. The template DNA is denatured by heating, followed by primer annealing and extension (incorporating ddNTPs) using *Taq*-polymerase. The cycle is then repeated. In this way, several didesoxy-terminated chains are synthesized from one template strand. The thermal denaturation step is carried out in the presence of primers, preventing reannealing of double-stranded templates. The large amount of product copied from a

single template strand means that this technique is far more sensitive than the standard Sanger sequencing method.

Sequencing primers had the same base-sequence as PCR-primers but were marked by a covalently bound fluorescing-pigment (IRD-800) at the 5'-terminus. When excited by laser rays the primer emits light that is registered by a camera.

Tab. 16: Thermal cycle-sequencing reaction profiles for the investigated gene fragments.

gene	temperature	time	no. of cycles	function
16 S rDNA	94°C	2 minutes	1	Inital denaturation
	94°C	25 sec		Denaturation
	52°C	25 sec	30	Annealing
	70°C	35 sec		Extension
	94°C	45 sec	1	Denaturation
COI	94°C	2 minutes	1	Inital denaturation
	94°C	25 sec		Denaturation
	42°C	25 sec	30	Annealing
	70°C	45 sec		Extension
	70°C	45 sec	1	Final extension
COI	94°C	2 min	1	Inital denaturation
	94°C	25 sec		Denaturation
	52°C	25 sec	30	Annealing
	70°C	45 sec		Extension
	70°C	45 sec	1	Final extension
ITS1	94°C	2 min	1	Inital denaturation
	94°C	25 sec		Denaturation
	52.5°C	25 sec	30	Annealing
	70°C	45 sec		Extension
	70°C	45 sec	1	Final extension

Thermal cycling was performed on a Primus 96^{Plus} Thermocycler of the company 'MWG-Biotech' AG using the 'Cycle Sequencing kit' of the company 'Amersham Pharmacia Biotec' according to the manufacturer's instructions. 1 – 3 µl DNA were used for the thermal cycling procedure. Cycling conditions are listed in tab. 16.

Two automated sequencers of the company 'Licor Incorporations' ('Li-Cor 4000' and 'Li-Cor 4200'), were used for sequencing. 1 µl of the thermo-cycled and fluorescent marked samples were added onto the polyacrylamide gel heated at 50°C (to prevent single strands from hybridizing).

7.2.3 Analysis methods

DNA sequences were analysed in several steps using various computer programs. These steps will be discussed in the following section.

Examination of the sequenced DNA fragment

Initially, raw sequence data were corrected using the Software Image-Analysis (Li-Cor, version 1.1). Thereafter, sequence data were stored and further correction of the sequence was performed by using the alignment software 'AlignIR' (Li-Cor, version 1.2). At a second step, the consensus sequence was built, aligning the sequences received from the forward and reverse sequencing primer. Contradictory sequence data were corrected by comparing the gel images of the sequences. The terminal parts of the sequences, where the primers bound for amplification, were cut off. This ensures the exclusion of the primer sequences, which may differ due to mismatches from the template sequence.

Alignment

For phylogenetic research, only homologous characters are allowed for comparison. That counts for molecular data as well as for morphological characters. The first step in both methodological different fields consequently is the postulation of a homology hypothesis and estimating its probability. Criteria for homologous characters in morphology are well defined (see e.g. Wägele 2001), and fundamentally, these are valid for homologising sequences and molecules as well. In contrast to morphological characters, sequence data can be quantified more precisely and mathematical operations can be performed much more easily (Wägele 2001).
Two sequences are homologous, if they are derived from one ancestral gene. In different descent DNA sequences of a particular gene, not the nucleotides are homologous but their sites ('positional homology'). In order to find the most probable sites to propose a homology hypothesis, sequences have to be aligned with the help of mathematical algorithms.
The corrected set of sequence data was exported into text format ('fasta') and aligned using the programs 'ClustalX' version 1.83 (Thompson et al. 1997) and 'ProAlign' version 0.5 (Löytynoja and Milinkovitch 2003). The latter searches for highly variable sites among sequences using different parameters for gap opening and gap extension penalty and therefore produces a more intersubjectively comprehensible result. Sites, where alignment was ambiguous were excluded from analysis. Resulting differences of the 16S gene were compared with a secondary structure model (see fig. 23) of *Drosophila melanogaster* (GenBank Accession number X53596, (Gutell et al. 1993), since no secondary structure model for a closer related

taxon exists. Special attention was paid to base-pairing regions ('stem regions') and non-pairing-regions ('loop regions'), as nucleotide substitutions are likely to occur within loop regions, since they often do not have a definite function and consequently lack selection pressure. Important is that rRNA allows non-canonical base pairing (e.g. U – G) thus hindering unambiguous reconstruction (Ouvrard et al. 2000). Contradictory sequence data were again compared to the gel band pattern and errors resolved. Using the program 'Genedoc', version 2.6.002 (Nicolas and Nicolas 1997) the protein coding COI sequences were translated into amino acids and aligned. Afterwards the protein alignment was retranslated into nucleotide data and substitutions analysed more precisely.

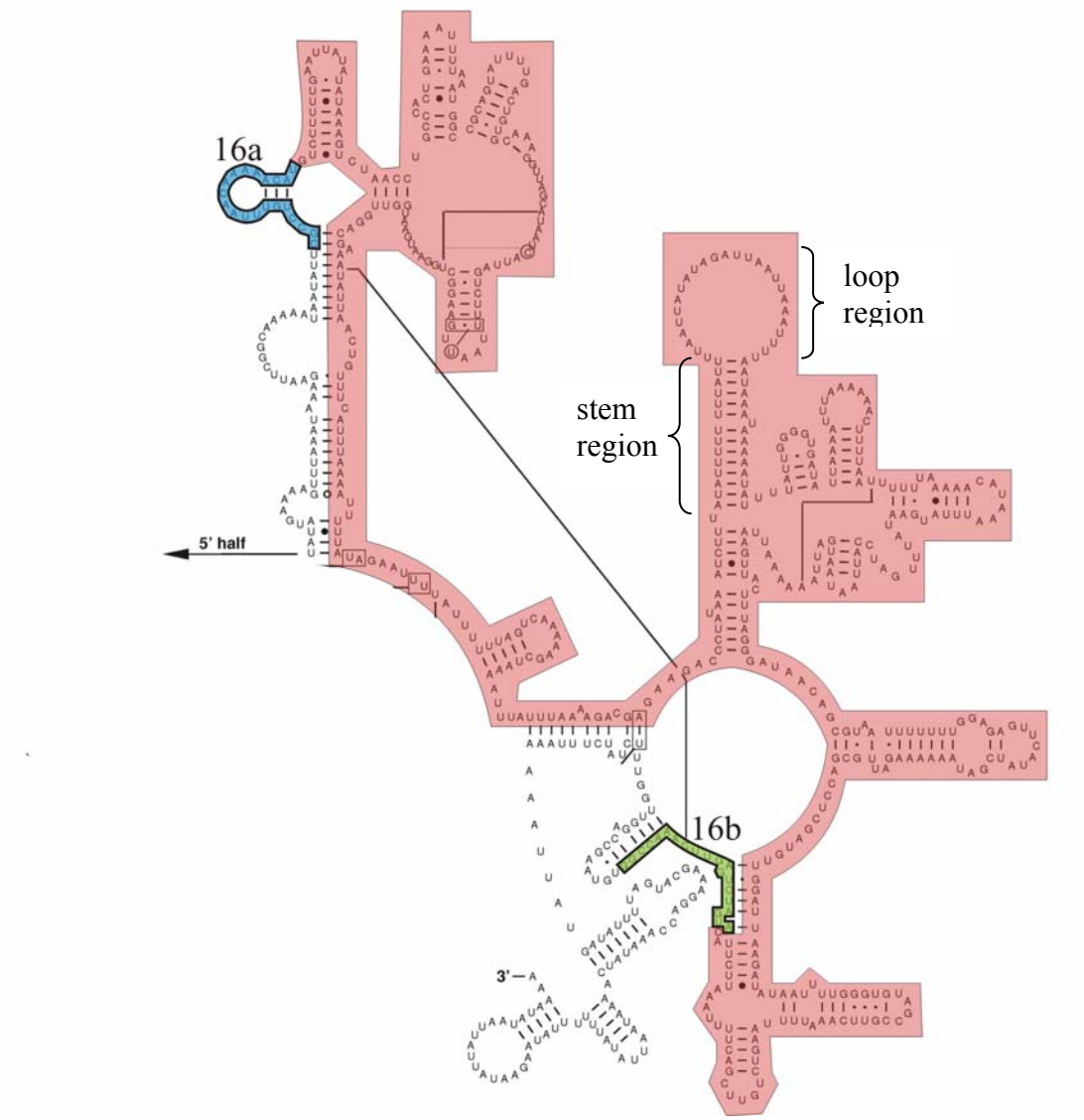

Fig. 23: Secondary structure of mitochondrial large subunit (3'-part) of *Drosophila melanogaster* (http://www.rna.icmb.utexas.edu/). Accession number of sequence: X53506 (NCBI, Genbank). The fragment of the amplified 16S rRNA gene (shaded) is situated between forward (16a) and reverse (16b) primers.

Blast

Before starting phylogenetic analysis, the sequences obtained were sent to the database GenBank of the National Center for Biotechnology Information. Using the BLAST[6] algorithm (Altschul et al. 1990) the database was searched for similar published sequences. Only if similarity with related sequences was proven the sequence was accepted for further phylogenetic analyses. Since no sequences for the 16S rDNA and the ITS-1, 5.8S or ITS-2 genes were published for trichopteran taxa until now, the sequences were used for phylogenetic analyses, if high similarity to sequences from closely related insect taxa was proven (Lepidoptera, Mecoptera).

[6] Basic Local Alignment Search Tool (Altschul et al. 1990)

Phylogenetic and population genetic analyses

Molecular genetic foundations

The basis for phylogenetic and population genetic investigations in order to resolve relationships between taxa constitute the rules affecting DNA evolution that lead to sequence differences. The consequences for phylogenetic inferences will be discussed briefly in the following.

DNA molecules consist of two groups of nucleotides: purines (adenine and guanine) and pyrimidines (cytosine and thymine). Evolution on DNA level is regarded as alterations in nucleotide sequence (chiefly substitutions, deletions or insertions of nucleotides) and rearrangements of genes in time. Concerning nucleotide substitutions, two kinds can be distinguished: transitions (purine → purine, or pyrimidine → pyrimidine) and transversions (purine ↔ pyrimidine) (see fig. 24).

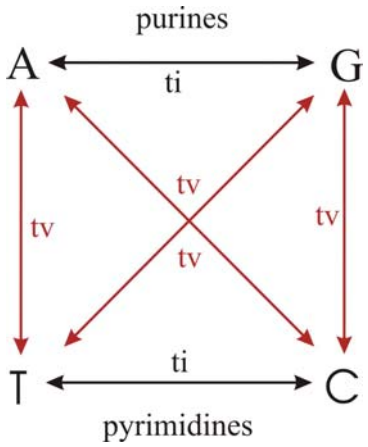

Fig. 24: Different nucleotide substitution types; transitions = ti (four possibilities); transversions = tv (eight possibilities) (see Huelsenbeck and Crandall 1997).

Regarding protein coding genes, synonymous and non-synonymous substitutions can be distinguished. Whereas synonymous substitutions are 'silent' and do not alter the amino acid sequence, non-synonymous substitutions lead to a replacement of a particular amino acid, thus changing the primary structure of the protein (see tab. 17).

Tab. 17: Examples for positions (shaded) of non-synonymous and synonymous (*) substitutions within protein-coding mitochondrial invertebrate genes. The coded amino acids can be found on the right of each codon triplet.

codon triplet	amino acid	codon triplet	amino acid	codon triplet	amino acid
ATA	methionine	TAA	stopp-codon	TTA*	phenyl-
GTA	valine	TGA	tryptophane	TTG*	alanin
CTA*	leucine	TCA	serine	TTC*	leucin
TTA*		TTA	leucine	TTT*	

Phylogenetic investigations

Phylogenetic investigations aim at the reconstruction of the historical relationships between particular taxa, often called OTUs[7]. Fundamentally, two processes are involved in this inference: first, the estimation of the topology of a tree (branching pattern) and second, the estimation of branch length (representing genetic distance) for a given tree topology. Whilst the latter is relatively simple, if a model of sequence evolution is estimated and lineages concerned are not too old (Nei 1996, Wägele, pers. comm.), the estimation of topology is still a challenging task. The number of possible topologies increases exponentially with increasing sequence number. Several problems exist estimating phylogenies from sequence data: (i) if substitution rate of the gene fragment is relatively high compared to the time intervall, multiple substitutions might have occurred, leading to discrepancies between the observed (D_{obs}) and real (evolutionary) distance (D_{tot}). Consequently, the latter has to be estimated (D_{est}) using accurate correction procedures. In character state methods (see below), multiple substitutions will obscure shared character information (synapomorphies) and create false character state matches, leading to high homoplasy[8] and low character consistency. (ii) Variable positions in different OTUs might not be the result of different history but of polymorphic variation within the common ancestor ('shared ancestral polymorphisms') (Simon et al. 1994). Investigation of different, unlinked genes and an estimation of the intraspecific variability are needed to prevent these two problems. (iii) Maximum Likelihood methods use artificial models of sequence evolution that always represent a hypothetical construct of which the existence cannot be proved. (iv) Symplesiomorphies might possibly be recognized as synapomorphies in a given data set and thus misrepresent phylogeny (Wägele 2001).

Methods of phylogenetic inference currently used in molecular phylogenetics can be classified into two basic categories. At first those that operate on pairwise distances among taxa ('Distance Methods') and second, those that operate on individual character data ('Character state Methods'). The latter can at least be subdivided into Maximum Parsimony (MP) and Maximum Likelihood (ML) methods (Swofford et al. 1996; Nei 1996; Wägele 2001).

In the present study, distance, ML, and MP methods were chosen to reconstruct the phylogenies of the sericostomatid DNA sequences and are therefore briefly described.

[7] OTUs = Operational Taxonomic Units.

[8] A homoplasy is a similarity that is not a result of common history. Similarity is caused by parallel, convergent or reverse mutations, or simply by the wrong tree (Avise 1994; Posada and Crandall 2001).

Distance methods

Distance methods aim at the estimation of the real, evolutionary distance D_{tot} from the observed distance D_{obs} between taxa (whereas logically $D_{obs} \leq D_{tot}$) for all pairs of sequences. The estimated evolutionary distance D_{est} results from the correction of D_{obs} and is an approximation of D_{tot} and in general $D_{est} \leq D_{tot}$ and $D_{obs} \leq D_{est}$. A phylogenetic tree is constructed from pairwise distances using a defined criterion (e.g. Minimum Evolution, Neighbor Joining, UPGMA and further more; (see Wägele 2001)). Theoretically, if the total number of substitutions between any pair of sequences is known, all the above distance methods produce the correct unique phylogenetic tree. In practice, however, this number remains almost always unknown, and thus many different methods for correcting the observed distances to gain the real number of substitutions (D_{tot}) have been proposed (see Swofford et al. 1996), for example Kimura's (Kimura 1980) and Hasegawa et al.'s (Hasegawa et al. 1985) methods. In this work a distance tree was calculated using solely an cluster method - the Neighbor Joining method (NJ) (Saitou and Nei 1987). This method was preferred due to its fast computational time in contrast to other methods (e.g. Minimum Evolution). With NJ a bifurcating tree with a minimal sum of branch lengths (S) is constructed. Pairwise comparison of distance data and computation of S is not performed for all possible topologies, selecting the topology for which S is minimal but only for selected topologies. Initially pairwise estimated distances d_{ij} between all terminal taxa are calculated. Then NJ starts constructing a suboptimal tree and calculates S_0 for this topology. In the following steps two sequences i and j at a randomly chosen node are paired and then separated from the rest of the sequences that still form a star tree. If i and j are neighbours connected by only one node than S_{ij} is smaller than S_0 and will be the preferred tree for the next node comparison. For an overview of the algorithms used by this method and other distance matrix based methods, see Nei (1996). Another advantage of NJ is that it can be applied to distance data being not ultrametric (no uniform molecular clock; (Saitou and Nei 1987)). A severe disadvantage of this method is that the resulting topology is influenced by the order of sequences. Therefore, NJ trees have to be calculated several times using alternatively ordered sequences.

ML methods

The aim of ML methods is to find a tree for a given data set representing most likely the phylogeny of the OTUs under a particular model of sequence evolution estimated *a priori* (Wägele 2001). The ML method uses both, character (nucleotide) data and branch length between taxa and chooses the tree with the highest likelihood value as the preferred tree. This

requires a sequence evolution model, e.g. the assumption that substitution rates are equal from one nucleotide to the other, or that the expected number of substitutions is equal for different sites and that the expected number of substitutions on a branch is given by the substitution rate and the branch length (Swofford et al. 1996).

MP methods

The principle of parsimony in science maintains that simpler hypotheses are preferable to more complicated ones. Maximum parsimony methods in molecular systematics use character state data with a global optimality criterion: the smaller the number of evolutionary changes required by a tree, the better the tree. In practice, in MP methods a given alignment of nucleotide sequences is considered and the nucleotides of ancestral sequences for a hypothetical topology are inferred under the assumption that mutational changes can occur in all directions among the four different nucleotides. The smallest number of nucleotide substitutions that explains the entire topology is then computed. Heuristic computation is performed and the topology that requires the smallest number of substitutions is chosen as the best tree (Yang 1996). In contrast to distance and ML methods, *ad hoc* hypotheses are avoided in MP methods.

Models of sequence evolution

For the inference of phylogenies from molecular data using ML and distance methods, it is essential to make assumptions about the process of molecular evolution. If DNA is concerned, assumptions have to be made about substitution rates. Assuming that evolution of DNA sequences can be described using mathematical models probabilities of substitutions for specific nucleotides can be calculated. Always being aware that mathematical models may not truly represent the process of evolution one can try to search for models of sequence evolution that most accurately explain the present data set.

All these models of sequence evolution require rates of substitutions according to which nucleotides are replaced in time. Some models use one specific constant rate for all substitution types and sites, e.g. the ‘Jukes-Cantor-Model’ (Jukes and Cantor 1969). Other models, e.g. the ‘Kimura-2-parameter Model’ (Kimura 1980), distinguish between transitional and transversional substitution rates.. Some models consider each possible type of substitution separately, even for separate sites; e.g. the ‘General Time Reversible Model’ (GTR) (Rodriguez et al. 1990). For a detailed overview, see Swofford (1996).

To select a particular model of sequence evolution that fits best to the present 16S rDNA data set, the program 'Modeltest', version 3.06 (Posada and Crandall 1998) was used. 'Modeltest' is a simple computer program designed for systematical comparison of different nested models of DNA substitutions in a hierarchical hypothesis-testing framework using likelihood ratio tests (Goldman 1993). In each operational step, the program compares two models, representing each a hypothesis of molecular evolution, by calculating the likelihood of each for the present data set. The model with significant lower likelihood is rejected and the alternative model is compared to the next model in the hierarchical process. With this it can be estimated whether additional parameters of a certain model explain more accurately evolution of a given sequence alignment despite the growing uncertainty due to the increased number more parameters. To obtain an appropriate substitution model and model parameter values the data set was evaluated using 56 models of sequence evolution (Posada and Crandall 1998). Models were evaluated with and without rate heterogeneity. Rate heterogeneity was accommodated in three ways: using a gamma model, using an invariant sites model, and using a gamma plus invariant sites model.

Today it is consensus among scientists that substitutions do not occur independently at different sites. For example the pairing sites in stem regions of ribosomal genes are not independent because a substitution in one nucleotide influences the probability that a compensatory substitution will occur in its corresponding pairing partner (Dixon and Hillis 1993; Huelsenbeck and Crandall 1997). In protein coding genes, substitutions that do not change the phenotype of a protein (synonymous substitutions) are much more likely to occur than non-synonymous substitutions that alter the structure of the protein. Due to the degeneration of the genetic code, synonymous substitutions are much more likely to occur at third codon position than at first or second.

With a particular model of sequence evolution, a matrix can be constructed that informs about the probability that a nucleotide i is replaced by a nucleotide j in time the time interval dt.

Alignment gaps

A frequently asked question is whether alignment gaps should be considered or not. Wägele (2001) proposes to exclude gaps if positional homology is ambiguous. Nevertheless, in some regions gaps are informative, e.g. if insertions or deletions have only occurred in one group of taxa and it can therefore be considered as an apomorphy. Problematic is the case when n nucleotides ($n > 1$) were inserted or deleted, since no empirically founded weighting scheme for handling them as one or n characters exist.

In the following, gaps are considered both as missing and fifth state. Differences in results are compared.

Revealing population substructure

The extent to which natural populations are subdivided into genetically differentiated sub-populations, resp. potential new sibling species, can be investigated comparing patterns of genetic differences. Fundamentally, one compares the differences within populations (intrapopulation divergence) to the differences between populations. If populations separated by a reduced gene flow are concerned it must be expected that variability within populations is smaller than variability between populations. If distinct species are concerned, no haplotype sharing is likely to be observed concerning fast evolving genes, in particular nuclear genes.

Population substructure can be analysed predominately with phenomenological methods using tree and network constructing algorithms or statistically evaluated for example using the F_{ST} (Wright 1951) and – suited better for sequence data - K_{ST} statistics (Hudson et al. 1992). These statistical methods offer only valid and comparable data if the sample size is high (> 50 individuals per population) and are thus not performed in this work.

Tree versus network methods

Scientists face the challenge of exponentially increasing molecular and analyzing techniques available for investigating molecular data (e.g. Swofford et al. 1996). The main problem often seems to be the adequate selection of techniques for particular phylogenetic analyses and the accurate visualization of the results.

Phylogenies are usually presented by dichotomous tree models, which are graphs, composed of nodes and branches (see fig. 25). Terminal nodes represent the different taxonomic units (e.g. orders, families, genera, species, populations, or simply genes) being investigated. Internal nodes denote the divergence point of an ancestral taxonomic unit, sometimes referred to as hypothetical taxonomic units (HTUs). A rooted tree model defines a hypothetical phylogeny. Branches (sometimes called edges) describe the descent and ancestry relationship among different nodes. Each edge of the tree has a certain amount of evolutionary divergence associated to it, defined by some measure of distance between sequences. In general, the real path of evolution of a particular gene remains unknown since ancestral nodes can rarely be precisely estimated and several alternative hypotheses might coexist. In one phylogenetic tree, only one of these hypotheses can be visualized. Problematic is the fact that sequence data often contain a number of different and sometimes conflicting signals and thus do not always clearly sup-

port a unique “best” tree. Especially using intraspecific data sets there are instances of reticulate evolution such as gene transfer, hybridization, and recombination. For these cases a phylogenetic network (e.g. see fig. 26), should be preferred, since reticulated relations and signal ambiguities can be visualized more accurately. Networks usually represent more of the phylogenetic information present in a given data set. A severe problem is that networks may become multidimensional due to incompatible data and difficult to visualize. In particular for mtDNA this is not the case (Bandelt 1995, 1999). Investigating species and subspecies-level phylogenies one should prefer networks to trees (Röhl 1999; Posada and Crandall 2001).

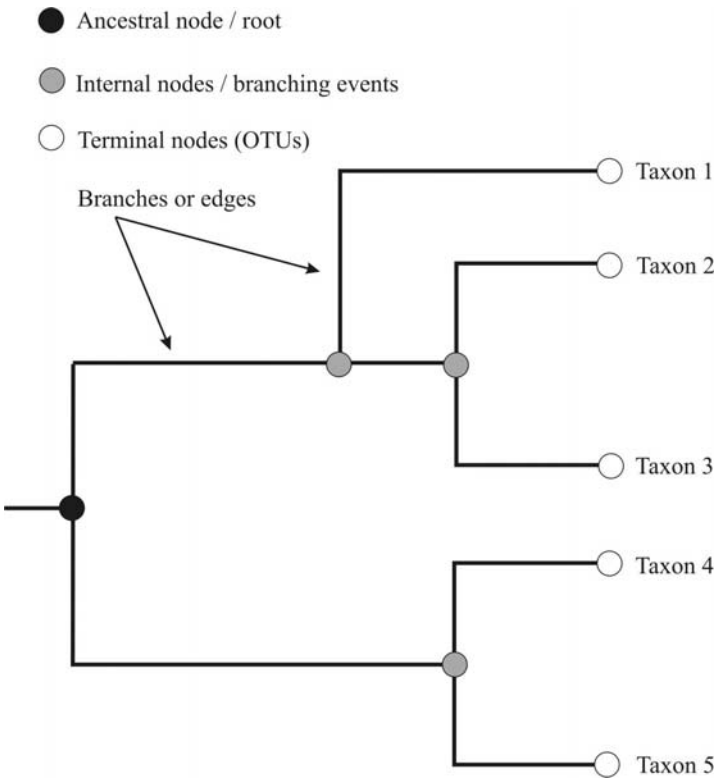

Fig. 25: Components of a typical, fictive bifurcating phylogenetic tree. Terminal nodes represent different taxonomical hierarchies (e.g. families, genera, or species).

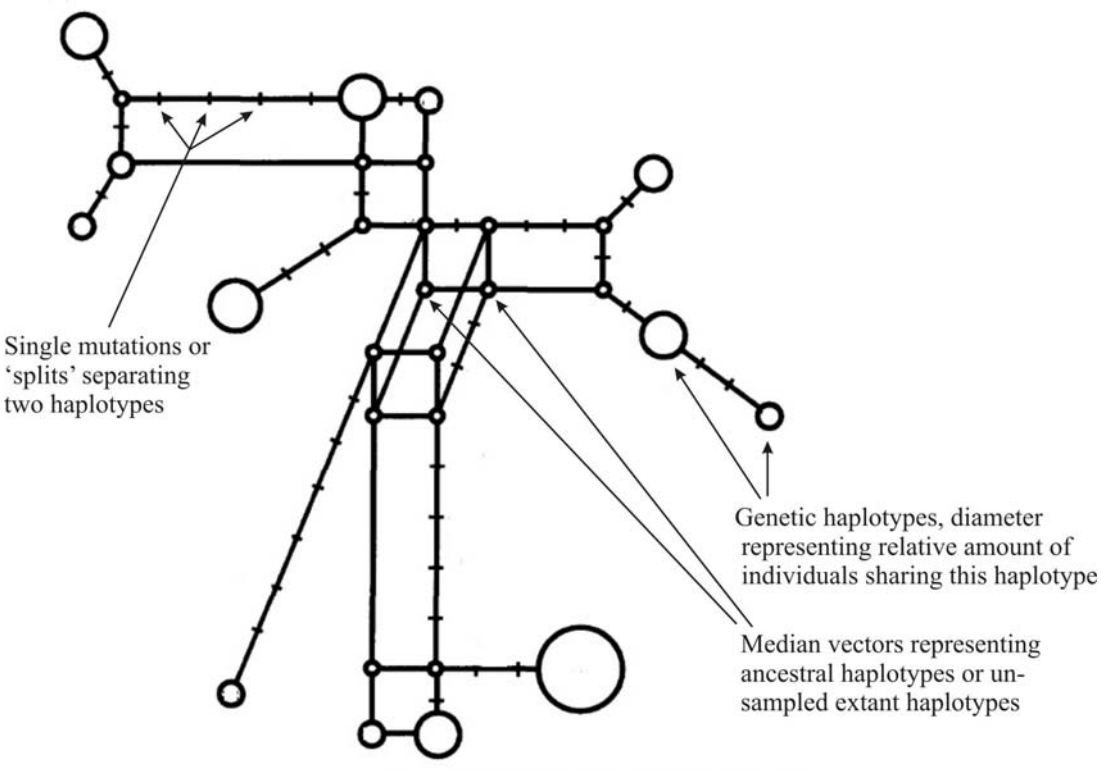

Fig. 26: Components of a fictive network (mod. after Bandelt 1995). Circles represent alternative evolutionary hypotheses (homoplasies).

7.2.4 Performing analyses

As mentioned in the introduction, two different subjects were examined using the molecular data sets: (i) genetic sub-structuring of the European *Sericostoma* complex, with special emphasis on the *S. personatum* and *S. schneideri* 'species', and (ii) phylogenetic relationships among the four predominating West European sericostomatid genera.

(i) Analysis of the *Sericostoma* complex – a population genetic approach

For all *Sericostoma* specimens (n = 46) 16S rDNA sequence data were examined. For 22 of these specimens COI sequence data were gained and analysed separately and in combination with the 16S rDNA. To derive relationships between the potential *Sericostoma* species two main problems arise: (i) it is uncertain whether different species or regionally differentiated populations are concerned, (ii) sample size is small (one to three individuals per sampling site) and therefore population genetic analysis is restricted. Since individuals are obviously very closely related phylogenetic network methods were chosen for analysis to visualize homoplasies.

To assess mitochondrial genetic diversity within the European *Sericostoma* complex, number of haplotypes, polymorphic (nonconstant) sites, haplotype diversity (π) with standard deviation (SD), and mean number of pairwise differences were calculated for the 16S, the COI, and the combined data sets using the program 'Arlequin', version 2.0 (Schneider et al. 2000) and 'DnaSP', version 4.0 (Rozas et al. 2003). Additionally, nuclear DNA from the ITS-1 region was analysed for n = 3 specimens.

Combined investigation of the mitochondrial genes

Circumstances under which separate data sets may or may not be combined are subject of controversial debates (e.g. Carpenter and Nixon 1996). None of the different viewpoints on this topic objects to combining data sets that are not demonstrably incongruent. This was tested using the 'Incongruence Length Difference Test' (ILD) introduced by Farris et al. (1995), implemented as the 'Partition Homogeneity Test' in 'PAUP' (Swofford 2000), with 1000 replicates, 10 random-addition tree searches per replicate, and invariant sites excluded. This test compares tree length of trees calculated from the separate data sets to tree length of trees calculated from the combined data set. If length of the combined data set increases significantly, the resulting p value decreases indicating incompatible signals of the different

genes. If length of trees is almost identical the resulting p value is 1, whereas incongruence is proven if $p < 0.05$.

Network methods

As mentioned before, network methods are suited better for investigation of the reticulated relationships among different populations (Bandelt 1994; Röhl 1999; Posada and Crandall 2001). To describe relationships and geographical partitioning among haplotypes within the *Sericostoma* complex, median-joining networks (Bandelt et al. 1999) were computed using the program 'Network', version 4.0 (Röhl 2003). Split-decomposition networks (Bandelt and Dress 1992) were constructed using the program 'Splitstree', version 4.0 beta 3 (Huson 1992). Differences of both methods are explained below. Since variability between populations was relatively low it, can be considered that multiple substitutions are most unlikely. Therefore no different weighting for tansversions and transitions was performed. Upweighting of transversions or slowly evolving sites is usually done to reduce introduced 'noise' in a data set (Swofford et al. 1996).

Split decomposition (Bandelt and Dress 1992): Any data set can be partitioned into sets of 'splits'[9]. A network can be built by taking in turn these splits defined by the characters and combining them successively (Bandelt and Dress 1992). Each split will define a branch connecting the two partitions delimited by the split. For ideal data, this graph is a tree, whereas less ideal data will give rise to a tree-like network. When splits are incompatible (e.g. they define contradictory groupings) a circle is introduced indicating alternative splits. The split decomposition method is fast, which means that a reasonable number of haplotypes (>50) can be analyzed.

Median-joining networks (Bandelt et al. 1999): The median-joining networks algorithm (Bandelt et al. 1999) allows analyses of large data sets and of multistate characters (not only binary characters) of recombination-free data sets. Since the present data consists of 16S and COI mtDNA sequences, the assumption of absence of recombination is not violated. Since some splits were not binary, this method is preferable to the reduced median algorithm (Bandelt et al. 2000) and other ones. In a first step a minimum spanning network is con-

[9] A split represents the division of the haplotypes into two exclusive sets. For n sequences, there are $2n - 1$ possible splits (Bandelt and Dress 1992).

structed by combining all minimum spanning trees (MST)[10]. MST were calculated using the Kruskal algorithm (Kruskal 1956) modified in that all (not only one) MST are computed. With a heuristic search, median vectors representing missing intermediates or extant unsampled haplotypes are added to the graph. For the algorithm a tolerance ε is specified up to which distances are not distinguished. Increasing ε leads to additional new median vectors and also relaxes the distance criterion. By this, a higher amount of homoplasies is considered. Networks were calculated for different ε values.

(ii) Phylogenetic analysis of the sericostomatid data sets

Analyses of the relationships between the sericostomatid genera were performed for the 16S rDNA data set using distance, ML and MP methods. Phylogenetic analyses of the relationship among the sericostomatid taxa *Notidobia*, *Oecismus*, *Schizoplex* and *Sericostoma* were performed using the 16S rDNA data set. Sequences from the caddisflies *Potamophylax luctuosus* and *P. cingulatus* (see tab. 11) and further, the lepidopteran taxon *Melitaea didyma* were used as outgroup in all phylogenetical analyses.

Distance methods

Distance methods were performed using the computer program 'PAUP' version 4.0beta (Swofford 2000) using the NJ algorithm (Saitou and Nei 1987). Nodal support of the phylogenies was estimated using the bootstrap approach (Felsenstein 1985) with 100 pseudoreplicates with 100 random-addition replicates per pseudoreplicate.

MP analyses

Parsimony analyses were performed using the computer program 'PAUP', version 4.0beta using the heuristic search option with the TBR branch-swapping algorithm[11]. Parsimony-uninformative characters were excluded. To insure that multiple 'islands' of most parsimonious trees were identified, 100 random-addition replicate analyses were carried out for weighted (tv:ti 3:1, resp. 5:1) and unweighted characters, gaps included and excluded analyses. Bootstrapping (Felsenstein 1985) under parsimony utilizing 100, 200, resp. 1000 pseu-

[10] A minimum spanning tree for a given sequence set connects all given OTUs without creating any cycles or additional nodes, such that the total length is minimal.
[11] TBR = Tree Bisection-Reconnection. Using this algorithm a given topology is subdivided at an internal edge and reconnected at another, randomly chosen internal edge. Length of both resulting topologies is compared and the topology fitting better to the chosen optimality criterion.

doreplicates (depending on computation time) with 10, respectively 100 random-addition replicates per pseudoreplicate, was performed to evaluate the trees.

ML analyses

The search for optimal trees was conducted with a heuristic search, TBR branch swapping and 100 random sequence addition replicates. Nodal support of the phylogenies was estimated using the bootstrap approach (Felsenstein 1985) with 100 pseudoreplicates with 10 random addition replicates per pseudoreplicate. Analyses were as well performed using 'PAUP', version 4.0beta. The model of sequence evolution was estimated before using 'modeltest' (Posada and Crandall 1998).

Evaluation of the phylogenetic signal

Beside tree constructing methods, the quality of the data set was evaluated using a phenomenological approach described by Wägele and Rödding (1998). With the program 'PHYSID', version 1998 (Wägele 1996; Wägele and Rödding 1998) the phylogenetic signal of the 16S rDNA data set was estimated. All splits of the alignment supporting the division of the data set into two groups were computed in order to detect patterns supporting the phylogenetic relationships. Ambiguous regions were excluded from analysis as proposed by Wägele and Rödding (1998). With 'PHYSID' it is possible to visualize supported splits that separate the data set into two groups (these named 'ingroup' and 'outgroup') and evaluate the phylogenetic signal by comparison of the number of supporting symmetrical (binary), asymmetrical (multistate characters in in- or outgroup) and noisy (multistate characters in in- and outgroup)[12] positions. Originally, a spectrum of supporting splits could be computed with 'PHYSID'. Unfortunately, this function did not work in the version available. Consequently, no spectra were by computed but solely supporting splits controlled manually with an allowed noise for in- and outgroup columns and rows of 25% or below.

[12] Noisy positions within a given alignment are referred to positions with potential consisting of convergencies or identity resulting by chance (see Wägele and Rödding 1998).

7.3 Results

The results part focuses on the analyses of the mitochondrial 16S rDNA and COI genes, since only three sequences of the nuclear ITS-1, 5.8S, ITS-2 gene fragment were obtained. The latter are briefly presented. *Sericostoma* species complex analyses are presented separately from the phylogeny of Sericostomatidae.

7.3.1 Descriptive sequence characteristics

(i) Species and populations of *Sericostoma*

The alignment of *Sericostoma* 16S rDNA sequences resulted in 458 bp including one gap. Of these, 12 characters were variable (2.6%) and ten parsimony-informative (2.2%). Substitutions occured only in regions, stem regions were conserved.

Alignment of the COI sequences (n = 22) was trivial and produced 439 bp without gaps. Of these, 30 characters (6.8%) were variable and 29 parsimony-informative (6.6%). Observed variability of the COI fragment was predominantly observed at third codon positions (23 sites, resp. 76.7% of variable positions) and a minor amount at first codon positions (seven sites, respectively 23.3%). No variable sites were observed at the second codon position (see fig. 27). For comparisons on protein level, sequence data were translated into amino acid data using the genetic code for invertebrate mitochondrial DNA. 25 of the 30 observed substitutions were synonymous ('silent'), resulting in no change of the transcribed amino acid. All variable sites at third and two sites at first codon position were synonymous. The five non-synonymous substitutions were solely found at first codon position. In two cases, substitution resulted in an exchange of the amino acid valine to isoleucine, at two sites valine was substituted by methionine and at one site serine was substituted by proline.

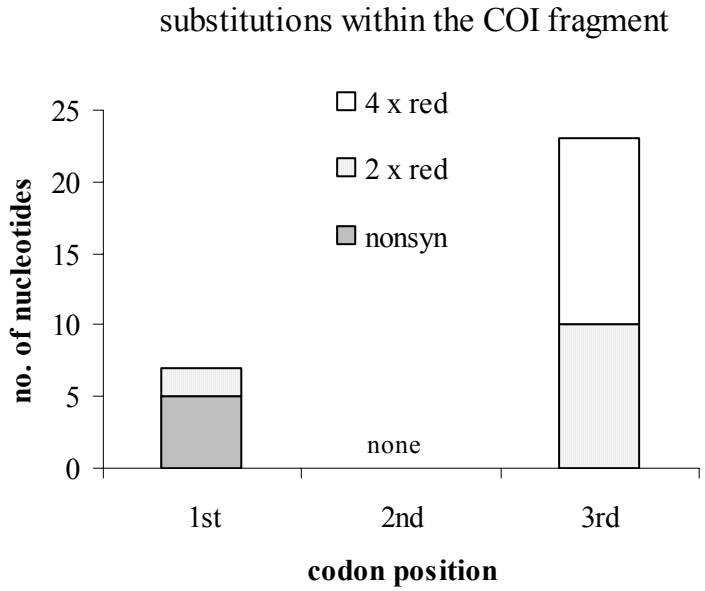

Fig. 27: Amount of codon specific synonymous and non-synonymous substitution within the COI fragment (439 bp). Synonymous substitutions are subdivided into amounts of two and four fold redundant amino acids for which they code.

(ii) Family Sericostomatidae

The alignment constists of 16S rDNA sequences of all sericostomatid specimens, three *Potamophylax* (Trichoptera: Limnephilidae) specimens and one lepidopteran outgroup species (*Melitaea didyma).* The alignment resulted in 480 bp including gaps. Of these, 33 positionss were excluded due to ambiguity of positional homology. Of the remaining 447 characters, 310 were constant, 137 variable (30.6%), and of these 103 characters (23.0% of total alignment) were parsimony-informative. Substitutions occurred chiefly in non-pairing loop regions, but in particular comparing different genera pairing stem regions also exhibited substitutions. In these cases, always both pairing nucleotides were substituted not affecting the secondary structure.

Combined genes

The results of the 'Incongruence Length Difference Test' (Farris et al. 1995) using the *Sericosotma* 16S and COI data sets indicated that the null hypothesis of congruence could not be rejected ($p = 1.0$). This justified the combined investigation of both mtDNA data sets.

Base frequency homogeneity

16S and COI gene fragments both exhibited a remarkable high A + T frequency (tab. 19), which is consistent with other insect mitochondrial genes (Simon et al. 1994). Using a chi-

square (χ^2) test implemented in 'PAUP' (Swofford 2000), no inhomogenous (p = 1.0) base frequencies among the sequence alignment data were observed (tab. 18)

Fig. 18: Homogeneity of base frequencies among the different data sets; calculated using χ^2-test implemented in 'PAUP'; df = degrees of freedom.

genes	n	df	χ^2	p-value
16S - Sericostomatidae	58	171	11.40	1.00
16S - *Sericostoma*	46	135	1.43	1.00
COI - *Sericostoma*	22	63	3.89	1.00
COI + 16S *Sericostoma*	22	63	1.19	1.00

Tab. 19: Nucleotide composition (in %) of the investigated genes; minimum, maximum and mean nucleotide percentage of 16S and COI data sets, estimated for all sites and for variable sites only. * including gaps, highly variable alignment positions excluded; ** parsimony-informative sites.

gene	no. of sites	nucleotides			
		A	C	G	T
16S - Sericostomatidae	447*	38.1 – 43.7 (39.6)	6.6 – 7.9 (6.7)	11.9 – 14.4 (13.7)	37.4 – 40.6 (39.9)
16S - Sericostomatidae variable	137 / 103*	37.18 – 51.7 (39.0)	4.3 – 9.2 (4.7)	7.4 – 14.1 (11.9)	34.9 – 47.1 (44.4)
16S - *Sericostoma*	449	39.4 – 40.2 (39.9)	6.5 – 6.9 (6.5)	13.1 – 14.0 (13.6)	39.6 – 40.2 (40.1)
16S - *Sericostoma* variable	12 / 10**	18.2 – 54.5 (37.4)	0 – 18.2 (1.2)	18.2 – 54.5 (35.0)	9.1 – 27.3 (26.5)
COI - *Sericostoma*	439	28.5 – 30.3 (29.8)	23.0 – 24.1 (23.8)	14.4 – 15.9 (14.8)	31.2 – 32-6 (31.7)
COI - *Sericostoma* variable	30 / 29**	10.0 – 36.7 (29.1)	23.3 – 40.0 (34.8)	10.0 – 33.3 (16.1)	13.3 – 33.3 (20.0)
codonposition 1	147	27.2 – 28.6 (28.0)	22.4 – 24.5 (23.8)	24.5 – 25.9 (25.1)	22.4 – 24.5 (23.1)
codonposition 2	146	13.0 (13.0)	28.8 (28.8)	16.4 (16.4)	41.8 (41.8)
codonposition 3	146	45.2 – 50.7 (48.4)	17.8 – 19.9 (18.8)	1.4 – 5.5 (2.7)	28.1 – 31.5 (30.2)
Pos. 1 nonconst.	7	14.3 – 42.9 (29.9)	0.0 – 42.9 (29.2)	14.3 – 42.9 (27.3)	0.0 – 42.9 (13.6)
Pos. 2 nonconst.	0	-	-	-	-
Pos. 3 nonconst.	23	8.7 – 43.5 (28.9)	30.4 – 43.5 (36.6)	4.3 – 30.4 (12.6)	8.7 – 30.4 (21.9)

Substitution patterns

Modeltest

The model used for ML and NJ analyses was selected with a hierarchical likelihood ration test using 'Modeltest' (Posada and Crandall 1998). A general time reversible (GTR) model with a gamma[13] correction was calculated to 'fit' best for the 480 bp Sericostomatidae 16S rDNA data set. Parameters of the model: gamma shape parameter $\alpha = 0.2821$; proportion of invariant sites: 0; substitution rates: A-C: 1.0, A-G: 19.8, A-T: 8.8, C-G: 8.8, C-T: 19.8, and G-T: 1.0. This model of sequence evolution was used for the inference of phylogenetic relationships using distance and ML methods.

Saturation effects

In order to estimate the influence of multiple substitutions uncorrected pairwise patristic distances were plotted against the GTR + gamma corrected patristic distances. Saturation caused by too many multiple substitutions, was not observed between sericostomatid taxa (see fig. 28, A) but was observed to play a major role comparing distances between all sericostomatid genera and *Potamophylax* or *Melitaea,* and between *Potamophylax* and *Melitaea* (uncorrected distance 16.8 – 19.1%, see 28, B). Highest saturation, indicated by a plateau at roughly 20% uncorrected p-distances, was observed comparing *Schizoplex festifa* and *Melitaea* (C), resp. *Oecismus monedula* and *Melitaea* (uncorrected genetic distance 20.9 – 21.3%, see 28, C and D).

For a detailed analysis of saturation, transitions and transversions of the 16S rDNA data set including *Potamophylax* and *Melitaea* and the COI (*Sericostoma*) data set were plotted against uncorrected p-distances. For substitution processes occurring at same rate, one would expect a linear relationship. For the 16S rDNA data set, transversions increases proportionally to the p-distance values, whereas frequency of transitions reaches a plateau at roughly 20% p-distances (Fig. 29). This can be taken as a sign of saturated transitional substitutions. Consequently, for reconstruction of phylogenetically older events, in this case the relationship between Sericostomatidae and *Potamophylax* / *Melitaea*), available phylogenetic information of the data set is reduced due to multiple substitutions. Upweighting of transversions might compensate this phenomenon. Since the main interest of the present investigation focuses on relationships within the family Sericostomatidae, no additional attention was paid to these problems. These topics are discussed in detail in the works of Kjer et al. (2001 and 2002).

[13] The gamma correction adjusts different variation in rates.

For the COI data set, transitions increase proportionally to the p-distance values. The number of transversions was too small to yield a certain trend (Fig. 30). Thus saturation does not concern the *Sericostoma* COI and the *Sericostoma* 16S rDNA data set. As a consequence, the *Sericostoma* data sets were not weighted.

Transition : transversion ration was 7.1 : 1, resp. 28.7 for the *Sericostoma* COI, resp. 16S data set. For the 16S Sericostomatidae data set this ration was 0.7 : 1 signalling saturation of transitions

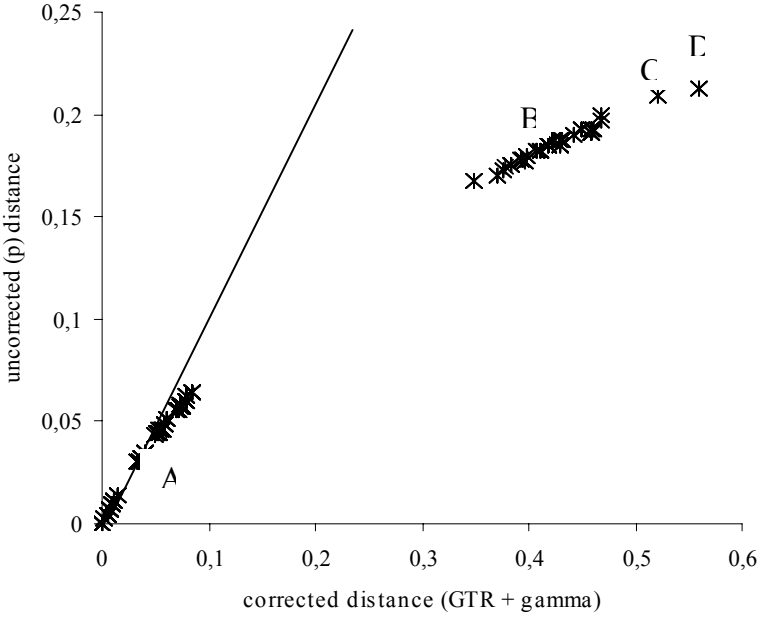

Fig. 28: Graphical representation of the accumulation of substitutions within the 16S rDNA data set. Uncorrected distance is plotted versus corrected distances, the diagonal line representing a 1:1 relationship. Letters indicate distances between: A: sericostomatid members, B: sericostomatid members and *Potamophylax* species, *Potamophylax* species and *Melitaea*, C: *Schizoplex* and *Melitaea*, D: *Oecismus* and *Melitaea*.

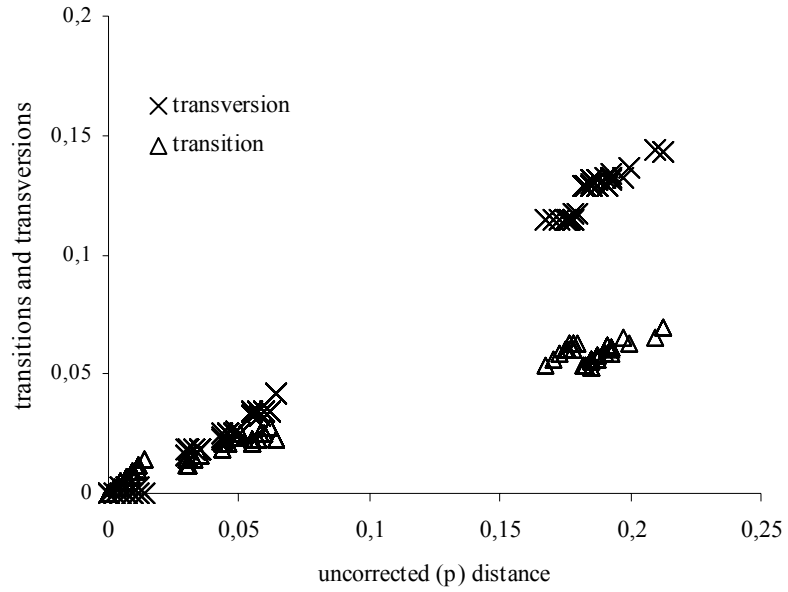

Fig. 29: Graphical representation of the accumulation of transitions and transversions of the Sericostomatidae 16S rDNA data set.

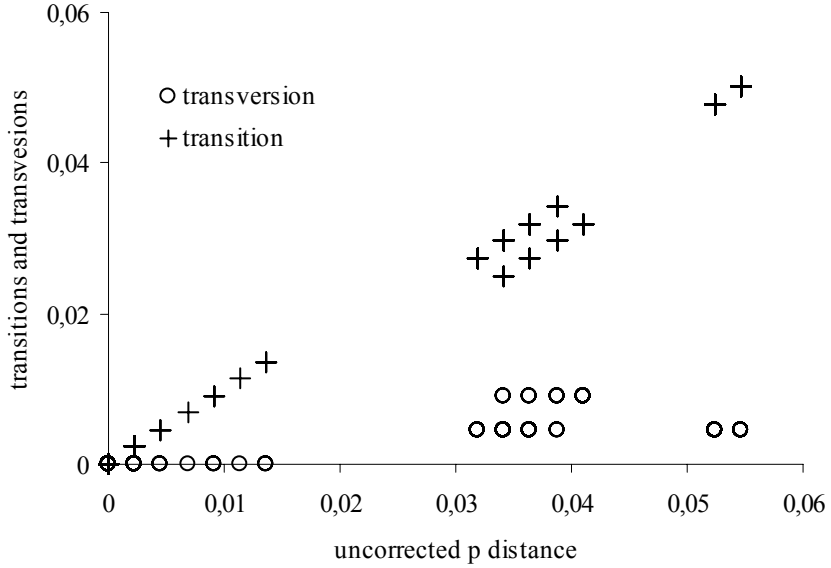

Fig. 30: Graphical representation of the accumulation of transitions and transversions of the *Sericostoma* COI data set.

7.3.2 Phylogenetic analyses

(i) The *Sericostoma* species complex

16S data set (449 bp, n = 46 specimens): Sequence data were found to be subdivided into nine different haplotypes (**H_{16S}1** – **H_{16S}9**). Haplotype diversity (π ± SD) was high: π = 0.792 ± 0.037. Variability was low with a maximal patristic distance D_p = 1.56% (seven variable positions) between *S. vittatum* specimens from Spain (E_MG_SV01, 02) and *S. personatum* specimens from Belarus (BY_SC_SP03).

Tab. 19: Haplotypes (H_{16S}1 – H_{16S}9), number and label of the sampled *Sericostoma* specimens for the 16S rDNA gene fragment (449 bp).

haplotype	n	specimens
H_{16S}1	15	N_JS_SP01, D_FL_SP20, D_PZ_SP01, D_HR_SP01, D_FL_SS02, D_FL_SP02, D_PN_SS07, D_PN_SP11, D_BR_SP02, D_FL_SP21, D_FL_SP03, D_BR_SP01, D_HR_SP02, D_BR_SP08_D_FL_SP10
H_{16S}2	1	N_JS_SP02
H_{16S}3	4	S_BM_SP01, D_FL_SS01, D_FL_SS03, D_PN_SP02
H_{16S}4	14	BY_SC_SP01, BY_SC_SP02, CZ_PK_SP01, CZ_PK_SP02, CZ_PK_SP09, CZ_PK_SP10, A_JW_SS06, D_FL_SP01, D_HR_SS02, FIN_JI_SP01, IRL_JC_SP01, IRL_JC_SP02, A_HM_SS01, D_BR_SS02,
H_{16S}5	1	BY_SC_SP03
H_{16S}6	5	CH_VL_SP01, CH_VL_SP02, CH_HG_SP01, F_HM_SP01, D_PN_SP01
H_{16S}7	2	E_MG_SPy03, E_MG_SPy04
H_{16S}8	2	E_MG_SV01, E_MG_SV02
H_{16S}9	2	TR_FS_SF01, TR_FS_SF02

Unweighted 16S haplotype data were used for the construction of median-joining networks (Bandelt et al. 1999) with different ε values (0 – 5). No differences between these networks were found. The calculated network exhibited solely one internal circle with two median vectors (fig. 31). *S. vittatum* collected in Spain (**H_{16S}8**, n=2), *S. pyrenaicum* from Spain (**H_{16S}7**, n=2), and *S. flavicorne* collected in Turkey (**H_{16S}9**, n=2) consisted each of only one haplotype, shared by no other individuals and were always separated from any other haplotype by at least two symmetrical sites. Individuals morphologically described as *S. schneideri* (n=8) revealed three different haplotypes (**H_{16S}1**, **H_{16S}2**, and **H_{16S}3**). None of these haplotypes consisted solely of *S. schneideri* individuals, but always other individuals, morphologically described as *S. personatum*, also shared these haplotypes. Specimens of *S. personatum* were subdivided

into six different haplotypes (**H_{16S}1 – H_{16S}6**). Two haplotypes, **H_{16S}2** and **H_{16S}5,** were represented by only one specimen: **H_{16S}2** being nearly identical with **H_{16S}1**, only separated by a mutation in one variable site, and **H_{16S}5** being identical with **H_{16S}4** except one mutation in a variable site. All specimens of *S. personatum* collected in the Alps (F_HM_SP01, CH_VL_SP01, 02, and CH_JG_SP01) and one of the two specimens from the Swabian Mountains (D_PN_SP01) represented one haplotype (**H_{16S}6**, n=5). This was separated from any other *S. personatum* specimens by mutations in at least four sites. Though genetically identical in the mtDNA sequences, these alpine *S. personatum* specimens exhibited expressive morphological variation at the Xth abdominal lateral sclerites and the maxillary palps (see Fig. 31). Specimens of *S. pyrenaicum* (**H_{16S}7**) differed in at least two sites and (**H_{16S}9**) in at least three sites from the Central European populations of *S. schneideri* (**H_{16S}1**, **3**, **4**). *S. pyrenaicum* and *S. flavicorne* were separated by two mutations.

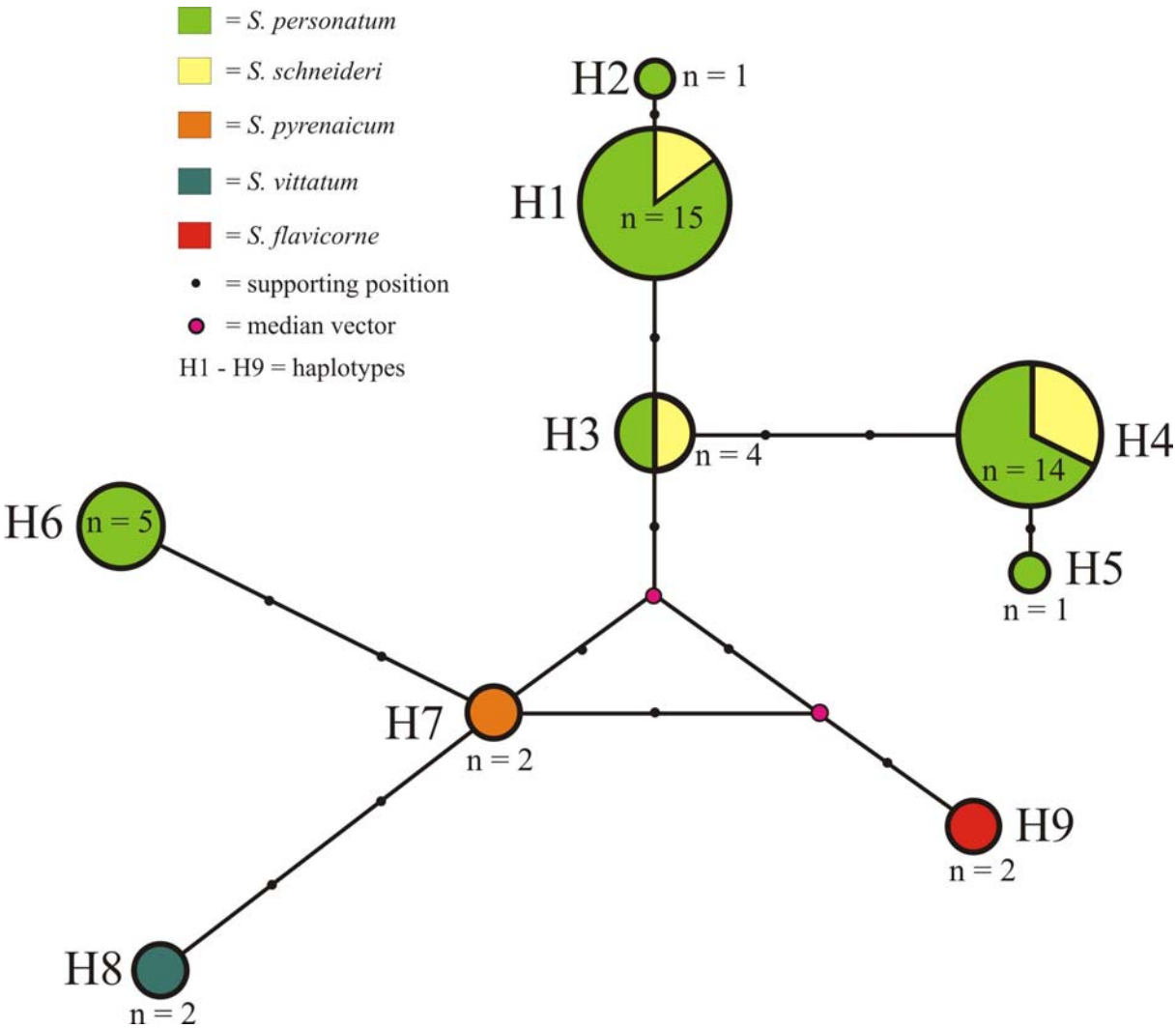

Fig 31: Median-joining network (ε = 0 – 5) of mitochondrial 16S rDNA haplotype data H_{16S}1 – H_{16S}9 (H1 – H9 in the graph) of all *Sericostoma* specimens collected (n = 46). Diameter of pie diagrams represents number of specimens (n = number of specimens). Coloured portions in pie diagrams represent the names of morphospecies that share the haplotype (see legend).

(ii) COI data sets (n = 22, respectively 23 specimens)

Variability within the 439 bp COI data set[14] of the investigated *Sericostoma* individuals (n = 22) was higher than within the 16S data set, with a maximal patristic distance of $D_p = 5.47\%$ between alpine *S. personatum* (e.g. CH_VL_SP01, 02) and *S. personatum* from Belarus (e.g. BY_SC_SP01). Within the 412 bp data set including the Spanish *Sericostoma* sequence obtained from GenBank (AF 436535), the maximal patristic distance was $D_p = 5.34\%$ for the same taxa couple and additionally for the pairwise distance between the Spanish *Sericostoma* and the alpine *S. personatum* and the Spanish *Sericostoma* and the *S. personatum* from Belarus. Sequences were found to be subdivided into nine different haplotypes (**$H_{COI}1$ – $H_{COI}9$**) (see tab. 20). Haplotype diversity ($\pi \pm SD$) was high: $\pi = 0.822 \pm 0.065$.

Tab. 20: Haplotypes ($H_{COI}1$ – $H_{COI}9$), number and label of the sampled *Sericostoma* specimens (including the Spanish Sericostoma sequence data obtained from GenBank) for the 412 bp COI gene fragment.

haplotype	n	specimens
$H_{COI}1$	3	CH_VL_SP01, CH_HG_SP01, F_HM_SP01
$H_{COI}2$	1	CH_VL_SP02
$H_{COI}3$	9	CZ_PK_SP01, CZ_PK_SP02, CZ_PK_SP09, CZ_PK_SP10, A_JW_SS06, IRL_JC_SP01, IRL_JC_SP02, D_BR_SS02, D_FL_SP01
$H_{COI}4$	3	BY_SC_SP01, BY_SC_SP02, BY_SC_SP03
$H_{COI}5$	3	D_BR_SP08, D_FL_SS02, D_FL_SP02
$H_{COI}6$	1	N_JS_SP02
$H_{COI}7$	1	D_FL_SS01
$H_{COI}8$	1	S_BM_SP01
$H_{COI}9$	1	*S. 'spain'* sp (AF 436535)

[14] The *S. 'spain'* sp. from Spain (GenBank Accession number AF 436535) excluded.

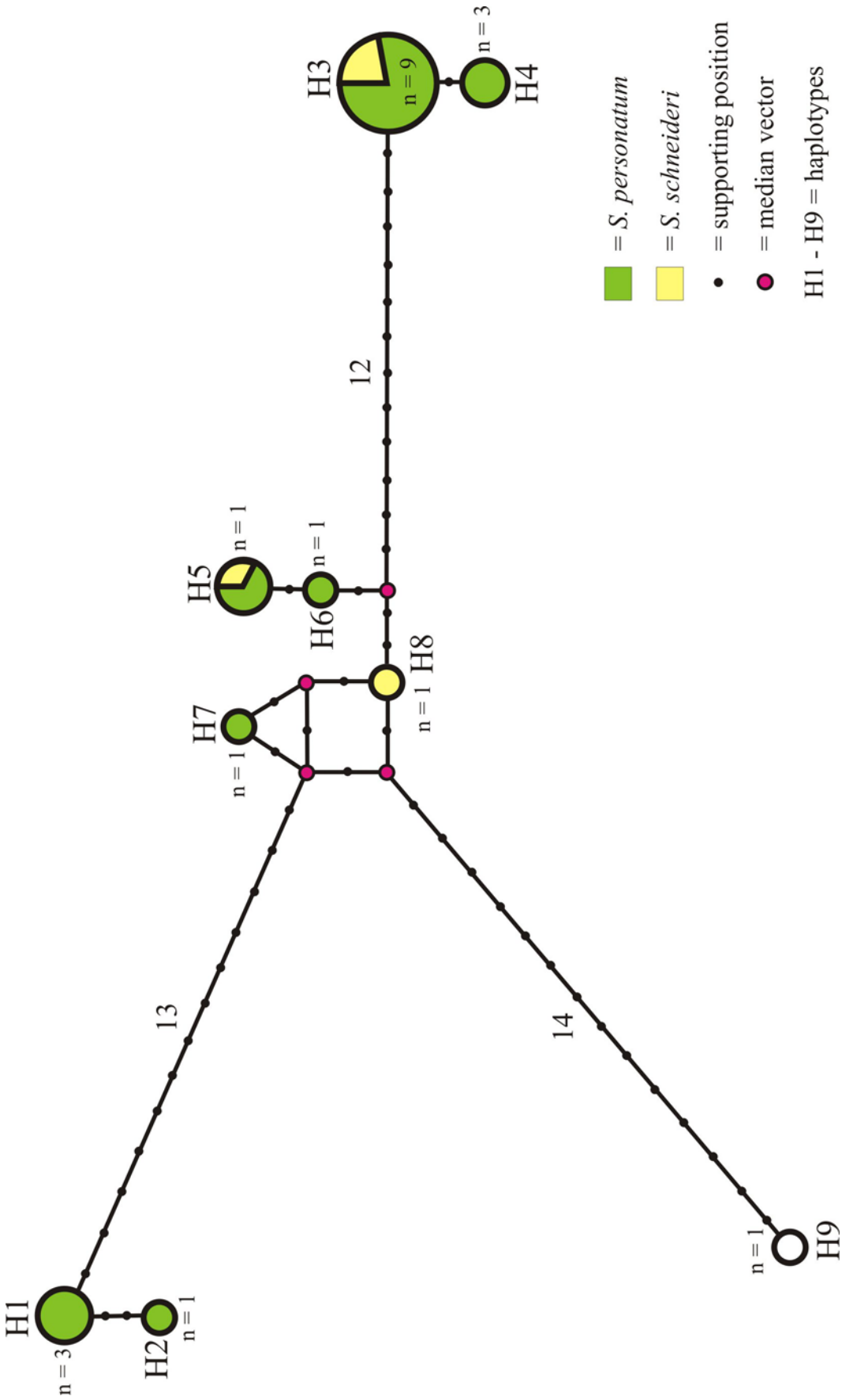

Fig. 32: Median joining network (ε = 0) of haplotypes $H_{COI}1$ – $H_{COI}9$ (H1 – H9 in the graph) of the mitochondrial COI (412 bp) data set (n = 23 *Sericostoma* species). Different colours within the haplotype pie diagrams represent the portion of *S. personatum* and *S. schneideri* specimens determined by morphological characters. Numbers on edges represent the number of supporting positions. H9 represents the unidentified Spanish *Sericostoma* specimen of the sequence obtained from Genbank (Accession number AF 436535).

(iii) Combined 16S and COI data set (n = 22 specimens)

The 439 bp data set for the 22 sampled *Sericostoma* specimens (see ii) and 16S rDNA sequence data for the same specimens (458 bp) were combined and found to be subdivided into nine different 'combined' haplotypes (**H_c1 – H_c9**) (see tab. 21). Haplotype diversity ($\pi \pm SD$) was rather high: $\pi = 0.814 \pm 0.071$. Variability of the combined 897 bp was located between variability of the separate data sets, with $p_{max} = 3.35\%$.

Unweighted haplotype data were used to create a median-joining network (Bandelt 1999) with $\varepsilon = 0$ (fig. 33) and a split-decomposition network (Bandelt and Dress 1992) (fig. 34). The calculated median-joining network contained five median vectors. Changing ε to 1 or values above added an extraordinary high number of homoplastic reticulations within the Central European *Sericostoma* complex (**H_c6 – H_c9**). The general haplotype distribution pattern was not altered by manipulating ε and therefore only the network for $\varepsilon = 0$ was visualized.

Tab. 21: Label and number of *Sericosotma* specimens per haplotype (**H_c1 – H_c9**) for the combined 16S rRNA and COI gene fragment (897 bp).

haplotype	n	specimens
H_c1	15	CH_VL_SP01, CH_HG_SP01, F_HM_SP01
H_c2	1	CH_VL_SP02
H_c3	14	CZ_PK_SP01, CZ_PK_SP02, CZ_PK_SP09, CZ_PK_SP10, A_JW_SS06, IRL_JC_SP01, IRL_JC_SP02, D_BR_SS02, D_FL_SP01
H_c4	1	BY_SC_SP01, BY_SC_SP02
H_c5	4	BY_SC_SP03
H_c6	5	D_BR_SP08, D_FL_SS02, D_FL_SP02
H_c7	2	N_JS_SP02
H_c8	2	D_FL_SS01
H_c9	2	S_BM_SP01

Analyses confirmed the previously observed haplotype sharing between *S. schneideri* and *S. personatum* specimens (see *figs. 31, 32*). No separate groupings of *S. personatum* and *S. schneideri* were found. Again, a unique distribution pattern of haplotypes was observed: **H_c6 – H_c9** consisted of Central European, Norwegian and Swedish specimens, which were separated by a maximum of six mutations. **H_c1** and **H_c2** were only found within the Alps and were separated by at least 19 mutations from the Central European haplotypes. **H_c3** (n=14) consisted chiefly of Eastern European and Austrian *S. personatum* and *S. schneideri* specimens

(Czech Republic, Austria), and of two Central European sericostomatids (D_FL_SP01 and D_PN_SS07). Both Irish *S. personatum* species shared this typical Eastern European haplotype. The haplotypes of the Belarus *S. personatum* specimens were separated from **H_c3** by only one (**H_c4**), resp. two (**H_c5**) mutations. The three Eastern European haplotypes (**H_c3** – **H_c5**) were separated from the Central European haplotype complex (**H_c6** – **H_c9**) by at least 16 mutations and from the alpine complex by at least 36 mutations.

The whole *Sericostoma* complex can be subdivided into three clearly distinct haplotypes groups: **H_c1** and **H_c2** only found within the Alps, **H_c3** and **H_c4** chiefly in Eastern European regions and Ireland, and finally into Central and Northern European regions (**H_c5 - H_c7**).

Sympatric *S. personatum* and *S. schneideri* specimens (D_PN_SP11, and D_PN_SS07) collected in Rhineland-Palatinate were genetically identical. Both specimens collected within the Breitenbach (Central Germany) differed significantly in their haplotype, one belonging to the eastern haplotype group, one belonging to the Central European haplotype group. The same pattern was already observed for the 16S data set concerning *S. personatum* specimens from the Swabian Mointains: D_PN_SP01 belonged to the alpine haplotype group and the sympatric specimen D_PN_SP02 had the Central European haplotype (see tab. 21).

Split-decomposition revealed the same pattern but exhibited some more homoplasies without altering the general pattern (see fig. 34).

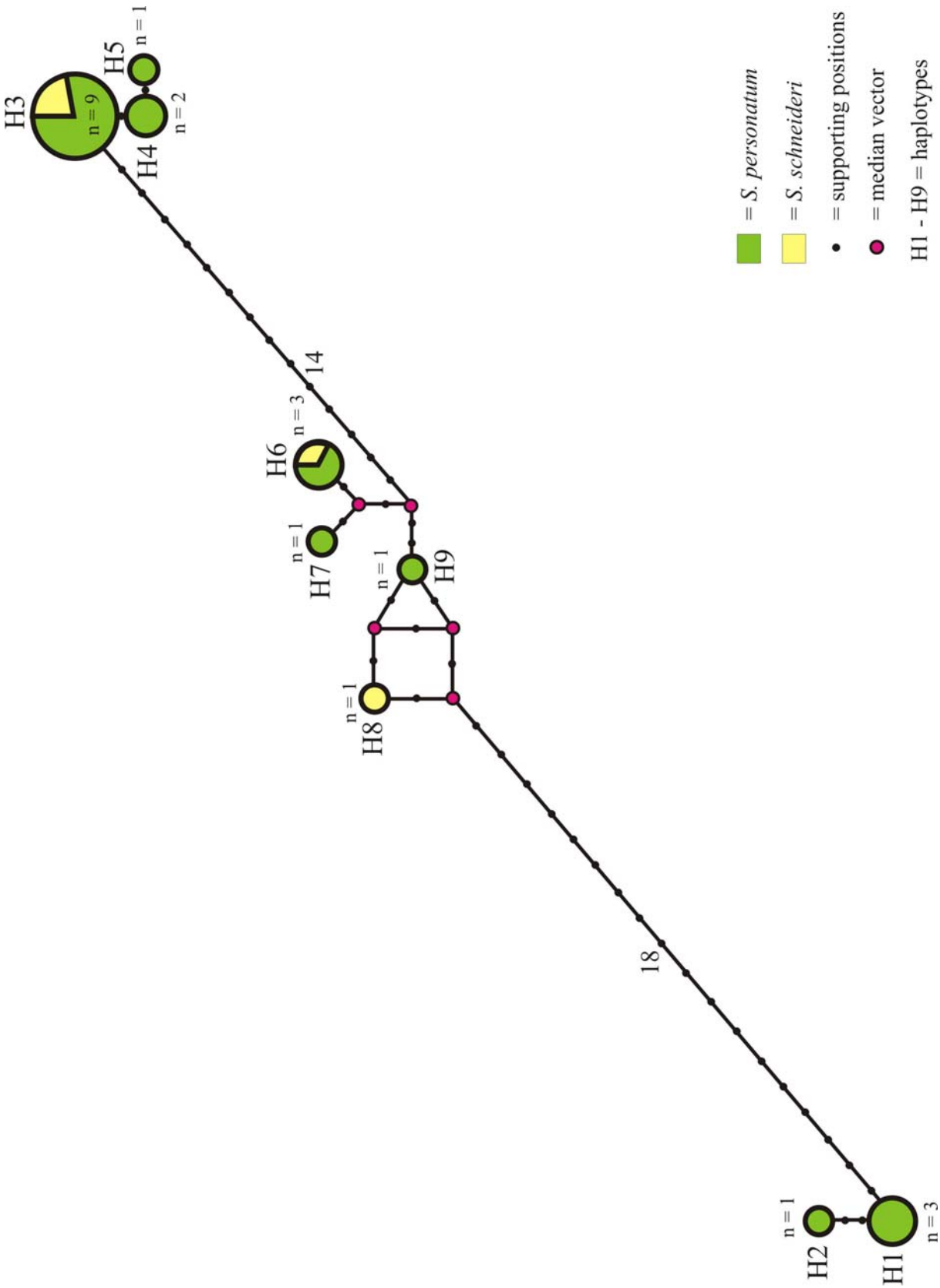

Fig. 33: Median joining network (ε = 0) of haplotypes H_c1 – H_c9 (H1 – H9 in the graph) of the combined mitochondrial 16S rDNA and COI (897 bp) data set. (n = 22 *Sericostoma* species). Diameter of pie diagramms represents number of specimens sharing this haplotype, different colours represent portion of morphospecies (*S. personatum* green, *S. schneideri* yellow). Numbers on edges represent number of supporting sites.

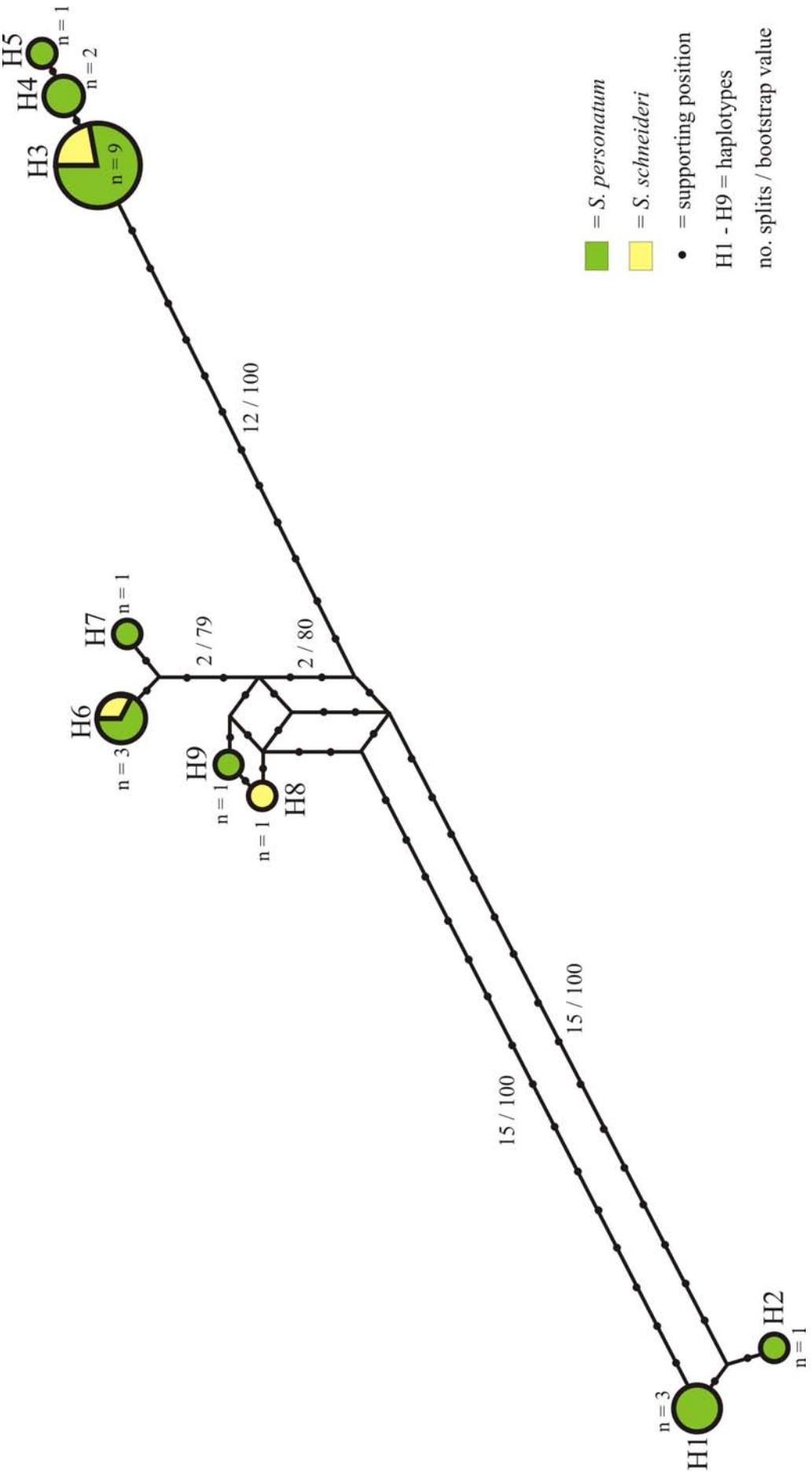

Fig. 34: Split decomposition network (Bandelt and Dress 1992), of haplotypes H_c1 – H_c9 (H1 – H9 in the graph) of the combined mitochondrial 16S rDNA and COI (897 bp) data set. (n = 22 *Sericostoma* species). Diameter of pie diagramms represents number of specimens sharing this haplotype, different colours represent portion of morphospecies (*S. personatum* green, *S. schneideri* yellow). Numbers on edges represent number of supporting sites. Numbers on the edges represent the number of representing splits (former) and (the latter) the node support calculated by the non parametric bootstrap test (Felsenstein 1985) with 1000 replicates.

Geographical haplotype pattern

Applying the geographical haplotype partitioning method proposed by Liebers and Helbig (2002), the haplotype data derived from mtDNA investigation were visualized on a map of Europe according to their predominant occurrence. Haplotypes of the 16S rDNA data set (fig. 31) that were not separated by more than one position from each other were pooled. For the COI and the combined COI / 16S data set (figs. 31 – 34), haplotypes that were not separated by more than seven positions were pooled. Both gene fragments, analysed separately and combined, offered consistent haplotype grouping patterns resulting in six different haplotype groups (**HG1** – **HG6**) (see tab. 22).

Tab. 22: Haplotype groups, related specimens and species identification according to morphological characters and their geographical location.

HG	specimen	'species'	geographical location
HG1	E_MG_Spy03, E_MG_SPy04	*S. pyrenaicum*	Iberian Peninsula
HG2	E_MG_SV01, E_MG_SV02	*S. vittatum*	Iberian Peninsula
HG3	CH_VL_SP01, CH_VL_SP02, CH_HG_SP01, F_HM_SP01, D_PN_SP01	*S. personatum*	Alpes (including Swabian Mountains)
HG4	N_JS_SP01, D_FL_SP20, D_PZ_SP01, D_HR_SP01, D_FL_SS02, D_FL_SP02, D_PN_SS07, D_PN_SP11, D_BR_SP02, D_FL_SP21, D_FL_SP03, D_BR_SP01, D_HR_SP02, D_BR_SP08, D_FL_SP10, N_JS_SP02, S_BM_SP01, D_FL_SS01, D_FL_SS03, D_PN_SP02	*S. personatum* and *S. schneideri*	Central Europe, including Norway and Sweden
HG5	BY_SC_SP01, BY_SC_SP02, BY_SC_SP03, CZ_PK_SP01, CZ_PK_SP02, CZ_PK_SP09, CZ_PK_SP10, A_JW_SS06, D_FL_SP01, D_HR_SS02, FIN_JI_SP01, IRL_JC_SP01, IRL_JC_SP02, A_HM_SS01, D_BR_SS02,	*S. personatum* and *S. schneideri*	Belarus, Finland, Czech Republic, Austria), Ireland (and three German specimens)
HG6	TR_FS_SF01, TR_FS_SF02	*S. flavicorne*	Turkey

The six haplotype groups could be related to five distinct European regions:

- **HG 1** and **2**: Iberian Peninsula
- **HG 3**: The Alps (including Swabian Mountains)
- **HG 4**: Central Europe (including Sweden and Norway)
- **HG 5**: Eastern Europe (Belarus, Czech Republic, Austria, Finland) and Ireland
- **HG 6**: Turkey

The Iberian and Turkish haplotypes could unambiguously be assigned to morphospecies *S. vittatum* (Spain), *S. pyrenaicum* (Spain) and *S. flavicorne* (Turkey). The alpine haplotype group (**HG3**) consisted only of members of *S. personatum* but genetic distance to the central

European *S. personatum* specimens was as high as between *S. vittatum* and the Central European complex. Morphological characters exhibited high variability within the alpine group. Additionally, one of the two specimens sampled in the Swabian Mountains (D_PN_SP02) could clearly be assigned to the Central European haplotype group (**HG4**). Furthermore, in Central Europe, where **HG4** represents the dominating haplotype group, three specimens were found that obviously belonged to the eastern haplotype group (**HG5**).

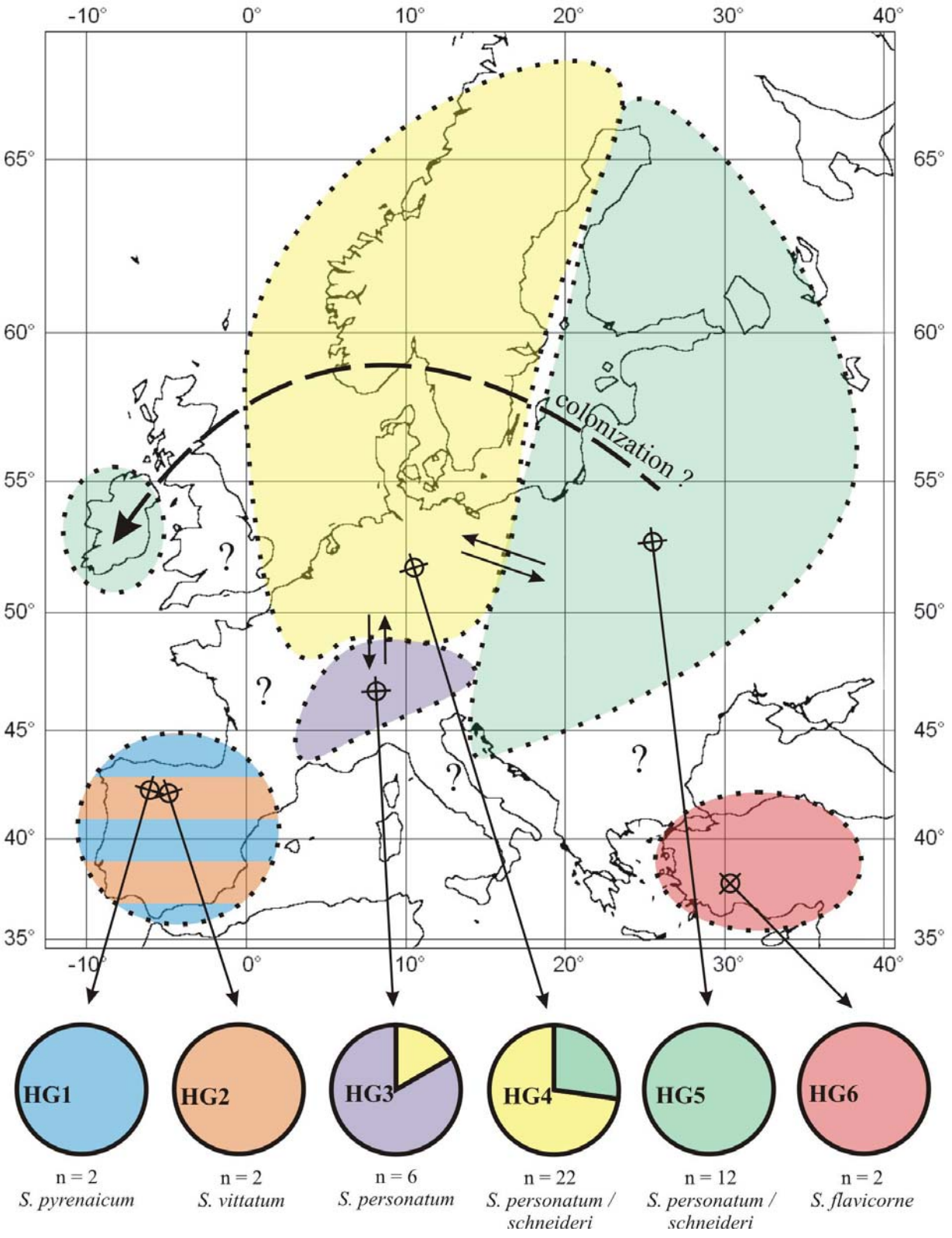

Fig. 35: Geographical distribution pattern of the six *Sericostoma* haplotype groups (HG1 – HG6). Pie diagrams indicate the portion of haplotype groups in a certain region. Question marks indicate regions not sampled. The interrupted arrow pointing from Eastern Europe at Ireland indicates haplotype sharing between both regions. Antiparallel arrows between Central Europe and the Alps, resp. between Central Europe and Eastern Europe indicate partial haplotype sharing between these regions.

(iii) ITS-1, 5.8S, ITS-2 data set

The 'Incongruence Length Difference Test' revealed no incongruence among the different genes, thus they were investigated in combination. The aligned data set consisted of 865 bp including gaps. 839 characters were constant and 26 were variable. Of these, seven sites exhibited substitutions (two transversions, five transitions), the resulting 19 sites contained at least one gap.

Tab. 23: Sequence characteristics of the ITS-1, 5.8S, ITS-2 sequence alignment. Minimum – maximum and average (in brackets) percentage of the separate nucleotides.

genes	no sites	A	C	G	T
ITS-1, 5.8S, ITS-2	865	26.1 – 26.2 (26.1)	22.8 – 23.0 (22.9)	25.7 – 25.8 (25.8)	25.2 – 25.3 (25.2)

Of the three sequences analyzed, two belonged to *S. personatum* species (Belarus: BY_SC_SP02 and Sweden: S_BM_SP01), and one belonged to *S. schneideri* species (Austria: A_JW_SS06). Variable sites were investigated using the split decomposition approach (Bandelt and Dress 1992). It was found, that variability between both *S. personatum* specimens was significantly higher (26 splits) than variability between BY_SC_SP02 and A_JW_SS06 (five splits).

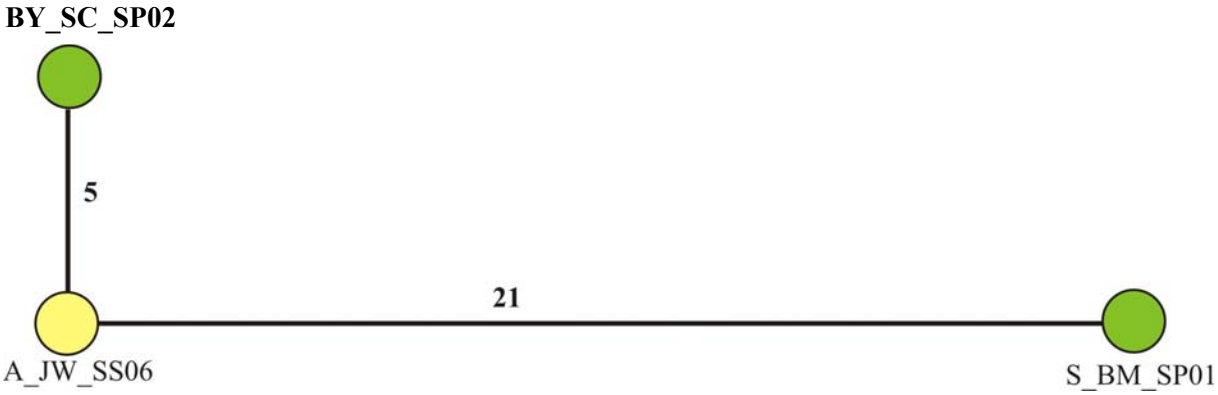

Fig. 36: Split decomposition network (Splitstree 4.0) of the three ITS-1, 5.8S, ITS-2 sequences. Numbers on edges indicate the number of split-supporting positions

This is almost consistent with the combined 16S and COI data set investigated by split decomposition: A_JW_SS06 (**H_c3**) is separated from BY_SC_SP02 (**H_c4**) by one split, but from S_BM_SP01 (**H_c9**) by 16 mutations (see fig. 34). Therefore, nuclear and mitochondrial character consistently prove this phenomenon.

Phylogeny of the European genera of the family Sericostomatidae

(i) Distance methods

Comparing the uncorrected genetic distances (including gaps) between the four genera reveals closest similarity between *Schizoplex* and *Sericostoma,* resp. *Notidobia* and *Sericostoma* (4.4 – 5.4 %, resp. 4.6 – 5.5 % sequence divergence), followed by *Notidobia* and *Schizoplex* (6.5 – 6.6 % sequence divergence) and *Oecismus* and *Sericostoma* (5.8 – 6.9 % sequence divergence). Highest sequence divergence was observed between *Oecismus* and *Notidobia* (6.9 – 7.4 % sequence divergence) and *Oecismus* and *Schizoplex* (7.0 – 7.1 % sequence divergence).

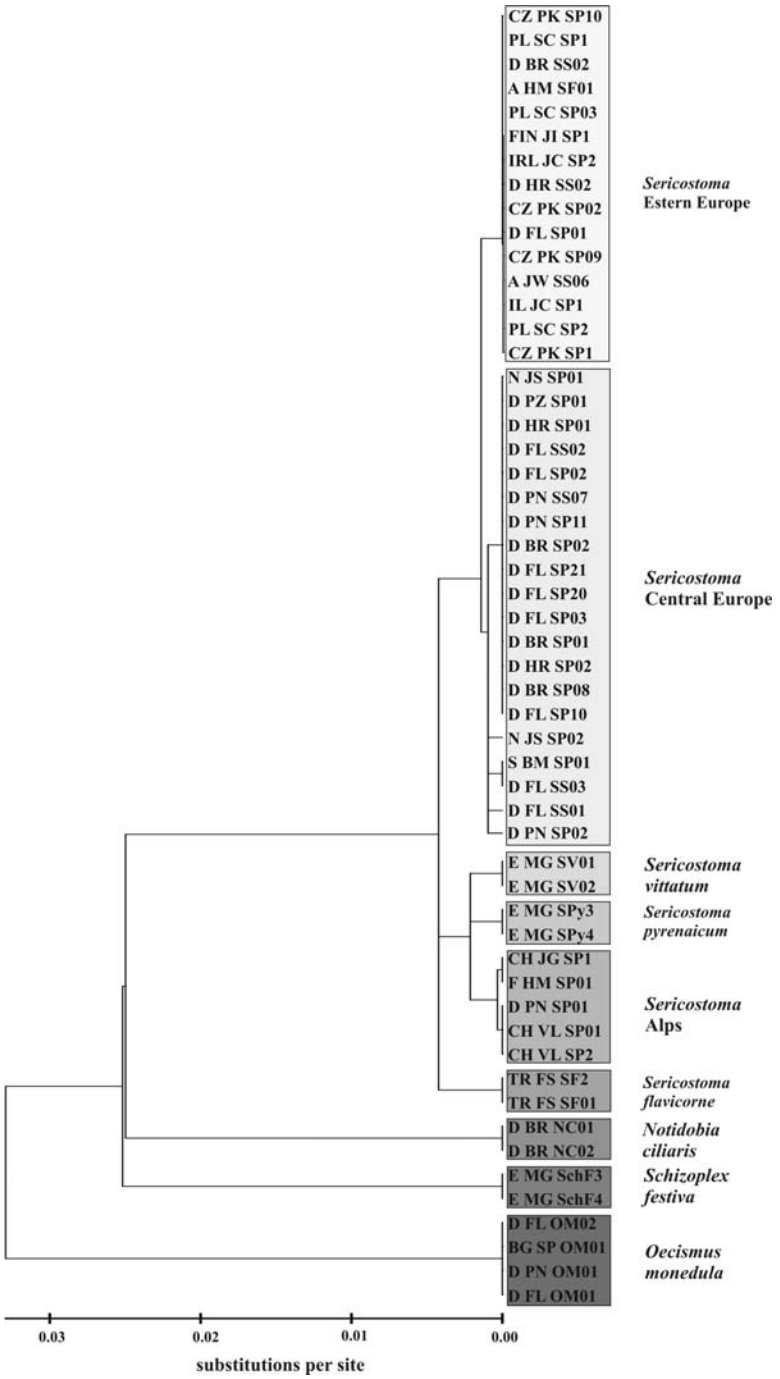

Fig. 37: Unrooted tree for Sericostomatid genera, calculated using uncorrected p-distances of the 16S rDNA data set and the neighbour-joining algorithm. The resulting tree was linearized using the program 'Mega' (Kumar et al. 2001).

The topology of the resulting tree (fig. 37) is unrooted and thus less informative, since for phylogenetic analyses it is necessary to add less related outgroup taxa to be able to distinguish between plesimorphic and apomorphic characters. Consequently, for further investigations limnephilid (Trichoptera) and lepidopteran taxa were used as outgroups.

Using the NJ algorithm a tree was estimated for the 16S rDNA from distances corrected with

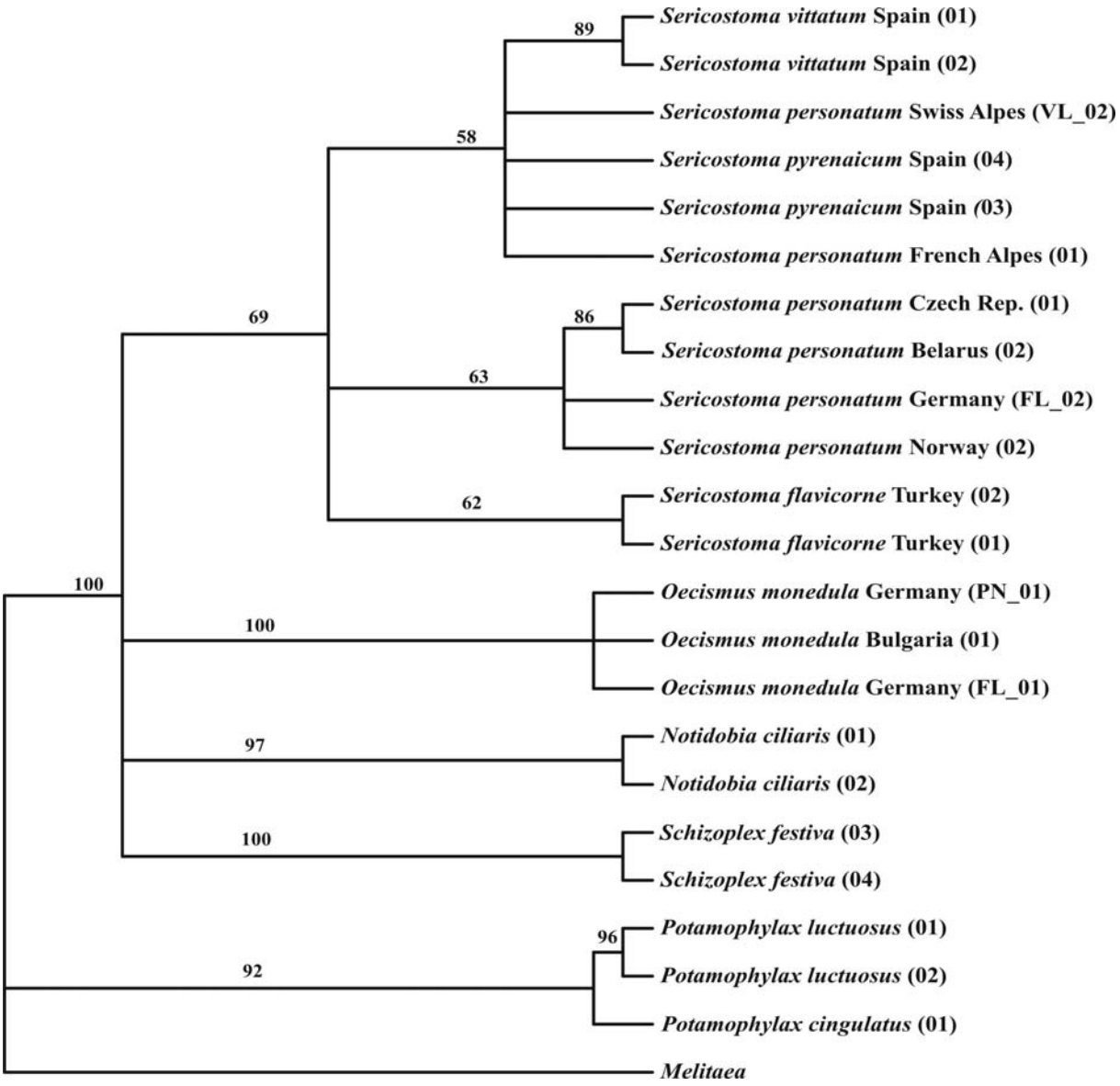

Fig. 38: Results of an unconstrained distance analysis of the 16S rDNA data set using the neighbor-joining algorithm. A GTR model with a gamma correction for among-site rate variation was used. Numerals above edges represent bootstrap proportions > 50 supporting the analysis. Information about the individual used is written in brackets behind morphospecies names (see tab. 11).

the GTR + gamma model. The data set included *Potamophylax cingulatus* as outgroup species. Gaps were excluded from analysis. The resulting tree supported all four investigated genera with high bootstrap values (fig. 38). The relation between the genera could not be resolved, but the genera are monomphyletic (bootstrap support ≥ 95).
In this reconstruction, the *Sericostoma* group was subdivided into two major clades, one including the Turkish *Sericostoma* specimens and the other including all other *Sericostoma* taxa. Bootstrap support for this division was low (see fig. 38).

MP

Parsimony analysis of the 16S rDNA data set without considering gaps identified four most-parsimonious trees (MPTs) each with a length of 184 steps. Homoplasy was low (HI = 0.1033), consistency (CI = 1 – HI = 0.8967), and retention index high (RI = 0.9387), indicating a high number of potential synapomorphies within the data set.
Parsimony analysis considering gaps as fifth character (fig. 39, B) identified four most parsimonious trees (MPTs) as well, each with a length of 213 steps. Homoplasy was low (HI = 0.0939) and consistency high. Retention index revealed high number of potential synapomorphies (RI = 0.9457). Upweighting of transversion (3:1, resp. 5:1) did not alter the tree topology.
The strict consensus tree calculated from the MPTs and the bootstrap tree for the data set considering gaps differed from the topology calculated without considering gaps (fig. 39 A). Although monophyly of all genera was well supported and topology of *Sericostoma* specimens was similar in both trees, the relationships between genera differed: Without considering gaps, only polytomic relationships were obtained in the bootstrap tree. Considering gaps as fifth character, *Notidobia* emerged as the sister group to S*ericostoma*, *Oecismus* and *Schizoplex*. *Sericostoma* emerged as sister group to *Schizoplex* and *Oecismus* that together constitute a sister group, although not well supported.

ML analysis

ML analyses using the GTR + gamma corrected data set yielded one best tree. Topology of this tree was similar to the tree reconstructed using the MP method considering gaps (fig. 39 B) except that the genus *Sericostoma* was subdivided into two major clades, the first consisting of the Turkish *Sericostoma* specimens and second containing all other *Sericosotma* specimens, as in the neighbor-joining tree (fig. 38). A bootstrap test did not support this to-

pology with values above 50 and the relationships between sericostomatid genera collapsed into polytomies, as in the MP tree without considering gaps.

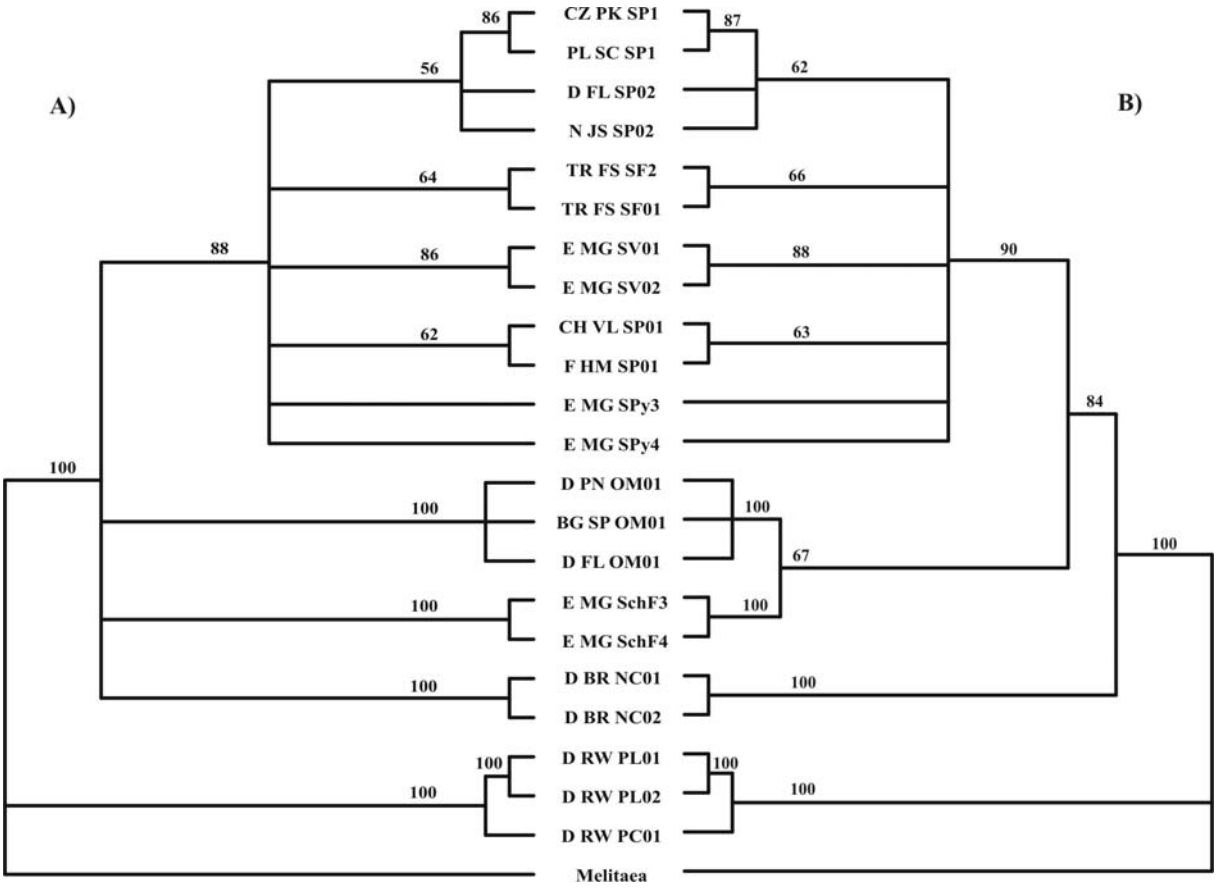

Fig. 39: Results of an equally weighted parsimony analysis of 16S rRNA data of Sericostomatidae including three *Potamophylax* (Trichoptera: Limnephilidae) and one *Melitaea* (Lepidoptera) species as outgroup. A) Analysis without considering gaps, 200 bootstrap replicates; B) Analysis considering gaps as fifth character, 1000 bootstrap replicates. Numbers above edges represent bootstrap proportions. Edges without numbers represent bootstrap proportions not recovered in the bootstrap analysis.

In summary, analyses incorporating a variety of assumptions al methods resulted in two trees, where either all genera appeared in polytomies, or *Notidobia* was placed at the stem of the Sericostomatidae, whilst the grouping the other genera was in general polytomic or low supported.

Phylogenetic signal

The basal position of *Notidobia* as calculated using the MP methods considering gaps was not supported.

Divergence time

No calibrated molecular clock rate is available for any trichopteran taxon yet. Therefore only rough approximations are allowed since no relative rate test was performed. Many valid estimations of evolutionary rates of mtDNA, in particular of COI and 16S rDNA have been made for other arthropod taxa (e.g. DeSalle et al. 1987; Brower 1994; Schubart et al. 1998) and resulted in rates of approximately 2% (COI) and 0.8% (16S) sequence divergence per 1 million years. Assuming this rate to be valid for the *Sericostoma* specimens al well, one can date divergence events separating the eastern haplotype group from the Central European groups. Sequence divergence is 3.16 – 3.64 %. This equals 1.58 – 1.82 million years. Sequence divergence between the alpine haplotype group and the Central European haplotype group is 3.4 – 4.1 %. This equals 1.7 – 2.05 million years. For the Spanish (species unidentified) haplotype group sequence divergence is 3.88 – 4.61 %. This equals 1.94 – 2.3 million years. Nevertheless these dates are not corrected for ancestral polymorphisms (Edwards and Beerli 2000).
For the 16S rDNA, a rate of 0.8% sequence divergence per million years can be expected. This implies an estimated separation time between genera of 6.1 to 8.9 million years B.P.

7.4 Discussion

7.4.1 Methodological aspects

Suitability of genes and primers

Amplification of the 16S rDNA gene fragment with primers 16a and 16b was trivial when DNA was present in an adequate amount. Loaned material was stored in alcohol of poor quality and therefore DNA isolation failed. Amplification of the COI and in particular of the ITS-1, 5.8S, ITS-2 gene fragments turned out to be an extremely challenging task. The COI reverse primer 'Pat_mod' was constructed to bind at a highly conserved site within the t-RNA coding gene (leucine). Because the t-RNA gene is located behind the COI gene (in *Drosophila*) it was thought to amplify almost the complete COI fragment when paired with the forward primer 'LCO-mod'. Unfortunately, amplification with 'Pat_mod' did only work for orthopteran DNA, but not for Trichoptera DNA. Rearrangement of the t-RNA gene within the mitochondrial genome might be a cause for this. PCR Mix B contained DNA stabilizing ingredients ('Q-solution' / Qiagen; 'MgAc' / Eppendorf) and improved results from degraded DNA samples. Problems of amplification of the ITS-1, 5.8S, and ITS-2 fragment might have occurred since the primers were primarily designed for molluscs. Amplification of the particular fragment did only work for three specimens. For all other ones no adequate amount of DNA or just a very short fragment (~ 400 bp) was amplified. Comparing the short fragment sequence data with sequences published in GenBank using the BLAST algorithm, the short fragment turned out to be protozoan DNA – probably a result of contamination.

Variability of the 16S rDNA gene fragment was adequate for generic level phylogeny but too low for species level phylogeny. For comparison between sericostomatid and outgroup taxa the 16S rDNA gene revealed too many substitutions thus reducing information about phylogenetically older events due to signal erosion (see fig. 29) (see Wägele 2001). COI revealed a significantly higher amount of variability that was adequate for the *Sericostoma* species level investigation.

Amino acid exchange within the COI fragment

Synonymous substitutions do not alter the phenotypic protein structure. In contrast to the 25 synonymous substitutions (two at first codon position, 23 at third codon position) five substitution alter the primary structure of the protein. In two cases, valine is replaced by isoleucine and methionine by valine (or vice versa). These types of replacements are functionally

equivalent (Knippers 2001) and occur often in sequences at low levels of divergence (Sperling et al. 1999).

A significant exchange is found at position 274. In Eastern European taxa the amino acid proline is transcribed, while the Central European and alpine specimens transcribe the amino acid serine. Proline is the only cyclic amino acid with an amino group consisting of nitrogen and solely one hydrogen atom. Serine belongs – due to its additional hydroxylgroup – to the polar amino acids. The phenotype of these proteins might differ significantly due to this amino acid replacement. Since proteins consist of domains with functional more or less important centres, it might be possible that this substitution concerns a protein domain of low functional evidence.

Combined investigation of gene fragments

There is considerable disagreement over whether data sets should be combined or considered separately in phylogenetic analyses (e.g. Carpenter and Nixon 1996). Hillis (1996) suggests that the best phylogenetic hypotheses are obtained by including all relevant data and that congruence among separate data sets enhances confidence in the overall phylogenetic hypothesis. Several studies have concluded that the most reliable phylogenetic hypotheses are obtained from combined data set analyses (e.g. Artiss et al 2001; Kjer et al 2002). Such kind of investigation increases the number of parsimony informative characters and offers a higher resolution of the phylogenetic relationship if data sets are congruent.

Substitution model

A GTR substitution model using a gamma correction fits best for the 16S rDNA data sets. The use of a gamma correction seems adequate for this data set, since empirical studies revealed that substitution rates vary between different alignment regions (e.g. Dixon and Hillis 1993; Simon et al. 1994; Misof et al. 2002). The shape parameter of the gamma correction was estimated as $\alpha = 0.2821$ indicating that substitutions concern only few characters, resp. certain regions and most characters change little. For the 16S gene fragment the functional stem regions are usually very constant due to high selection pressure and substitutions occur preferably at loop regions (e.g. Whitefield and Cameron 1998; Schultz, in prep.) To correct site specific substitution rates, the gamma distribution function is used. It may be problematic to use a constant gamma distribution for phylogenetic inferences, since this induces the assumption that substitution rates do not change in time (see Wägele 2001).

Which model of analysis is best suited for a particular data set is a question of current debate, since different methods may lead to conflicting genealogies. As we can never be certain about the past history of the organisms investigated, it is always a challenging task to interpret the data sets and to make careful assumptions.

7.4.2 Phylogenetic analyses

A first objection concerning the calculated phylogenetic hypotheses is that results may not be representative because (i) of the small number of specimens surveyed for each population, (ii) the investigation is founded primarily on results of mtDNA, and (iii) sequence data might contain pseudogene data or contaminant DNA.

(i) The problem of small sample sizes is severe since not all haplotypes can be detected, leading to a reduced or, when only very rare specimens are investigated, false resolution of the true relationships among taxa. Nevertheless, many similar publications are based on data sets containing only few specimens, as often specimens are rare or insensitive for the methods applied (Sperling 1993; Landry et al. 1999; Myers and Sperling 2002). For population genetic analysis the problem of sample size is much more severe than for phylogenetic investigations (Posada and Crandall 2001). In the present work, statistical tests to estimate population variation were excluded due to this deficiency. Variability was only determined using network methods and results were interpreted with reservation.

(ii) Since the mode of inheritance of mtDNA significantly differs form nuclear DNA it might be possible that the phylogenetical signal traced form nuclear DNA is different (e.g. Avise 1986; Sperling 1993; Simon et al. 1994). Introgression of distinct haplotypes might have led to the establishment of different mtDNA haplotypes whilst the corresponding nuclear genes have been sorted out due to lower fitness (Wägele, pers. comm.).

(iii) Pseudogenes are recognizable copies of a certain gene that have lost their original function. Recent studies revealed in arthropods the existence of so-called ‘paralogous’ copies of mitochondrial genes that were translocated to the nucleus after duplication (Bensasson et al. 2001). If translocation events occurred recently there is a high probability that the primers amplify these regions as well. The phylogenetical signal of pseudogenes is different from the ‘orthologous’ genes, since pseudogenes normally lack selection pressure. It is thus important to evaluate the observed sequence differences using a secondary structure model (for rDNA) or the amino acid sequence for protein coding genes. For pseudogenes, substitutions and indels are assumed to occur at any position with the same rates, thus differing from functional genes (see Bensasson et al. 2001). Trying to isolate chiefly mitochondrial DNA, comparing the observed substitutions with the reading frame and codon position for protein coding genes, resp. with the secondary structure for rRNA genes, the risk of nuclear pseudogene contamination can be minimized. In the present work no evidence for pseudogene contamination was observed.

The risk of contamination was reduced to a minimum due to sterile working conditions and comparison of sequence data with GenBank sequences using the BLAST algorithm. Nevertheless, database comparison does not detect cross-contamination from conspecific individuals. This constitutes the most severe problem and can only be excluded due to extremely sterile working conditions

(i) Population genetics

Evaluation of results

For the construction of median-joining networks, a 'tolerance' (see Bandelt et al. 1999) of ε = 0 was chosen since higher values for ε only led to an enormous increase of homoplastic events within the Central European haplotype group without changing the relationships between the genetically distinct haplotype groups.

Delimination of haplotype groups was done with respect to the the 16S *Sericostoma* data set. Since no haplotype was separated from any other by more than two supporting positions, no prominent mutation differences were detected for pooling of haplotypes. These differences could be detected more objectively for the combined 16S and COI analyses: A distance of a maximum of six mutations was observed between the Central German *S. schneideri* specimen D_FL_SS01 (**H_c8**) and the Norwegian *S. personatum* specimen D_JS_SP02 (**H_c9**) and the German *S. personatum* / *schneideri* specimens D_BR_SP08, D_FL_SS02, D_FL_SP02. If sample size can be increased, statistical tests (F and K statistics) would offer useful tools to underpin the results of this work.

7.4.3 Conclusions

To rephrase the four hypotheses stated in the introduction, the questions originally asked can be answered as follows:

1. Do S. personatum and S. schneideri represent genetically distinct evolving groups as is assumed by most scientists (Botosaneanu and Malicky 1978; Pitsch 1993; Schmidtke 1994; Neu 2002; Robert 2003)?

There is no evidence that allows distinction of two species based on the molecular results of this study. Haplotypes of both morphospecies overlap for example the sympatric *Sericostoma* specimens morphologically described as *S. schneideri* (D_PN_SS07) and *S. personatum* (D_PN_SP11) were found to be genetically identical. It might be that speciation occurred only recently and that variability of genes investigated is older than the speciation event (ancestral polymorphisms).

2. Does S. personatum consist of several detectable geographically separated 'races' (Wagner 1990)?

At least three clearly separate haplotype groups of *S. personatum* exist. One group was only found within the Alps and the Swabian Mountains, one group in Central Europe including Western Scandinavia, and one haplotype group is chiefly present in Eastern European regions (Czech Republic, Belarus, and Finland) and Ireland. The three specimens from the BTB revealed an uneven pattern. Two of these (D_FL_SP02, 03) obviously belonged to the Central European haplotype complex, but D_FL_SP01 obviously belonged to the eastern haplotype group. A similar situation was observed concerning the specimens from the Swabian Mountains: D_PN_SP01 exhibited the alpine haplotype and D_PN_SP02 the Central European haplotype. It thus cannot be excluded that in these cases both haplotypes represent two distinct species occurring sympatrically, but it is more likely that these three specimens belong to one species being affected by introgression of the Eastern European, resp. the alpine haplotypes (compare Sperling 1993; Sperling et al. 1999).

No valid information concerning the taxonomical status of these groups can be given. Genetic differences between two well defined trichopteran species (e.g. *Potamophylax luctuosus* to *P. cingulatus*) was always considerably higher than between the populations of *'S. schneideri'* and *'S. personatum'* indicating that we are dealing with intraspecific taxonomical units. Nevertheless, if we describe species using the biological species concept we need to prove that

gene flow is restricted to be able to postulate separate species. This can only be traced using nuclear genes.

3. Do S. schneideri and S. flavicorne represent separate evolving groups as proposed by Botosaneanu (2001)?

As mentioned above, using mtDNA data the problem it can only partially be decided whether 'species' or 'races' are concerned, since genetic distances do not inform about reproductively isolation of the observed specimens. Comparing the genetic results it has been proven by analysis of the 16S rDNA fragment that both morphospecies are separated by at least three synonymous mutations. *S. vittatum* that differed most from the *S. schneideri* specimens was separated from these by at least four splits. Although hybridization cannot be excluded, it seems likely that *S. schneideri* and *S. flavicorne* represent distinct evolving lineages. One could object that the genetic distance might solely exist due to geographical distance. The results of this investigation revealed, that conspecific specimens of geographically distant population in general share the same haplotype concerning the slowly evolving 16S rDNA gene: for example the geographical distance between the German and the Bulgarian *Oecismus monedula* specimens D_PN_OM01 and BG_SP_OM01 was almost 1600 km, nevertheless they were genetically identical.

4. Do *S. schneideri* and *S. pyrenaicum* represent one entity as proposed by Botosaneanu and Malicky (1978)?

S. pyrenaicum constitutes a single haplotype group separated by at least two supporting positions from the Central European *S. schneideri* sequences regarding the 16S gene. Thus, the situation seems almost similar as the situation with the Turkish *S. flavicorne* specimens: Genetical differences can be observed but their biological evidence remains uncertain.

Reasons for geographical haplotype grouping

Lineage separation within the genus *Sericostoma* might be explained with effects of the Quaternary ice ages (2.4 million years to the present with most severe glaciation starting 700.000 years B.P.). A rough estimation of lineage separation without correction for ancestral polymorphisms was estimated to have occurred approximately 1.5 – 2.3 million years before present. The observed patterns of haplotype distribution can possibly explained with these mechanisms.

Hewitt (1996; 2000) analysed the effects of recurrent ice ages and concluded that species ranges have contracted and expanded repeatedly during glaciation cycles. The consequences are loss of variation among northern taxa and divergence among populations due to isolation, genome reorganization and hybridisation of slightly divergent genomes (see Hewitt 1996; 2000). The results presented herein support this hypothesis, since variability (haplotype number) in southern regions was significantly higher than in northern regions. All haplotypes of northern regions (Finland, Ireland, Sweden and Norway) were found in more southern regions as well, a result that can be explained by colonization from South to North (see fig. 40).

Recolonization

In principle, geographic and genetic variability allow identification of recolonisation routes and hybrid zones after the last ice ages.

Irleand was often partially or completely covered with ice. Consequently, fauna and flora consist predominantly of species found in other countries as well (low degree of endemism). No endemic aquatic insects are found in Ireland. It can be assumed that during the last Würm–Weichsel-glaciation (70.000 – 10.000 B.P.) almost all insect taxa migrated towards the warmer southern refuges. To reconstruct the recolonization history of Ireland by *Sericostoma* species, more specimens from different areas should be investigated to verify or falsify the observation that Irish *Sericostoma* specimens share the Eastern and not the Central European haplotype.

As Scandinavia was ice-covered during the last glaciation, present-day populations of *Sericostoma* specimens must be descendants of lineages that persisted in southern ice-free refugia. For recolonization the Baltic Sea might not have acted as a severe geographical barrier, since on the one hand geographical distance is short between Denmark and southern Sweden potentially allowing recolonization by the by usage of the multiple, tightly connected islands. Additionally, salinity of the Baltic Sea dropped to freshwater levels (‘Ancylus Sea’) during certain periods (Heinsalu et al. 2000) making recolonization easier. On the other hand, recolonization of Scandinavia was possible from the eastern regions neighbouring Finland.

The observed pattern with *Sericostoma* populations in Norway and Sweden being closely related to the Central European haplotype complex and *Sericostoma* species in Finland being closely related to the Eastern European haplotype complex supports the hypothesis that recolonization of Scandinavia occurred twice indepently (see fig. 40). Nevertheless, more specimens should be investigated to resolve this question.

(i) Phylogeny of sericostomatid genera

Evalutation of the phylogenetic tree

Kumar (1996) stated that trees calculated using distance based methods will give only a rough estimation of evolutionary relationship. Consequently, only one distance based tree reconstruction algorithm (NJ) was used for inferring evolutionary relationship and these results were compared to the more detailed ML and MP based trees.

If all characters used for tree construction give the same signal (and are not homoplasies), the tree is more trustworthy (Swofford et al. 1996). The more agreement there is, the less homoplasy and more consistency the characters will show on the most parsimonious tree. The values of the homoplasy and retention indices indicate minor homoplastic reticulations and thorough support the calculated MP trees. Nevertheless, their value can not be used to verify the information content of a certain topology.

The ML and the NJ methods led to phylogeny hypotheses similar for the hypotheses recovered by MP analysis, considering and not considering gaps. Supporting positions for different topologies were compared using 'PHYSID'. The basal position of *Notidobia* as calculated using the MP methods considering gaps might be due to an insert (bp 325 – 327), shared by no other sericostomatid genera, but by the outgroup taxon *Melitaea*. It might be overestimated using the approach considering gaps. According to Malicky (pers. comm.), *Notidobia* is the only genus that morphologically differs clearly from the other genera, supporting the sister group relationship between *Notidobia* and all other genera as revealed by MP analysis considering gaps. Other species of these genera should be investigated to test monophyly of these genera. Oriental, Nearctic and Neotropical sericostomatid taxa should be included for further investigation.

Finally, all sericostomatid genera show almost equal distances (4 – 7%) and sequences of other genes would be useful to gain a higher resolution.

8. FINAL CONCLUSIONS

A comparison of the results derived from the different methodological approaches, seems to reveal their ultimate incompatibility. Hatching and diurnal activity unambiguously support the division of *S. personatum* and *S. schneideri* populations as separate evolving groups, while the molecular genetic results do not support a division into two *Sericostoma* morphospecies but favour a distinction of alpine, Central, and Eastern European *Sericostoma* groups with high morphological variability regardless of their morphological differences.

Concerning the genetic differences between alpine, Eastern, and Central European populations, a hypothetical recolonization history was reconstructed following the examples for rhithrobiontic Ephemeroptera (Haybach 2003) (see fig. 40). Recurrent ice ages seem to have established separate evolving lineages in refuges in South, West and East Europe. Haplotype overlapping in Germany may support the thesis of recolonization of Central Europe from both the East and the West European refuges.

The genetically similar alpine *Sericostoma* specimens probably have endured the ice ages in Italy and recolonized the Alps from southern refuges. On the other hand, some have possibly

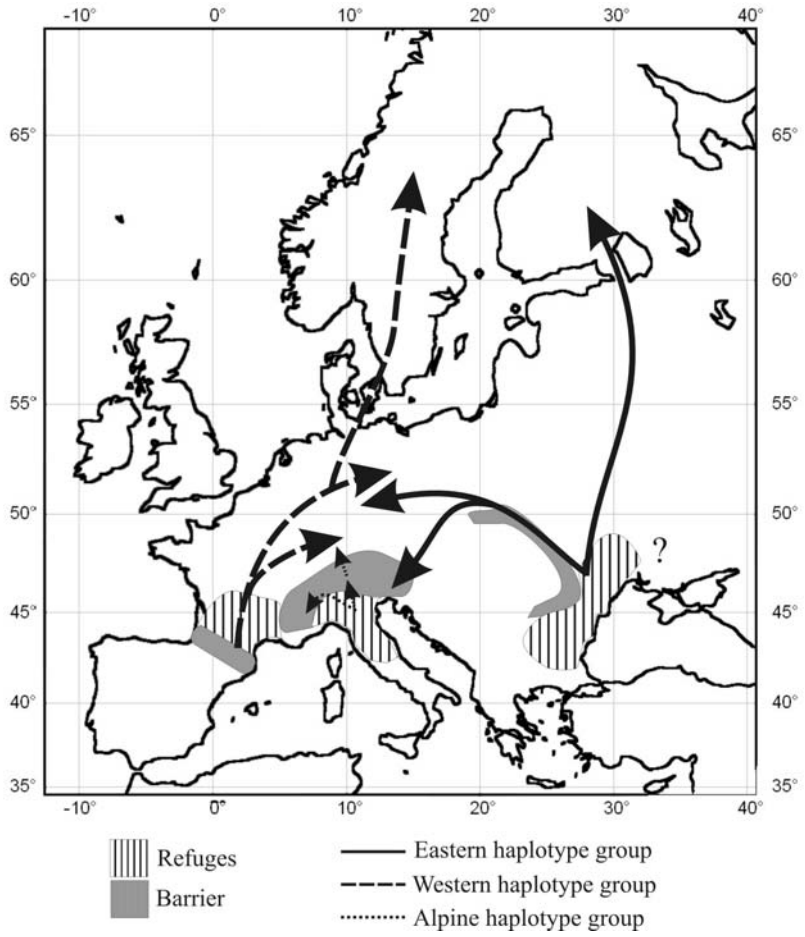

Fig. 40: Refuges and recolonization routes of the East, West, and alpine *Sericostoma* groups. Shaded areas indicate geographical barriers; lined areas indicate potential refuges for the *Sericostoma* populations (mod. after Haybach 2003).

endured the ice ages in some of the ice-free refuge within the Alps (Lubini, pers. comm.). Specimens from the eastern Alps (A_JW_SS06, A_HM_SS01) share the Eastern European haplotype, indicating that these regions were recolonized by specimens enduring the ice ages in South-Eastern refuges (see fig. 40). This observation is congruent with results of Pauls (in prep.) concerning the recolonization history of the caddisfly *Drusus annulatus.*

The genetic consequences of this hypothesis are visualized for the Central and the Eastern European Haplotype group (fig. 41 A).

Nevertheless, the differences concerning diurnal activity support the hypothesis that *S. personatum* and *S. schneideri* do represent separate evolving lineages. From the molecular genetic point this thesis can be explained with shared ancestral polymorphisms causing haplotype overlapping of *S. personatum* and *S. schneideri*, since both share the same mitochondria (see Fig. 41 B). Introgression in the common ancestral population might be the cause.

The fact that no supporting positions have been found separating *S. personatum* and *S. schneideri* may be due to recent speciation. COI and 16S might be too conserved to trace recent speciation. Techniques offering a higher resolution (e.g. AFLP) might detect differences concerning the postulated separation of both morphospecies.

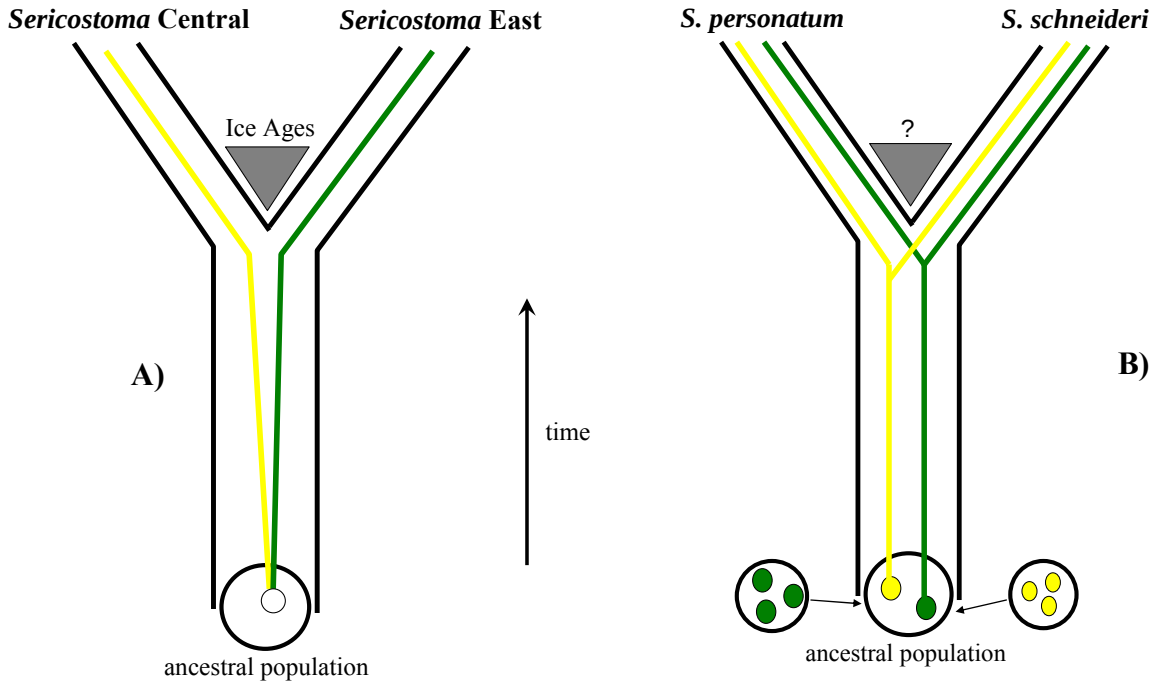

Fig. 41: Two competing hypotheses for the observed partitioning pattern of the Eastern and the Central European haplotype group. A) A division of Central and Eastern European *Sericostoma* populations occurred due to the recurrent ice ages, *S. schneideri* and *S. personatum* are not separated; morphological differences a result of polymorphisms or plasticity. B) *S. personatum* and *S. schneideri* represent distinct species; overlapping haplotype data is a result of shared ancestral polymorphisms due to introgression in the ancestral population. Speciation was traced by 16S and COI sequence data.

In 2004, chemotaxonomic and autecological approaches will be repeated to gain more information about this conflict. More sequences will be obtained from specimens collected at other sites to provide higher resolution.

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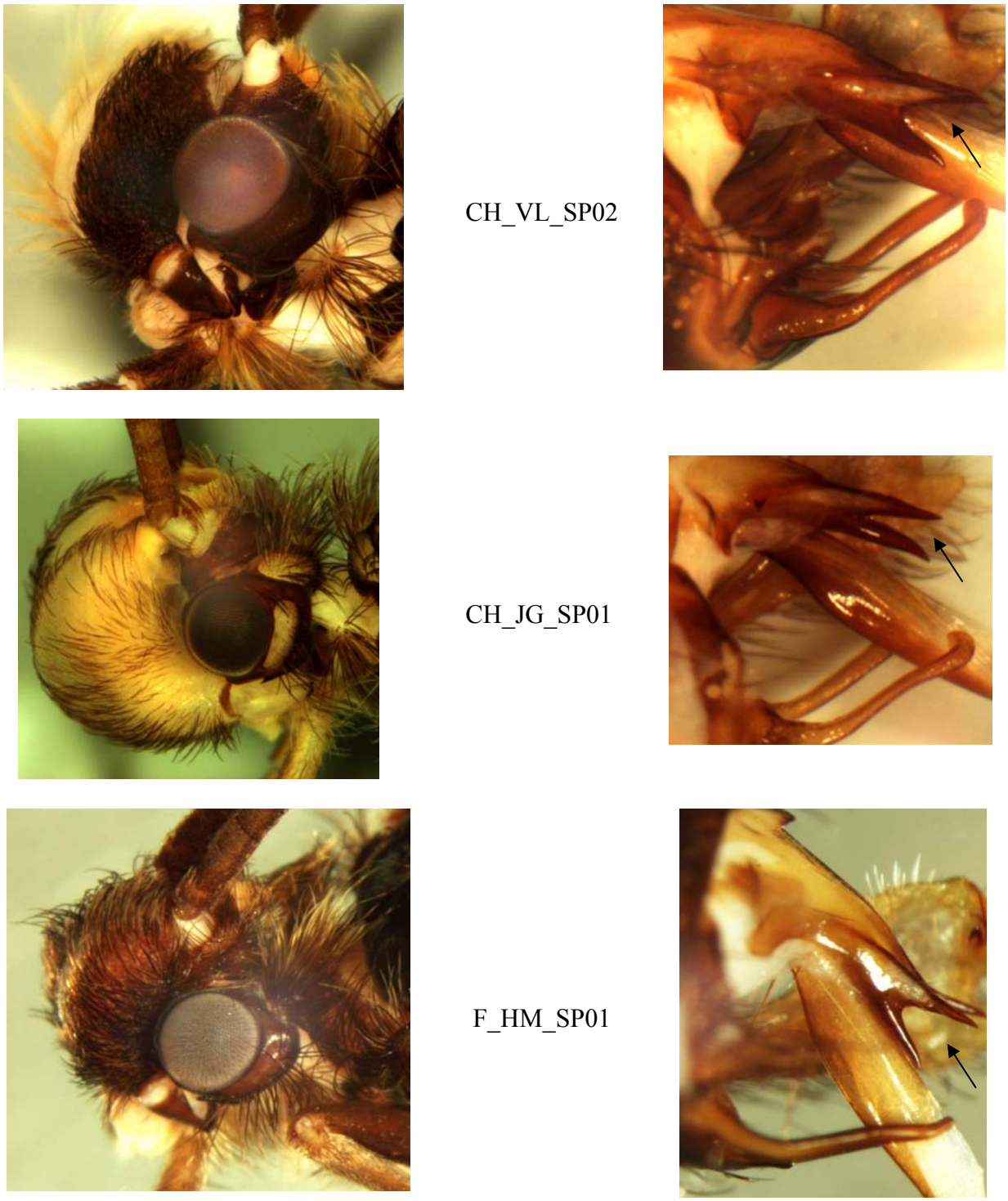
11. APPENDIX

11.1 Morphological variation



Morphological variability of maxillar palps and the lateral sclerite of the 10th abdominal segment (black arrows) of the genetical identic alpine *Sericostoma* specimens. Heads (left column) were amplified 50 fold and lateral sclerites (right column) 90 fold.

11.2 16S rDNA sequence alignment

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Melitaea     1   T-AAAGGGCTGCAGTATATTGACTGTACAAAGGTAGCATAATCATTAGTCTTTTAATTGAAGACTTGTATGAAAGATTTG 79
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D_RW_PL02    1   T-AAAGGGCTGCGGTATTTTAACCGTACAAAGGTAGCATAATAATTAGGTTTTTAATTGAAATCTAGAATGAATGATTGG 79
D_RW_PC01    1   T-AAAGGGCTGCGGTATCTTAACCGTACAAAGGTAGCATAATAATTAGGTTTTTAATTGAAATCTAGAATGAATGATTGG 79
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IRL_JC_SP2  156 AAGACCCTATAAATGTTTATATTTTTTTTATATGTAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
IL_JC_SP1   156 AAGACCCTATAAATGTTTATATTTTTTTTATATGTAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
A_HM_SF01   156 AAGACCCTATAAATGTTTATATTTTTTTTATATGTAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
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BY_SC_SP1   156 AAGACCCTATAAATGTTTATATTTTTTTTATATGTAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
D_BR_SS02   156 AAGACCCTATAAATGTTTATATTTTTTTTATATGTAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
S_BM_SP01   156 AAGACCCTATAAATGTTTATATTTTTTTTATATGTAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
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TR_FS_SF2   156 AAGACCCTATAAATGTTTATATTTTTTTTATATATAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
TR_FS_SF01  156 AAGACCCTATAAATGTTTATATTTTTTTTATATATAAAA--AAAGTATTTTATTGGGGTGATAAAAAAATTTAAAAAACT 233
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D_RW_PL02   238 TTTTAAA-TTAATTTACTAAAATTAAAGATTTTTA---AATAATGATCTATT----ATTAATAATTCAAAGAATAAGATA 309
D_RW_PC01   238 TTTTTAA-TTAATTTACTTAAATTAAAGATTTCTA---ATTTATGATCTATT----AATAATAATTCAAAGAATAAGATA 309
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TR_FS_SF2   234 TTTATTA--TTTTAATCATTAATTAATGAATTATATGTATTTATGATCTAT---AAGTT-GTAAATTAA-GATAAAGATA 306
TR_FS_SF01  234 TTTATTA--TTTTAATCATTAATTAATGAATTATATGTATTTATGATCTAT---AAGTT-GTAAATTAA-GATAAAGATA 306
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D_RW_PL02    310 CTTTAGGGATAACAGCGTAATTTTAATTTTAAGTTCTTATTAAAATTAAAGATTGCGACCTCGATGTTGGATTAAAATTT 389
D_RW_PC01    310 CTTTAGGGATAACAGCGTAATTTTAATTTTAAGTTCTTATTAAAATTAAAGATTGCGACCTCGATGTTGGATTAAAATTT 389
E_MG_SchF3   307 CTTTAGGGATAACAGCGTAATTTTAATTTTTAGTTCTTATAAAAATTAAAGATTGCGACCTCGATGTTGGATTAAAATTT 386
E_MG_SchF4   307 CTTTAGGGATAACAGCGTAATTTTAATTTTTAGTTCTTATAAAAATTAAAGATTGCGACCTCGATGTTGGATTAAAATTT 386
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BG_SP_OM01   310 CTTTAGGGATAACAGCGTAATTTTAATTTTTAGTTCTTATAAAAATTAAAGATTGCGACCTCGATGTTGGATTAAAATTT 389
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D_BR_NC02    308 CTTTAGGGATAACAGCGTAATTTTAATTTTTAGTTCTTATAAAAATTAAAGATTGCGACCTCGATGTTGGATTAAAATTT 387
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D_FL_SS01    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
D_FL_SS03    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
D_PN_SP02    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
D_PN_SP01    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTAAAA 433
CH_VL_SP01   387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTAAAA 433
CH_VL_SP2    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTAAAA 433
CH_JG_SP1    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTAAAA 433
F_HM_SP01    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTAAAA 433
E_MG_SV01    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
E_MG_SV02    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
E_MG_SPy3    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
E_MG_SPy4    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCTTTGAAA 433
TR_FS_SF2    387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCCTTGAAA 433
TR_FS_SF01   387 ATATTAAATGTAGATGTTTAAATATTGGGTCTGTTCGACCCTTGAAA 433
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11.3 COI sequence alignment

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CH_JG_SP01      1   CTTGTTCCCCTAATACTAGGGGCCCCCGATATGGCCTTCCCTCGTATAAATAACATAAGATTTTGACTTCTCCCACCTGC  80
CH_VL_SP01      1   CTTGTTCCCCTAATACTAGGGGCCCCCGATATGGCCTTCCCTCGTATAAATAACATAAGATTTTGACTTCTCCCACCTGC  80
CH_VL_SP02      1   CTTGTTCCCCTAATACTAGGGGCCCCCGATATGGCCTTCCCTCGTATAAATAACATAAGATTTTGACTTCTCCCACCTGC  80
F_HM_SP01       1   CTTGTTCCCCTAATACTAGGGGCCCCCGATATGGCCTTCCCTCGTATAAATAACATAAGATTTTGACTTCTCCCACCTGC  80
CZ_PK_SP10      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
IRL_JC_SP01     1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
CZ_PK_SP09      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
A_JW_SS06       1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
CZ_PK_SP02      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
D_BR_SS02       1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
IRL_JC_SP02     1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
CZ_PK_SP01      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
D_FL_SP01       1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
BY_SC_SP01      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTTCCACCCGC  80
BY_SC_SP02      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTTCCACCCGC  80
BY_SC_SP03      1   CTTATTCCCCTAATACTAGGAGCCCCCGATATAGCCTTTCCCCGTATAAATAACATAAGATTTTGACTTCTTCCACCCGC  80
D_BR_SP08       1   CTTGTTCCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
D_FL_SP02       1   CTTGTTCCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
D_FL_SS02       1   CTTGTTCCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
N_JS_SP02       1   CTTGTTCCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
D_FL_SS01       1   CTTGTTCCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCTGC  80
S_BM_SP01       1   CTTGTTCCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCACCCGC  80
S. spain sp.    1   ??????????????????????????????GATATAGCCTTCCCCCGTATAAATAACATAAGATTTTGACTTCTCCCGCCCGC  80

CH_JG_SP01     81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
CH_VL_SP01     81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
CH_VL_SP02     81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
F_HM_SP01      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
CZ_PK_SP10     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
IRL_JC_SP01    81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
CZ_PK_SP09     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
A_JW_SS06      81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
CZ_PK_SP02     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
D_BR_SS02      81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
IRL_JC_SP02    81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
CZ_PK_SP01     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
D_FL_SP01      81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
BY_SC_SP01     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
BY_SC_SP02     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
BY_SC_SP03     81   ACTAAATTTTCTTCTATTCAGAAGAATAATTGAAAGAGGAACAGGAACAGGATGAACAGTCTATCCCCCCCTATCCAGTA 160
D_BR_SP08      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTTTATCCCCCCCTATCCAGTA 160
D_FL_SP02      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTTTATCCCCCCCTATCCAGTA 160
D_FL_SS02      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTTTATCCCCCCCTATCCAGTA 160
N_JS_SP02      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTTTATCCCCCCCTATCCAGTA 160
D_FL_SS01      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTTTATCCCCCCCTATCCAGTA 160
S_BM_SP01      81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACAGTTTATCCCCCCCTATCCAGTA 160
S. spain sp.   81   ACTAAATTTTCTTTTATTCAGAAGAATAGTTGAAAGAGGAACAGGAACAGGATGAACTGTCTATCCCCCTCTATCCAGTA 160

CH_JG_SP01    161   ATTTAGCCCATATGGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGGGCA 240
CH_VL_SP01    161   ATTTAGCCCATATGGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGGGCA 240
CH_VL_SP02    161   ATTTAGCCCATATGGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGGGCA 240
F_HM_SP01     161   ATTTAGCCCATATGGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGGGCA 240
CZ_PK_SP10    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
IRL_JC_SP01   161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
CZ_PK_SP09    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
A_JW_SS06     161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
CZ_PK_SP02    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
D_BR_SS02     161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
IRL_JC_SP02   161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
CZ_PK_SP01    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
D_FL_SP01     161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
BY_SC_SP01    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
BY_SC_SP02    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
BY_SC_SP03    161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
D_BR_SP08     161   ATTTAGCCCATATAGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
D_FL_SP02     161   ATTTAGCCCATATAGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
D_FL_SS02     161   ATTTAGCCCATATAGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
N_JS_SP02     161   ATTTAGCCCATATAGGGTCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
D_FL_SS01     161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
S_BM_SP01     161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
S. spain sp.  161   ATTTAGCCCATATAGGATCATCCGTAGATCTTTCAATCTTTTCATTACATTTAGCAGGAGTATCCTCAATTTTAGGAGCA 240
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CH_JG_SP01     241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGCATACCTCTATTCGCATGATCAGT 320
CH_VL_SP01     241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGCATACCTCTATTCGCATGATCAGT 320
CH_VL_SP02     241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGCATACCTCTATTCGCATGATCAGT 320
F_HM_SP01      241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGCATACCTCTATTCGCATGATCAGT 320
CZ_PK_SP10     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
IRL_JC_SP01    241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
CZ_PK_SP09     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
A_JW_SS06      241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
CZ_PK_SP02     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
D_BR_SS02      241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
IRL_JC_SP02    241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
CZ_PK_SP01     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
D_FL_SP01      241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
BY_SC_SP01     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
BY_SC_SP02     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
BY_SC_SP03     241 ATTAACTTTATTACAACAATAATAAACATACGACCATTGGGAGTATCCTATGATCGTATGCCTCTCTTTGCATGATCAGT 320
D_BR_SP08      241 ATTAACTTTATTACAACAATAATAAATATACGATCATTGGGAATATCCTATGATCGTATGCCACTCTTCGCATGATCAGT 320
D_FL_SP02      241 ATTAACTTTATTACAACAATAATAAATATACGATCATTGGGAATATCCTATGATCGTATGCCACTCTTCGCATGATCAGT 320
D_FL_SS02      241 ATTAACTTTATTACAACAATAATAAATATACGATCATTGGGAATATCCTATGATCGTATGCCACTCTTCGCATGATCAGT 320
N_JS_SP02      241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGTATGCCACTCTTCGCATGATCAGT 320
D_FL_SS01      241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGCATGCCGCTCTTCGCATGATCAGT 320
S_BM_SP01      241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGATCGCATGCCACTCTTCGCATGATCAGT 320
S. spain sp.   241 ATTAACTTTATTACAACAATAATAAACATACGATCATTGGGAATATCCTATGACCGCATGCCTCTCTTCGCATGATCAGT 320

CH_JG_SP01     321 TCTTATTACAGCTGTACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGTAATC 400
CH_VL_SP01     321 TCTTATTACAGCTGTACTACTATTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGTAATC 400
CH_VL_SP02     321 TCTTATTACAGCTGTACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGTAATC 400
F_HM_SP01      321 TCTTATTACAGCTGTACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGTAATC 400
CZ_PK_SP10     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
IRL_JC_SP01    321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
CZ_PK_SP09     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
A_JW_SS06      321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
CZ_PK_SP02     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
D_BR_SS02      321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
IRL_JC_SP02    321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
CZ_PK_SP01     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
D_FL_SP01      321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
BY_SC_SP01     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
BY_SC_SP02     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
BY_SC_SP03     321 TCTTATCACAGCTATACTACTACTACTCTCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
D_BR_SP08      321 CCTTATTACAGCTGTACTACTACTACTATCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
D_FL_SP02      321 CCTTATTACAGCTGTACTACTACTACTATCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
D_FL_SS02      321 CCTTATTACAGCTGTACTACTACTACTATCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
N_JS_SP02      321 CCTTATTACAGCTGTACTACTACTACTATCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
D_FL_SS01      321 CCTTATTACAGCTGTACTACTACTACTATCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
S_BM_SP01      321 CCTTATTACAGCTGTACTACTACTACTATCACTTCCTGTTTTAGCTGGAGCTATTACCATATTATTAACAGATCGTAATC 400
S. spain sp.   321 TCTTATTACAGCCGTACTACTACTACTCTCACTTCCTGTTCTCGCTGGGGCTATTACTATATTATTAACAGATCGTAACC 400

CH_JG_SP01     401 TCAATACATCTTTCTTTGACCCAGCAGGGGGAGGTGACC                                  439
CH_VL_SP01     401 TCAATACATCTTTCTTTGACCCAGCAGGGGGGGGTGACC                                439
CH_VL_SP02     401 TCAATACATCTTTCTTTGACCCAGCAGGGGGAGGTGACC                                439
F_HM_SP01      401 TCAATACATCTTTCTTTGACCCAGCAGGGGGAGGTGACC                                439
CZ_PK_SP10     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
IRL_JC_SP01    401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
CZ_PK_SP09     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
A_JW_SS06      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
CZ_PK_SP02     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
D_BR_SS02      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
IRL_JC_SP02    401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
CZ_PK_SP01     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
D_FL_SP01      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
BY_SC_SP01     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
BY_SC_SP02     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
BY_SC_SP03     401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
D_BR_SP08      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
D_FL_SP02      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
D_FL_SS02      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
N_JS_SP02      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGGGGAGACC                                439
D_FL_SS01      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGAGGAGACC                                439
S_BM_SP01      401 TCAATACATCTTTCTTTGACCCAGCAGGAGGAGGAGACC                                439
S. spain sp.   401 TCAATACATCTTTCTTTGATCCAGCAGGAGGAGGAGACCCAATTCTTTATCAACATTTATTTTATTTT 468
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