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PARTE III
Atti delle sessioni patrocinate dalla STSN al congresso annuale dell'Accademia svizzera delle scienze naturali, 23-25 settembre 1998, Airolo, San Gottardo, Piora

Presentazione

L'Accademia Svizzera delle Scienze Naturali (ASSN) è stata fondata nel 1815 come *Società Elvetica delle Scienze Naturali* e costituisce l'organizzazione mantello delle società scientifiche nazionali, suddivise in sette sezioni: fisica, chimica, ambiente, scienze della terra, biologia I e II, matematica. Essa conta attualmente 28'000 aderenti, raggruppati in 43 società nel ramo scientifico e 28 società regionali e cantonali.

Dal 23 al 28 settembre 1998 ad Airolo-San Gottardo-Piora abbiamo accolto l'assemblea generale ed il congresso annuale dopo un'assenza di 25 anni dal Ticino. La nostra Società di scienze naturali, fondata nel 1903 ed affigliata all'ASSN ha ritenuto importante presentare in questa occasione la ricerca svolta in Ticino nell'area delle scienze naturali tramite il simposio di cui sono riportati i contributi in queste pagine. L'organizzazione della sessione specialistica: "Approccio molecolare alla biodiversità" e "Idrobiologia microbica e Lago di Cadagno" è stata effettuata in collaborazione con la Società Svizzera di Microbiologia, la Società Svizzera di Zoologia, l'Associazione Ticinese Economia delle Acque, l'Associazione della Svizzera Italiana per la Ricerca Biomedica ed il Centro di Biologia Alpina di Piora.

Gli scopi di questo simposio erano molteplici:

1) Mediante le presentazioni, mostrare l'attività di ricerca nel campo della biologia
2) Illustrare i temi nei settori clinici, ambientali e della gestione delle risorse naturali svolti in collaborazione con le università svizzere ed istituti di ricerca del Nord Italia.
3) Evidenziare l'unitarietà metodologica raggiunta nello studio della biodiversità grazie allo sviluppo delle nuove tecniche di biologia molecolare nei diversi campi.
4) In una Svizzera italiana recentemente divenuta universitaria mediante la creazione dell'Università della Svizzera italiana (USI), presentare un'importante attività di ricerca in un settore non ancora contemplato nelle facoltà recentemente istituite. Attività consolidata nel tempo mediante una buona tradizione.

Il riscontro di pubblico delle giornate in alta Leventina è stato notevole in quanto abbiamo registrato la partecipazione di più di 1000 persone con 45 società scientifiche e commissioni che hanno tenuto le loro sessioni specialistiche sullo stesso "campus accademico" ai piedi del S. Gottardo favorendo così fruttuosi scambi interdisciplinari.

La raccolta degli interventi di Airolo rappresenta pure la continuazione della serie di pubblicazioni iniziata nel 1996 con il simposio "La politica della scienza di Stefano Franscini" al Monte Verità, proseguita poi con la manifestazione Ticino Universitario allo scopo di illustrare l'evoluzione dell'attività di ricerca nella Svizzera Italiana.

La Commissione della Svizzera Italiana del Fondo Nazionale Svizzero per la Ricerca Scientifica perseguendo quale scopo l'incremento dell'attività scientifica nella Svizzera italiana partecipa alla pubblicazione degli atti.

Raffaele Peduzzi
Mauro Tonolla
Presidente annuale ASSN-98 e
Membro del comitato scientifico ASSN-98

Introduzione al simposio «Approccio molecolare alla biodiversità» e «Idrobiologia microbica e Lago di Cadagno»

Claudio Valsangiacomo, Laboratorio Cantonale, Lugano

Patrocinato da: Società Ticinese di Scienze Naturali, Società Svizzera di Microbiologia, Società Svizzera di Zoologia, Associazione Ticinese Economia delle Acque, Associazione della Svizzera Italiana per la Ricerca Biomedica, Centro di Biologia Alpina di Piora

In occasione dell'assemblea annuale dell'Accademia svizzera di scienze naturali (ASSN), svoltasi ad Airolo dal 23 al 26 settembre 1998, la STSN ha organizzato un simposio sui temi «*Approccio molecolare alla biodiversità*» e «*Idrobiologia microbica e Lago di Cadagno*».

Fil-rouge delle presentazioni orali di questo simposio è stata la caratterizzazione molecolare degli organismi viventi. Fin dagli albori le scienze naturali si sono occupate della classificazione e catalogazione degli organismi viventi. I sistemi di classificazione codificati da Linneo e i principi evoluzionistici proposti da Darwin hanno conservato fino ai nostri giorni il loro valore scientifico. Il secolo nel quale viviamo ha fatto conoscere alle scienze biologiche un progresso geometrico, le conoscenze dei sistemi biologici sono aumentate a pari passo con lo sviluppo di nuove tecnologie messe al servizio dei ricercatori. Con l'avvento della biologia molecolare nel dopoguerra e dell'ingegneria genetica negli anni ottanta la biologia ha conosciuto lo sviluppo di nuove discipline destinate a rivoluzionare usi e costumi della nostra società. Lo studio del materiale genetico nella sua struttura e funzione è stato possibile con l'aiuto di potenti mezzi informatici per l'analisi e lo stoccaggio dell'informazione genetica. Traguardi fantasiosi solo 10 anni fa, ora sono realtà: già nel recente 1995 fu possibile sequenzare *in primis* per intero il genoma di un organismo vivente (il batterе *Haemophilus influenzae*), entro la fine del secolo saranno decine i genomi batterici sequenzati, e nei primi anni del 2000 il genoma intero di un essere umano sarà disponibile su supporto informatico.

Pure la biologia organismica ha beneficiato di queste nuove tecnologie: in particolare i campi della biologia sistematica e evoluzionistica. Mentre l'approccio classico a queste discipline avveniva con la quantificazione e l'analisi di caratteri prettamente morfologici, è ora possibile raccogliere dati genetici direttamente dal DNA, sede del patrimonio genetico. La tecnica della PCR verso la fine degli anni ottanta e il sequenzaggio automatico del DNA verso l'inizio degli anni novanta hanno permesso di generare e arricchire le banche dati con una enorme quantità di informazione genetica. L'analisi molecolare degli organismi viventi ha quindi permesso di affinare, confermare oppure correggere teorie evoluzionistiche e di biologia sistematica elaborate in precedenza con analisi di tipo morfometrico. I costi di queste nuove tecnologie non permettono ancora di sostituire il retino e la lente di ingrandimento, ma nel giro di pochi anni l'approccio molecolare alla caratterizzazione di ogni essere vivente sarà senza dubbio semplificato e reso accessibile anche ai più piccoli gruppi di ricerca. Ne è prova la ricerca svolta in gran parte in istituti del nostro Cantone e oggetto di presentazione nell'ambito di questo simposio. Tutte le presentazioni del simposio illustrano la caratterizzazione molecolare di organismi viventi, spaziando fra le diverse discipline quali la zoologia, la botanica, la microbiologia clinica e ambientale, l'idrobiologia.

The use of mitochondrial DNA sequences in insect taxonomy: examples from the *Scathophagidae* (Diptera, Calyptratae)

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Key Words: Scathophagidae, Phylogeny, mitochondrial DNA (mtDNA), Subunit I of the cytochrome oxidase gene (COI)

INTRODUCTION

The use of mitochondrial DNA as molecular markers for a wide range of taxonomic, phylogenetic, population and evolutionary investigations in animals has become well established (AVISE *et al.* 1987, HILLIS 1987, MORITZ *et al.* 1987, PACKER 1989, QUICKE 1993, AVISE 1994, SIMON *et al.* 1994, HILLIS *et al.* 1996). The positive attributes of mitochondrial DNA can be summarised as follows: (i) Its small size is associated with relative manageability and ease of extraction. (ii) Most cells contain many mitochondria, so that a significant quantity of mtDNA is available even from small tissue samples. (iii) A general conservation of gene order and composition across metazoa. (iv) A range of mutational rates in different regions of the molecule. (v) Because of its maternal inheritance, differences that occur in mtDNA are due entirely to mutation and are not the result of independent assortment or recombination (QUICKE 1993, MORITZ *et al.* 1987, WOLSTENHOLME 1992, AVISE *et al.* 1987).

The mtDNA in higher animals is a circular, double-stranded molecule. It comprises approximately 16'000 base-pairs encoding two rRNAs, 22 tRNAs and usually 13 other genes, mostly coding for proteins involved in the electron transport system located on the inner mitochondrial membrane. Animal mitochondrial genes lack introns and intergenic sequences are generally small or lacking. Also absent are repetitive DNA and pseudogenes. Further, the genetic code employed in mitochondria is slightly different from that of nuclear genes. (CLARY & WOLSTENHOLME 1985, AVISE *et al.* 1987, MORITZ *et al.* 1987, WOLSTENHOLME 1992, CROZIER & CROZIER 1993).

Although mtDNA-sequence data have proven valuable in phylogenetic analysis, gene choice is of crucial importance (SIMON *et al.* 1994, LUNT *et al.* 1996). Among the coding genes in the mitochondria genome, the subunit I of the cytochrome oxidase (COI) gene possesses features making it particularly suitable for evolutionary studies (LUNT *et al.* 1996). First, it has been well-studied at the biochemical level and its size and structure appears to be conserved across all animals investigated. Then, because the COI gene is one of the largest protein-coding genes in the animal mitochondrial genome, it is possible to amplify and sequence a large portion of DNA within the same functional complex. Furthermore, highly conserved and variable regions are closely associated.

Using examples taken from the *Scathophagidae* (Diptera), we illustrate the usefulness of mtDNA sequences in elucidating taxonomic and phylogenetic questions. Scathophagids flies, with more than 250 species, are mainly confined to the Holarctic region. Only about 5 species occur south of the equator, in South America and Africa, mostly at high altitudes, whereas in the Oriental region one species occurs in the Malay Peninsula (VOCKEROTH 1956, VOCKEROTH 1989). Individuals of most species feed on vertebrate dung or decomposing carcasses, performing the ecologically-important function of resource recycling. One species, *Scathophaga stercoraria*, has been used extensively to investigate questions in animal ecology and evolution (SIGURJONSDOTTIR & PARKER 1980, AMANO 1983, PARKER & SIMMONS 1991, SIMMONS & WARD 1991, WARD & SIMMONS 1991, SIMMONS & PARKER 1992, PARKER *et al.* 1993, WARD 1993, WARD & HAUSCHTECK-JUNGEN 1993; MÜHLHÄUSER *et al.* 1996, SIMMONS *et al.* 1996, BLANCKENHORN 1997, OTRONEN *et al.* 1997, BLANCKENHORN 1998, WARD 1998). However, the taxonomy and phylogeny of this family still remain unclear. The use of morphological characters, including the structure of male genitalia which seems to be very variable for some species are usually enough for the correct identification of genera and species but are still insufficient to infer precise phylogeny. The integration of the new molecular data with previous morphological studies should provide therefore a powerful tool for the inference of correct phylogeny of this fly family.

MATERIALS AND METHODS

After DNA extraction and standard PCRs, a portion of 810 bp of the terminal region of the COI gene was sequenced in 40 Scathophagid species covering a wide geographic area, as well as a diverse spectrum of ecological habitats. Moreover, three other flies species of the *Mus-*

coidea superfamily, *Musca domestica* (family *Muscidae*), *Lasiomma seminitidum (Anthomyiidae)* and *Fannia armata* (*Fanniidae*) were also sequenced. Outgroup comparisons were made with published COI gene sequence of *Drosophila yakuba* (EMBL database access X03240), and translations to amino acid sequences used the *Drosophila* code (DE BRUIJN 1983). Sequence data were analysed by pairwise sequence alignment and by multi alignment with the Lasergene program Megalign (DNAstar 1994) and phylogenetic analysis were performed using MEGA (Molecular Evolutionary Genetics Analysis 1.01, KUMAR *et al.* 1993). Several Neighbour Joining trees were generated using different methods for distance estimation. Some Maximum Parsimony trees obtained with heuristic search were also examined.

RESULTS AND DISCUSSION

The topologies of the several phylogenetic trees generated are overall very similar (Fig. 1). In general, molecular data are in agreement with previous morphological information. But some peculiarities reveal discrepancies between the two approaches. Within the *Scathophagidae* two subfamilies are currently recognised: the *Delinae* (represented here by the species *Delina nigrita* and *Phrosia albilabris*) and the *Scathophaginae* (GORODKOV 1986, VOCKEROTH 1989). However, various authors have proposed a different number of subfamilies or phylogenetic groups (ranging from 2 to 5; SACK 1976). The molecular data do not confirm the separation in 2 subfamily-groups currently recognised and the relationships among the groups proposed in the past

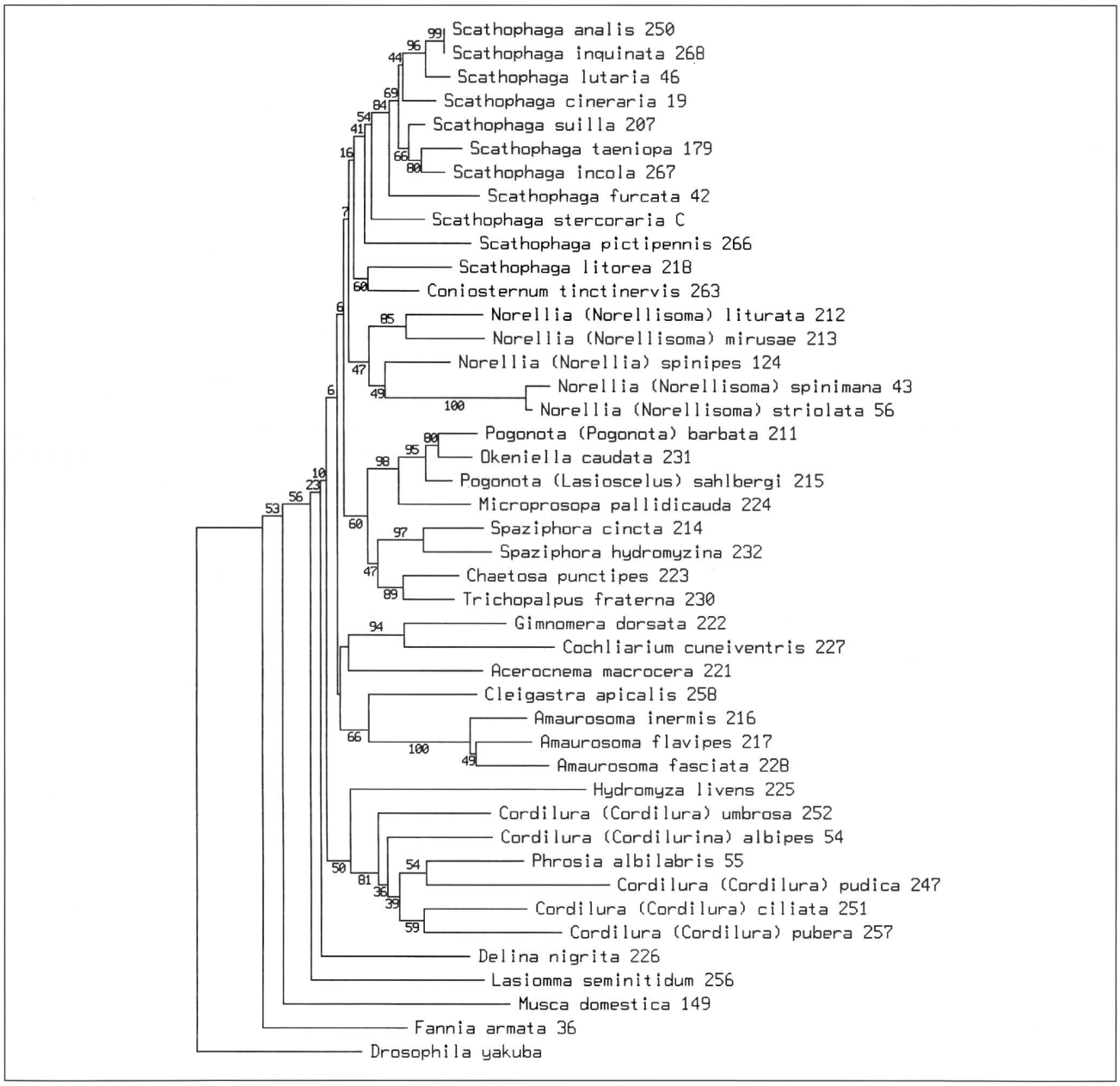

Fig. 1 - Rooted Neighbour Joining tree for 40 Scathophagids species and 3 other Muscoidea species (*Musca domestica* belongs to the *Muscidae*, *Lasiomma seminitidum* to the *Anthomyiidae*, and *Fannia armata* to the *Fanniidae*) based on the Tamura-Nei model and gamma corrected distances (with default gamma parameter a=0.5). *Drosophila yakuba* was chosen as outgroup. Bootstrap values for 500 replicates are shown.

are not always confirmed. Only the *Delina* genus appears sometimes to be isolated relative to the other genera. Nevertheless, this information is not supported by all the trees generated and the bootstrap values are usually low. Concerning the genus *Phrosia*, its membership of the *Delinae* is controversial even from the morphological point of view. As a matter of fact some authors consider this genera as a *Scathophaginae* and cannot understand why *Phrosia* should be placed in the *Delinae* subfamily (F. Püchel, personal communication 1997). In particular, adult individuals of *Phrosia albilabris* are morphologically very similar to the species belonging to the genus *Cordilura* (R. Vockeroth, personal communication 1998). Molecular data not only support the idea that *Phrosia* should be considered as a true *Scathophaginae* but also place this species unequivocally within the *Cordilura* genus.

Pogonota, *Lasioscelus* and *Okeniella* were once treated at the genus rank and considered as belonging to the same phylogenetic group (HACKMAN 1956). Modern taxonomists have now placed *Lasioscelus* as a subgenera of *Pogonota* (GORODKOV 1986) The molecular data confirm the relationship among the 3 groups relative to the *Scathophagidae*. However, it seems not correct to consider *Lasioscelus* as a subgenera of *Pogonota*, since no tree supports this interpretation. Even the generally high bootstrap values indicate a more strict relationship between *Pogonota* and *Okeniella* than between *Pogonota* and *Lasioscelus*.

Scathophaga taeniopa and *S. suilla*, are morphologically very similar species. In particular, a detailed study based on traditional morphological characters and comparing also the genitalia of the two species concluded that *S. taeniopa* and *S. suilla* are identical (SIFNER 1975, 1995). The major problem related to these two species is the risk of error by morphological determination, perhaps because of the lack of clearly distinctive chaetotaxy which could be applied to all the individuals. In this context the molecular data are very useful, allowing a sure characterisation of the specimens. Even if the case is not to be considered closed, the molecular data unequivocally suggest that *S. taeniopa* and *S. suilla* are different and must therefore be considered as distinct taxa.

S. analis, even if present in classical works on *Scathophagidae*, is considered as a doubtful species in the Catalogue of Palaearctic Diptera (GORODKOV 1986). Molecular data seems to indicate that it is probably a synonym of *S. inquinata*. In the determination key, the discriminating point is represented by the anterior cross-vein which should be dark-shadowed in *S. analis* but not in *S. inquinata* (SACK 1976). It is possible that the cross-vein character used to separate the two fly types is not valid, even if analysing the specimens from a morphological point of view, some other showy differences appear, like for example the colour of the shoulders. Perhaps under the name *S. analis* and *S. inquinata* entomologists have separated individuals belonging to the same species. The only way to clarify the question is to get sequence information generated from specimens which have been previously identified without any doubt by morphological characters.

In conclusion, as shown by these examples, the integration of the molecular data derived from mtDNA sequences, together with previous morphological studies, provides a keystone for the inference of correct phylogeny and taxonomy of an insect family.

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REFERENCES

AMANO K., 1983. Studies on the intraspecific competition in dung breeding flies. 1. Effects on larval density in yellow dung fly, *Scathophaga stercoraria* (Diptera: Scathophagidae). Jap J Sanit Zool 34:165-175.

AVISE J.C., 1994. Molecular Markers, Natural History and Evolution. Chapman & Hall New York-London, xiv, 511.

AVISE J.C., ARNOLD J., BALL R.M., BERMINGHAM E., LAMB T., NEIGEL J.E., REEB C.A., SAUNDERS N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Ann Rev Ecol Syst 18: 489-522.

BLANCKENHORN W.U., 1997. Effects of temperature on growth, development and diapause in the yellow dung fly-against all rules? Oecologia 111: 318-324.

BLANCKENHORN W.U., 1998. Adaptive phenotypic plasticity in grouth, development, and body size in the yellow dung fly. Evolution 52: 1394.

CLARY D.O. & WOLSTENHOLME D.R., 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organisation, and genetic code. J Mol Evol 22: 252-271.

CROZIER R.H. & CROZIER Y.C., 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organisation. Genetics 133: 97-117

DE BRUIJN M.H., 1983. *Drosophila melanogaster* mitochondrial DNA, a novel organization and genetic code. Nature 304 (5923): 234-241.

GORODKOV K.B., 1986. Catalogue of palaearctic Diptera, Scathophagidae-Hypodermatidae, 11: 11-41.

HACKMAN W., 1956. The Scatophagidae (Dipt.) of eastern Fennoscandia. Helsingsforsiae, 1-67.

HARRISON R.G., 1989. Animal Mitochondrial DNA as a genetic marker in population and evolutionary biology. Trend Ecol Evol 4: 6-11.

HILLIS D.M.. 1987. Molecular versus morphological approaches to systematics. Ann Rev Ecol Syst 118: 23-42.

HILLIS D.M., MORITZ C., MABLE B.K., 1996. Molecular systematics, Second edition, Sinauer Associates, Inc. Publishers, Sunderland Massachusetts USA. xvi, 655.

KUMAR S., TAMURA K., NEI M., 1993. Molecular evolutionary genetics analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802.

LUNT D.H., ZHANG D.-X., SZYMURA J.M., HEWITT G.M., 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol Biol 5(3): 153-165.

MORITZ C., DOWLING T.E., BROWN W.M., 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann Rev Ecol Syst 18: 269-292.

MÜHLHÄUSER C, BLANCKENHORN W.U., WARD P.I., 1996. The genetic component of copula duration in the yellow dung fly. Anim Behav 51: 1401-1407.

OTRONEN M., REGUERA P., WARD P.I., 1997. Sperm storage in the yellow dung fly *Scathophaga stercoraria*: Identifying the sperm of competing males in separtate female spermathecae. Ethology 103, 844-854.

PACKER C., 1989. Molecular Ecology, Trend Ecol Evol 4: 156-157.

PARKER G.A. & SIMMONS L.W., 1991. A model of constant random sperm displacement during mating: evidence from *Scatophaga*. Proc R Soc Lond B 246: 107-115.

PARKER G.A., SIMMONS L.W., WARD P.I., 1993. Optimal copula duration in dungflies: effects of frequency dependence and female mating status. Behav Ecol Sociobiol 32:157-166.

QUICKE D.L.J., 1993. Principles and techniques of contemporary taxonomy. Blackie Academic & Professional. xii, 311.

SACK P., 1976. 62a Cordyluridae. In Lindner, E. (ed.) Die Fliegen der Palaearktischen Region, E. Schweizerbart'sche Verlagsbuchhandlung Stuttgart, 7: 1-103.

SIFNER F., 1975. Scatophagidae (Diptera) de Mongolie. Annales Historico-Naturales Musei Nationalis Hungarici 67: 219-227.

SIFNER F., 1995. Additions to the Catalogue of flies of the Palaearctic region (Diptera: Scatophagidae). Eur J Entomol 92: 513-515.

SIGURJONSDOTTIR H. & PARKER G.A., 1980. Dung fly struggles: evidence for assessment strategy. Behav Ecol Sociobiol 8: 219-230.

SIMMONS L.W. & PARKER G.A., 1992. Individual variation in sperm competition success of yellow dung fly *Scathophaga stercoraria* (L.). Evolution 46: 366-375.

SIMMONS L.W. & WARD, P.I., 1991. The heritability of sexually dimorphic traits in the yellow dung fly *Scathophaga stercoraria* (L.). J Evol Biol 4: 593-601.

SIMMONS L.W., STOCKLEY P., JACKSON P., PARKER G.A., 1996. Sperm competition or sperm selection: no evidence for female influence over paternity in yellow dung fly *Scathophaga stercoraria*. Behav Ecol Sociobiol 38: 199-206.

SIMON C., FRATI F., BECKENBACH A., CRESPI B., LIU H., FLOOK, P., 1994. Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. Ann Entomol Soc Am 87(6): 651-701.

VOCKEROTH J.R., 1989. Scathophagidae. In McAlpine (ed) Manual of nearctic Diptera 2: 1085-1097.

VOCKEROTH J.R., 1956. Distribution patterns of the Scatomyzinae (Diptera, Muscidae). Proceedings tenth international congress of entomology 1: 619-626.

WARD P.I., 1993. Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria* (L.). Behav Ecol Sociobiol 32: 313-319.

WARD P.I., 1998. A possible explanation for cryptic female choice in the yellow dung fly, *Scathophaga stercoraria* (L.). Ethology 104: 97-110.

WARD P.I. & HAUSCHTECK-JUNGEN E., 1993. Variation in sperm length in the yellow dung fly *Scathophaga stercoraria* (L.). J Insect Physiol 39 (7): 545-547.

WARD P.I. & SIMMONS L.W. 1991. Copula duration and testes size in the yellow dung fly, *Scathophaga stercoraria* (L.): the effects of diet, body size, and mating history. Behav Ecol Sociobiol, 29: 77-85.

WOLSTENHOLME D.R., 1992. Animal mitochondrial DNA: structure and evolution. Int. Rev. Cytol. 141: 173-216.

Biodiversity and bacterial pathogenicity

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The aim of this contribution is to illustrate how intraspecific as well as interspecific biodiversity plays a key role in the complex event of bacterial pathogenesis. *Helicobacter pylori* and *Escherichia coli* were chosen as examples for bacterial species with heterogeneous (*H. pylori*) and conserved (*E. coli*) genomic structures.

PATHOGENICITY OF *HELICOBACTER PYLORI*

Helicobacter pylori, first described by WARREN & MARSCHALL in 1983, is known to be involved in the pathogenesis of upper gastrointestinal diseases. *H. pylori* establishes a long-term colonization of the mucosa of the human stomach (BLASER, 1997). The chronic infection often occurs without symptoms, but some individuals develop severe features such as peptic ulcer disease (lifetime risk 15%), gastric adenocarcinoma, and mucosa associated lymphoid tissue lymphoma (lifetime risk 0.1%) (FENNERTY, 1996). The development of the disease is influenced by the virulence of the infecting bacterial strain, the genetic susceptibility of the host and environmental co-factors.

In the 1990s with the aid of powerful molecular techniques, our knownledges of the genetic of *H.pylori* genome has expanded exponentially. Physical and genetic maps of the genome have been constructed, physiologically and pathologically important genes have been cloned and characterized. The complete genome sequence of *H. pylori* strain 26695 has been unmasked (TOMB *et al.,* 1997) and published on the internet (http://www.tigr.org). The genetic heterogeneity among different isolates is high and can be assessed by various methods, such as restriction fragment length polymorphism analysis and PCR fingerprinting (TAYLOR *et al.,* 1992, KANSAU *et al.,* 1996, GIBSON *et al.,* 1998). The genetic variation of *H.pylori* is greater than that of other bacteria that have been studied. Comparison of the genetic maps of five *H. pylori* strains demonstrated that there is no characteristic arrangement of 17 known genes on the chromosome conserved by these strains (macrodiversity) (JOANG *et al.,* 1996). Mechanisms causing genomic diversity are poorly understood at present. For macrodiversity, one hypotesis suggests that this diversity reflects a long evolutionary association with the human host. Different strains remain within each individual human for many generations and may indipendently undergo evolution. Alternatively, the diversity could result from gen-rearrangement within the chromosome caused by transposon-mediated gene mobility and recombination between repeated sequences (GE & TAYLOR, 1998). Analysis of this overall genomic variability is useful for epidemiological fingerprinting, but this genomic variability is not related to the pathogenicity of *H.pylori* strains.

Virulence genes of *H. pylori*

Recently, specific bacterial genes that are associated with phenotypic strain virulence have been described. Approximately 50 to 60% of the *H.pylori* strains contain the cytotoxin-associated *(cagA)* gene and consequently produce the 128-kDa cagA protein. CagA was first described as a protein which was expressed more commonly by toxigenic than non-toxigenic strains. The presence of cagA is associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer. *CagA* is part of a larger genomic entity, designated the pathogenicity (cag) island of about 40 kbp, which contains a collection of about 40 genes (CENSINI *et al.*, 1996). This multiple genes are related to the virulence and the pathogenicity of the strain, for exemple, by inducing cytokine production by the host, and are associated with higher levels of inflammation. Therefore the presence of *cagA* can be considered a marker for this genomic pathogenicity (RUDI *et al.*, 1998). The *cagA* region has much in common with pathogenicity islands in other bacteria: it appeared to be involved in virulence, has a different nucleotide composition to other *H. pylori* genes, is flanked by direct nucleotide repeats, and is relatively genetical instable. Like other bacterial pathogenicity islands, it is thought to have been acquired relatively late in the evolution from an external source, perhaps a bacteriophage or a plasmid. In many strains it is present in his enterely, but in others it is re-organized or even partially deleted following interruption by a novel insertion sequence which is though to be involved in chromosomal reorganisation.

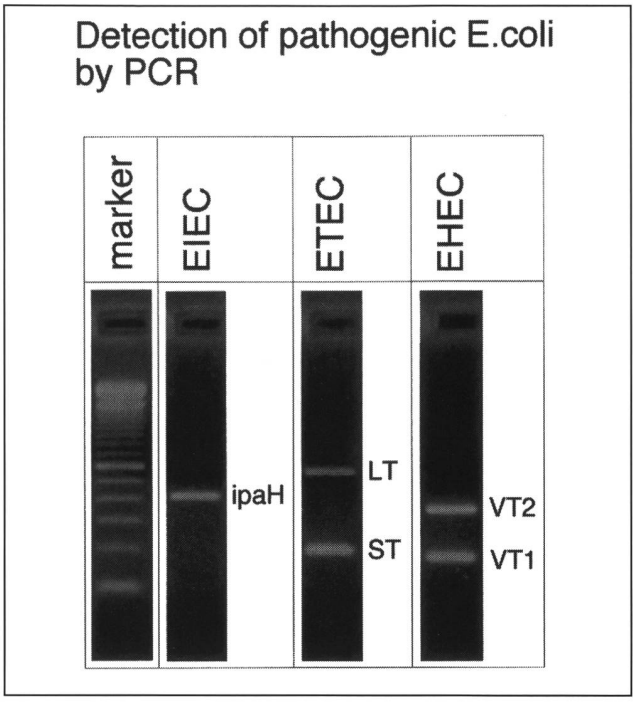
Another virulence factor, produced by approximately 50% of the *H. pylori* strains, is a cytotoxin that induces the formation of vacuoles in mammalian cells in vitro

and that leads to cell death. This toxin is encoded by the *vacA* gene. Although *vacA* is present in all *H. pylori* strains, it contains at least two variable parts. Recently different allelic variants in these two parts has been described (VAN DOORN *et al.*, 1998). The VacA protein has a short N-terminal signal peptide (or signal sequence) which is recognized and cleaved during export of the toxin across the bacterial cytoplasmic membrane. Further cleavage of the C-terminal third of the toxin precursor occurs during passage across the outer membrane to leave an 87 kDa secreted polypeptide. The N-terminal signal(s) region occurs as either an s1a, s1b, or s2 allele. Recently, a novel subtype, designated s1c, was found (STROBEL *et al.*, 1998). However this subtype was observed exclusively in isolates from East Asia and appears to be the major s1 allele in that part of the world. The middle (m) region is present as an m1 or an m2 allele. The mosaic structure of the *vacA* gene accounts for the differences in the cytotoxin production between strains. The particular *vacA* s and m genotype is a marker of the pathogenity of an individual strain, since in vitro production of the cytotoxin, in vivo epithelial damage, and peptic ulcer disease are all related to the vacA genotype (DONATI *et al.*, 1999). In a study group of 106 patients with duodenal ulceration *H.pylori* of genotype s1 was isolated from 96% , whereas genotype s2 was only present in 4% indicating a strong correlation between the *vacA* genotype and peptic ulceration. Particular midregion genotypes were not associated with clinical manifestations.

PATHOGENICITY OF *ESCHERICHIA COLI*

Escherichia coli is the predominant facultative anaerobe of the human colonic flora. *E.coli* usually remain harmlessly confined to the intestinal lumen; however, in the debilitated or immunosupressed host, or when gastrointestinal barrieres are violated, even normal *E.coli* strains can cause infection. Three general clinical syndromes result from infections with pathogenic *E.coli* strains: (i) urinary tract infections, (ii) sepsis/menengitis; (iii) enteric diarrheal disease. This article will focuse on the diarrheagenic *E.coli* strains in particular on their differentiation on the basis of pathogenic features.

Identification of diarrheagenic *E.coli* strains requires that these organisms be differentiated from nonpathogenic members of the normal flora. Serotypic markers correlate, sometime very closely, with specific categories of diarrheagenic *E.coli*; however, these markers are rarely sufficient to reliably identify a strain as diarrheagenic. Those *E.coli* strains were among the first pathogens for which molecular diagnostic methods were developed. Indeed, molecular methods remain the most popular reliable techniques for differentiating diarrheagenic strains from nonpathogenic members of the stool flora, and distinguishing one category from another. PCR is a major advance in molecular diagnostics of pathogenic microorganisms and has been successfully performed for several diarrheagenic *E.coli.*



E. coli virulence

The most highly conserved feature of diarrheagenic *E.coli* strains is their ability to colonize the intestinal mucosal surface. Once colonization is established, the pathogenic strategies exhibit remarkable variety. Three general paradigms have been described by which *E.coli* may cause diarrhea: (i) enterotoxin production (ETEC and EAEC), (ii) invasion (EIEC), and/or (iii) intimate adherence with membrane signalling (EPEC, EHEC). The versatility of the *E.coli* genome is conferred mainly by two genetic configurations: virulence related plasmids and chromosomal pathogenicity islands. Such islands have been described for uropathogenic *E.coli* strains (DONNENBERG & WELCH, 1996) and systemic *E.coli* strains as well (BLOCH & RODE, 1996) and may represent a common way in which the genomes of pathogenic and nonpathogenic *E.coli* strains diverge genetically.

In the following sections the pathogenicity of three categories of diarrheagenic *E.coli strains* (ETEC, EIEC, and EHEC) will be presented.

Enterotoxinogenic *E. coli*

ETEC is defined as containing the *E.coli* strains that produce at least one member of the two defined groups of enterotoxins: ST (heat-stable toxins) and LT (heat-labile toxins) (LEVINE, 1987). The LTs of *E.coli* are large, oligomeric and closely related in structure and function to the cholera enterotoxin (CT) expressed by *Vibrio cholerae.* In contrast, the STs are small, monomeric and contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins.

ETEC strains are associated with two major clinical syndromes: diarrhea among childrens in the developing countries, and traveller's diarrhea. Epidemiologic investi-

gations have implicated contaminated food and water as the most common vehicles for ETEC infections. The diarrhea is watery, usually without blood, mucus or pus; fever and vomiting are present in a minority of patients. ETEC diarrhea may be mild, brief, and self-limiting or may be severe and similar to that seen in *V. cholerae* infections.

Enteroinvasive *E. coli*

EIEC strains are biochemically, genetically, and pathogenetically related closely to *Shigella* spp.; like Shigella spp. EIEC strains are generally lysine decarboxylase negative, nonmotile, and lactose negative (BRENNER *et al.*, 1973). Genes necessary for invasiveness are carried on a 120-MDa plasmid in *Shigella sonnei* and a 140-MDa plasmid in other *Shigella* serotypes and in EIEC. The invasion related plasmid has been designated pInv. EIEC strains can be difficult to distinguish from *Shigella* spp. and from other *E.coli* strains, including nonpathogenic strains. Molecular techniques such as PCR with specific primers overcome this problem. It should be noted that EIEC strains may lose all or part of the pInv plasmid on in vitro passage or storage. Therefore PCR assay with primers derived from genes which are located on the plasmid as well as on the chromosom may increase the detection level. The *ipaH* (multicopy invasion plasmid antigen gene) gene is unique in that five complete or partial copies are present on the invasion plasmid of the various *S. flexneri* serotypes and multiple copies are also found on the invasion plasmids of other *Sighella* species and EIEC but not on the chromosome of non pathogenic *E. coli* strains. EIEC infections present most commonly as watery diarrhea, which can be indistinguishable from the secretory diarrhea seen with ETEC.

Enterohemorrhagic *E. coli*

E. coli strains, causative agens of bloody diarrhea in humans associated with hemorrhagic colitis, hemolytic uremic syndrome, produce one or more toxins of the Stx (Shiga-like toxin) family. The Stx family contains two major, immunologically non-cross-reactive groups called Stx1 and Stx2. A single EHEC strain may express Stx1 only, Stx2 only or both toxins. In recent years, there have been significant advances in our understanding of the pathogenesis of these *E.coli* infections, and these are contributing to the development of improved diagnostic methods, as well as to the development of therapeutic and preventive strategies. The alternative nomenclature «verotoxigenic *E. coli*» or «Verotoxin producing *E. coli*» was derived from the observation that these strains produced a toxin that was cytotoxic for Vero cells. Representative for this group is serotype O157:H7 isolated from large and severe outbreaks occured after ingestion of undercooked hamburgers at a fast food restaurant chain (RILEY *et al.*, 1983). In conventional diagnostic to trace for Verotoxin producing *E. coli* O157:H7 MacConkey medium supplemented with sorbitol was used. More recently other methods such ELISA and immunomagnetic separation were also used to detect O157:H7. However, serological cross reaction with other enterobacteriaceae has been observed. MacConkey Agar Sorbitol is not enough discriminating because part of the O157:H7 can ferment sorbitol and it has been demonstrated that other serotypes can cause diarrhea. Virolence test on HeLA cells or Verocells can not be easely performed in a routine laboratory. Large scale of meat favorizes the spread of EHEC contaminations. In the USA serotype O157:H7 plays a dominant role. In beef from the Swiss Market VT producing serotypes other than O157:H7 must be expected. Therefore the diagnosis with high specific molecular techniques such as PCR is highly recommended.

REFERENCES

BLASER M.J. Ecology of *Helicobacter pylori* in the human stomach. J. Clin. Invest 1997; 100: 759-62.

BLOCH C.A., RODE C.K. Pathogenicity island evaluation in *Escherichia coli* K1 by crossing with laboratory strain K12. Infect. Immun. 1996; 64:3218-23.

BRENNER D.J., FANNING G.R., MIKLOS G.V., STEIGERWALT A.G. Polynucleotide sequence reletedness among *Shigella* species. Int. J. Syst. Bacteriol. 1973; 23: 1-7.

CENSINI S., LANGE C., XIANG Z., CRABTREE J.E., CHIARA P., BORODOVSKY M., RAPPUOLI R., COVACCI A. cag, a pathogenicity island of *Helicobacter pylori*, encodes typeI-specific and disease-associated virulence factors. Proc. Natl. Acad.Sci. USA 1996; 93: 14648-53.

DONATI M., STORNI E., D'APOTE L., MORENO S., TUCCI A., POLI L., Cevenini R. PCR-based restriction pattern typing of the *vacA* gene provides evidence for a homogeneous group among *Helicobacter pylori* strains associated with peptic ulcer disease. J. Clin. Microbiol. 1999; 37:912-5.

DONNENBERG M.S., WELCH R.A. Virulence determinants of uropathogenic *Escherichia coli*, p.135-174. In H.L.T. Mobley and J.W. Warren (ed), Urinary tract infections: molecular pathogenesis and clinical menagement. American Society for Microbiology , Washington D.C. 1996.

FENNERTY M.B. Is the only good *H. pylori* a dead *H. pylori*? Gastroenterology 1996; 111: 1773-4.

GE Z, TAYLOR D.E. Helicobacter pylori-molecular genetics and diagnostic typing. Britisch Medical Bull 1998; 54: 31-8.

GIBSON J.R., SLATER E., XERRY J., TOMPKINS D.S., OWEN R.J. Use of an amplified-fragment length polymorphisms technique to fingerprint and differentiate isolates of *Helicobacter pylori*. J. Clin. Microbiol. 1998; 36: 2580-5.

JOANG Q., HIRATSUKA, TAYLOR D.E. Variability of gene in different *Helicobacter pylori* strains contributes to genome diversity. Mol. Microbiol. 1996; 20: 833-42.

KANSAU I., RAYMOND J., BINGEN E., COURCOUX P., KALACH N., BERGERET M., BRAIMI N., DUPONT C., LABIGNE A. Genotyping of *Helicobacter pylori* isolates by sequencing of PCR products and comparison with the RAPD technique. Res. Microbiol. 1996; 147: 661-9.

LEVINE M.M. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 1987;155: 377-89.

MARSHALL B.J., WARREN J.R. Unindentified curved bacilli on gastric epithelium in active gastritis. Lancet 1983; 1: 1273-5.

RILEY L.W., REMIS R.S., HELGERSON S.D., MCGEE H.B., WELLS J.G., DAVIS B.R., HERBERT R.J., OLCOTT E.S., JOHNSON L.M., HARGRETT N.T., BLAKE P.A., COHEN M.L. Hemorragic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. 1983; 308: 681-5.

RUDI J., KOLB C., MAIWALD M., KUCK D., SIEG A., GALLE P.R., Stremmel W. Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. J. Clin. Microbiol. 1998; 36: 944-8.

STROBEL S., BERESWILL S., BALIG P., ALLGAIER P., SONNTAG H.G., KIST M. Identification and analysis of a new vacA genotype variant of *Helicobacter pylori* in different patient groups in Germany. J. Clin. Microbiol. 1998;36: 1285-9.

TAYLOR D.E., EATON M., CHANG N., SALAMA S.M. Construction of a *Helicobacter pylori* genom map and demonstration of the diversity at the genome level. J. Bacteriol. 1992; 174:6800-6.

TOMB J.F., WHITE O., KERLAVAGE A.R. The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature 1997; 388: 539-47.

VAN DOORN L.J., FIGUEIREDO, ROSSAU R., JENNES G., VAN ASBROECK M., SOUSA J.C., CARNEIRO F., QUINT W.G.V. Typing of *Helicobacter pylori vacA* gene and detection of *cagA* gene by PCR and reverse hybridization. J. Clin. Microbiol. 1998; 36: 1271-6.

Symbiotic relations between bacteria and cephalopods

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INTRODUCTION

Chronic heterotrophic symbiosis is known from many invertebrate taxa, including cephalopods. The symbionts can be intracellular or extracellular, and entertain with the host parasitic, commensal or mutualistic reltionships. The transmission from one generation to the other is either hereditary (vertical), or non-hereditary (horizontal), the infection occurring after birth.

In cephalopods, two types of symbiosis are now well documented: the sepiolid *Euprymna*/luminous bacterium *Vibrio fisheri* association, and the accessory nidamental glands/microbial community symbiosis.

(1) Many cephalopods are bioluminescent, and in some species of sepiolids and loliginids the light is emitted by extracellular symbiotic bacteria, which live in the crypt of a light organ located on the ink sac. In this case, the host provides an adequate environment for the heterotrophic bacterium, and the host derives luminescence from the bacteria. At birth, the sepiolid is aposymbiotic and acquires free-living *Vibrio fisheri* from the surrounding sea water within hours (WEI & YOUNG, 1989; MCFALL-NGAI & RUBY, 1991, 1998; MONTGOMERY & MCFALL-NGAI, 1994). Only some tissues of the light organ are influenced by the presence of *Vibrio*, which induce light organ morphogenesis (DOINO & MCFALL-NGAI , 1995).

(2) As far as the accessory nidamental glands (ANG) microbial community symbiosis is concerned, we know that these organs harbour a dense bacterial community, colourless in immature animals, which becomes red-orange in mature females (BLOODGOOD, 1977). Sequencing of bacterial 16S DNA has allowed to show that various bacteria species are present in the ANG of *Loligo pealei* (BARBIERI *et al.* 1996). ANG symbiotic bacteria are present in sepioids and in myopsid squids, but their role is not yet totally understood. The ANG are closely associated with nidamental glands and oviduct, and their role might be to coat the eggs with bacteria in charge of protective antimicrobial activity (BARBIERI *et al.*, 1997). In agreement with this suggestion, similarities in the microbial community of both ANG and egg cases were shown. As far as the transmission is concerned, bacterial colonization of ANG is thought to occur after hatching (LUM-KONG & HASTINGS, 1992; KAUFMAN *et al.*, 1998).

In this paper, we investigate the presence of symbiotic bacteria in different organs of the sepioid *Sepia officinalis,* the nautiloid *Nautilus macromphalus* and of the octopod *Eledone sp.* the sepioid *Sepia officinalis* is known to harbour symbiotic bacteria in the ANG, and Schipp *et al.* (1985) suggest the presence of bacteria in the pericardial appendages of *Nautilus.* In *Eledone* no symbiotic association is known so far.

MATERIAL AND METHODS

Sepia officinalis: 8 adult specimens were collected in the English Channel (Luc-sur-mer). Various organs were obtained by aseptic dissection: the accessory nidamental gland (ANG); the nidamental gland, the anterior and posterior renal sac, the heart, the branchial heart, the gills, the digestive gland, the blood, the pericardial gland, the spermatophore.

Nautilus macromphalus: 9 adult specimens were collected in New Caledonia. Various organs were analysed: the gills, the heart, the pericardial appendages, the digestive gland, the renal appendages.

Eledone sp.: 3 adult specimens were collected in the Mediterranean (Banyuls-sur-mer). The organs analysed were the renal sac, the coelomic liquid, the branchial heart, the gills.

Samples for **PCR analysis** were stored in TE buffer pH8 (10 mM tris/HCl pH7.2, 1mM EDTA) at -20°C until use. Total DNA extraction was performed by classic phenol purification and ethanol precipitation after 10mg/ml Lysozyme and 10mg/ml Proteinase K digestion. DNA was resuspended in 50µl of TE and stored at -20°C.

The amplification primers are listed in table 1. We used the universal bacterium-specific 27F-1385R or UNIL-UNIR, which produced almost the entire 16S rRNA gene fragment and the eubacteria specific EUB-UNIL pair. Two other amplification primer pairs were used: the AMOR-AMOF pair specific for ammonifying bacteria (amplification of the gene coding for the ammonia-monoxygenase), and the NARGF-NARGR pair specific for denitrifying bacteria (amplification of the gene coding for the nitrate reductase).

PRIMER	SEQUENCE
27F	5'GAGTTTGATCCTGGCTCAG3'
1385R	5'GCCACACATGTTCCGGG3'
UNIR	5'ATGGTACCGTGTGACGGGCGGTGTA3'
UNIL	5'ATTCTAGAGTTTGATCATGGCTCA3'
EUB	5'GCTGCCTCCCGTAGGAGT3'
AMOR	5'AGACTCCGATCCGGACTACG3'
AMOF	5'TGGGGCATAACGCATCGAAAG3'
NARGF	5'TTACTTCAAACAGAAGGGTGAAACCTTT3'
NARGR	5'TTTCGCTTTATCGGCGTCTTCAATGAT3'

Tab. 1 - Amplification primers sequences.

PCR was conducted with dNTP, *Taq* polymerase and buffer (Eurogentec S.A.) in a Thermojet Thermocycler (EquiBio, Belgium) with a denaturing step at 94°C for 5 min, 32 cycles of 94°C (30 sec), 55°C (30sec) anf 72°C (1 min) and a final elongation step of 72°C for 7 min. For amplification with NARGF-NARGR primers, Kloos *et al.* (1995) protocol was followed. Length of PCR products was controlled by electrophoresis in 1.5% agarose gel.

RESULTS AND DISCUSSION

Several organs were tested for bacterial symbiosis based on PCR amplification, in three species of cephalopods, *Sepia officinalis, Nautilus macromphalus, Eledone sp.*, using 4 different primer pairs. The results obtained are presented in table 2.

Table 2. Results of PCR amplifications of different bacterial strains from various organs of three cephalopods. *: positive results; **: highly positive results; -: negative results; nd: not done. ANG: accessory nidamental gland; Ant.RS: anterior renal sac; BH: branchial heart; Coel.Liq.: coelomic liquid; DG: digestive gland; NG nidamental gland; P.App: pericardial appendages; P.Gl: pericardial gland (branchial heart appendages); Post.RS: posterior renal sac; Renal App: renal appendages; Sperm: spermatophore.

(A) *Sepia officinalis*

Primers\ organs	Gills	BH	Heart	Blood	NG	ANG	Sperm	P.Gl	DG	Ant. RS	Post. RS
Universal bact.	*	-	*	-	-	**	-	-	-	*	**
EUB	*	-	*	-	-	**	-	-	-	**	**
bAMO	-	-	-	-	nd	-	nd	-	nd	-	*
NAR-G	nd	nd	nd	nd	nd	*	nd	-	nd	-	-

(B) *Nautilus macromphalus*

Primers\ organs	Gills	BH	Heart	P.App	DG	Renal App
Universal bact.	**	-	-	**	-	**
EUB	nd	-	-	**	-	**
bAMO	-	-	-	-	nd	*
NAR-G	nd	nd	nd	nd	nd	-

(C) *Eledone sp.*

Primers\ organs	Gills	BH	Coel.Liq	Renal Sac
Universal bact.	*	-	-	-
EUB	**	-	-	-
bAMO	*	-	nd	-
NAR-G	nd	nd	nd	nd

As expected the accessory nidamental glands of *Sepia officinalis* give highly positive results (universal bacteria, eubacteria, NAR-G). But also some other organs of this species appear to harbour dense bacteria populations, in particular the renal sacs (universal bacteria, eubacteria, bAMO).

The presence of symbiotic bacteria in the pericardial appendages of *Nautilus* was suggested by Schipp *et al.* (1985), based on electron microposcopy observations. This is confirmed here: the results obtained with universal bacteria and eubacteria specific primers are highly positive. In addition, this species also appears to have, like *Sepia*, bacteria associated to the renal appendages. In both species, the excretory organs harbour among eubacteria, ammonifying bacteria.

As far as *Eledone* is concerned, the only positive result concerns the gills and might be fortuitous. Preliminary results with in situ hybridization do not reveal the presence of bacteria in the gill tissues of *Eledone* (unpubl. res.). Accordingly, the positive result obtained with PCR is considered as doubtful. The origin of the bacteria present in the gills of the three taxa might be environmental, since these organs are in contact with external sea water.

Thus, bacterial symbiotic associations appear to concern several organs of *Sepia* and *Nautilus*, whereas no significant association could be identified, so far, in *Eledone*. This might be related to a different buoyant system. *Eledone* is a benthic species with no need for buoyancy. *Sepia* and *Nautilus* live in the sea water column and both have a chambered shell, either internal (*Sepia*) or external (*Nautilus*), playing an important part in the regulation of buoyancy by accumulating gas, mainly N_2, in the chambers. Due to these shelled species low excretion rates compared to non shelled ones, it was suggested that the origin of N_2 accumulation might be the result of protein catabolism end-product (NH_4^+) recycling by symbiotic bacteria (BOUCHER-RODONI & MANGOLD, 1994). The results obtained here are in agreement with this hypothesis, since only the two shelled species *Sepia* and *Nautilus* appear to harbour symbiotic bacteria, including ammonifying or denitrifying strains. In parallel to in situ hybridization aimed to localize the bacteria within the tissues, the specific identification by 16S rRNA sequencing of the bacteria present in the various organs of *Sepia* and *Nautilus* is currently underway and should confirm if some of them could effectively be involved in the Nitrogen cycle.

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REFERENCES

BARBIERI E., GULLEDGE J., MOSER D. & CHIENG C.C., 1996. New evidence for bacterial diversity in accessory nidamental gland of the squid (*Loligo forbesi*). Biol. Bull., 191: 316.

BARBIERI E., BARRY K., CHILD A. & WAINWRIGHT N., 1997. Antimicrobial activity in the microbial community of the accessory nidamental gland and egg cases of *Loligo pealei* (Cephalopoda: Loliginidae). Biol. Bull., 193: 275-276.

BLOODGOOD R. A., 1977. The squid accessory nidamental gland: ultrastructure and association with bacteria. Tissue & Cell 9, 2: 197-208.

BOUCHER-RODONI R. & MANGOLD K., 1994. Ammonia production in cephalopods, physiological and evolutionary aspects. Mar. Fresh. Behav. Physiol., 25: 53-60.

DOINO J.A., & MCFALL-NGAI M.J., 1995. A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. Biol. Bull., 189: 347-355.

KAUFMAN M.R., IKEDA Y., PATTON C., VAN DYKHUIZEN G. & EPEL D., 1998. Bacterial symbionts colonize the accessory nidamental gland of the squid *Loligo opalescens* via horizontal transmission. Biol. Bull. 194: 36-43.

KLOOS K., FESEFELDT A., GLIESCHE C.G. & BOTHE H., 1995. DNA-probing indicates the occurrence of denitrification and Nitrogen fixation genes in *Hyphomicrobium*. Distribution of denitrifying and Nitrogen fixing isolates of *Hyphomicrobium* in a sewage treatment plant. FEMS Microbiol. Ecol., 18: 205-213.

LUM-KONG A. & HASTINGS T.S., 1992. The accessory nidamental glands of *Loligo forbesi* (Cephalopoda: Loliginidae): characterization of symbiotic bacteria and preliminary experiments to investigate factors controlling sexual maturation. J. Zool. Lond., 228: 395-403.

MCFALL-NGAI M.J. & RUBY E.G.,1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science 254: 1491-1494.

MCFALL-NGAI M.J. & RUBY E.G.,1998. Sepiolids and Vibrios: when first they meet. Bioscience, 48(4): 257-265.

MONTGOMERY M.K. & MCFALL-NGAI M.J., 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. Develoment, 120: 1719-1729.

SCHIPP R., MARTIN A. W., LIEBERMANN H. and MAGNIER Y., 1985. Cytomorphology and function of pericardial appendages of *Nautilus* (Cephalopoda, Tetrabranchiata). Zoomorphol., 105:16-29.

WEI S.L. & YOUNG R.E., 1989. Development of symbiotic bacterial bioluminescence in a nearshore cephalopod, *Euprymna scolopes*. Mar. Biol., 103: 541-546.

Phylogenetic biodiversity and *in situ* detection (whole cell hybridization) of the microbial flora from Lake Cadagno

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Riassunto: Il lago di Cadagno è un lago alpino situato ad un'altitudine di 1923 m nella val Piora, posta a sud delle Alpi svizzere (46°33' N, 8°43' E). Il lago ha una superficie di 26 x 10^5 m^2 ed una profondità massima di 21 m. L'infiltrazione di acqua percolata in rocce di dolomia saccaroide ricca di gesso ed immessa nel lago attraverso sorgenti sottolacustri determina uno stato di meromissi crenogenica. Il lago è caratterizzato dunque da una elevata salinità del monimolimnio e dalla presenza di un chemoclinio ad una profondità di 9-14 m (Peduzzi et al. 1998). Le analisi iniziali centrate sulla distribuzione verticale e temporale delle popolazioni di zolfobatteri del lago Cadagno sono state effettuate grazie all'utilizzo di fattori discriminanti quali la morfologia cellulare e l'autofluorescenza. Studi seguenti si sono focalizzati sull'analisi dei gruppi filogenetici maggiori quali le suddivisioni α, β, γ e δ del dominio *Bacteria* utilizzando le tecniche di ibridazione cellulare *in situ* combinata con l'analisi morfologica.

Alfine di descrivere la natura delle popolazioni batteriche osservate microscopicamente nel chemoclinio e nel monimolimnio sottostante, abbiamo amplificato (attraverso PCR utilizzando primers del dominio *Bacteria*) e clonato i geni 16S rDNA dei batteri del monimolimnio del lago Cadagno (campione di acqua prelevato a 17 m di profondità).

Gli inserti clonati sono stati riamplificati per confermare la presenza dell'inserto globale di 1.4 kb, che è stato detettato in 276 cloni e parzialmente sequenziato.

La distribuzione delle diverse popolazioni di zolfobatteri fototrofi nel chemoclinio è stata analizzata utilizzando 5 sonde diverse sviluppate specificatamente. La sonda oligonucleotidica **Cmok453** diretta contro l'ARN ribosomale 16S è stata progettata per la detezione specifica di *Chromatium okenii* DSM 169 come pure la sequenza dell'ARN ribosomale 16S inserita nel clone 359. La sonda **Apur453** effettua la detezione di *Amoebobacter purpureus* DSM 4197 e la sequenza nel clone 345. La sonda **Laro453** è specifica per *Lamprocystis roseopersicina* DSM 229 e la sequenza nel clone136. Le sonde **S453D** e **S453F** ibridizzano con l'ARN ribosomale 16S di batteri con sequenza uguale a quella presente rispettivamente nel clone 261 e nei cloni 335, 371.

Abstract: Lake Cadagno is an alpine lake situated 1923 m above sea level in the Piora valley in the south of Switzerland (46°33' N, 8°43' E). The lake has a surface area of 26 x 10^5 m^2 and a maximum depth of 21 m. Due to the infiltration of water through dolomite rich in gypsum, Lake Cadagno is a meromictic lake characterized by a high salinity of the monimolimnion and a permanent chemocline in a depth between 9 and 14 m (Peduzzi et al. 1998). Initial analyses on the spatial and temporal distribution of different populations of sulfur bacteria of the meromictic lake Cadagno where performed by means of distinctive parameters, namely cell morphology and autofluorescence. Further studies dealt with the analysis of major phylogenetic groups of bacteria, i.e. the α, β, γ and δ subdivisions of Proteobacteria, by *in situ* hybridization and a concomitant characterization of these populations by morphological criteria.

To resolve the nature of the bacterial populations observed by microscopy in the chemocline and in the underlying monimolimnion, we amplified (by PCR with universal primers for the domain *Bacteria*) and cloned the 16S rDNA genes of bacteria from the monimolimnion of the Lake Cadagno (water sample taken at 17 meters depth).

The cloned inserts were reamplified to confirm the presence of the 1.4 kb full-length insert, which was detected in 276 clones, and partially sequenced.

The distribution of the different populations of phototrophic sulfur bacteria in the chemocline could be analysed by the utilization of 5 probes which we had specifically developped.

16S rRNA-targeted oligonucleotide probe **Cmok453** was designed to detect *Chromatium okenii* DSM 169 as well as our clone 359. Probe **Apur453** targeted *Amoebobacter purpureus* DSM 4197, and also the sequence of our clone 345. Probe **Laro453** targeted *Lamprocystis roseopersicina* DSM 229, as well as the sequence of clone 136. Probes **S453D** and **S453F** targeted the 16S rRNA of bacteria harboring the sequence of clone 261 and those of clones 335 and 371, respectively.

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ANALYSIS OF BACTERIOPLANKTON BY DIRECT MICROSCOPICAL OBSERVATION

The Lake Cadagno was well stratified during the whole period of analysis (1993-1998), and only small spacial and temporal variations in the depth profiles of the different physico-chemical parameters were observed. The temperature profiles, the conductivity values (with over 300 µS cm^{-1} below a depth of 16 m), the changes in redox conditions (from 346 mV to 68 mV), the rapid decrease of oxygen (concentrations dropping below detection limit at a depth of approx. 10 m) and the concomitant increase in sulfide concentrations (from 10 to 100 mg l^{-1} below 11 m) with depth indicated the formation of a condensed chemocline in a depth between 10 and 14 m. In this layer the maximum turbidity was also found. The increase in turbidity in the chemocline was correlated to an increase in bacterial numbers determined after DAPI-staining. In mixolimnion between 5.9 (± 2.0) x 10^5 and 28.1 (± 3.7) x 10^5 cells ml^{-1} were found, while in the chemocline numbers increased to 1.3 (± 0.3) x 10^6 and 7.4 (± 1.6) x 10^6 cells ml^{-1}. In the monimolimnion the bacterial counts ranged between 2.2 (± 0.6) x 10^6 and 11.0 (± 1.7) x 10^6 cells ml^{-1}.

The analysis of Acridine Orange and DAPI-stained bacterial cells revealead very different morphologies especially at the upper sulfide border in the chemocline. Both total DNA staining methods demonstrated the presence of cells with volumes ranging from 0.1 to up to 70 µm³. Due to their distinct morphologies and their intensive autofluorescence, a major portion of the largest bacteria was identified as phototrophic sulfur bacteria, i.e. as *Chromatium okenii* or as *Amoebobacter purpureus* resembling morphotypes. These bacteria occurred in highest densities in a small layer of 1 to 2 meters at the upper sulfide border where light intensities during the day ranged from 0.1 to 4 µE m^{-2} sec^{-1} depending on the weather conditions. The *Cm. okenii* morphotype reached concentrations of up to 2.0 (± 0.5) x 10^5 cells ml^{-1} in July corresponding to approx. 3% of the total number of bacteria at this depth.

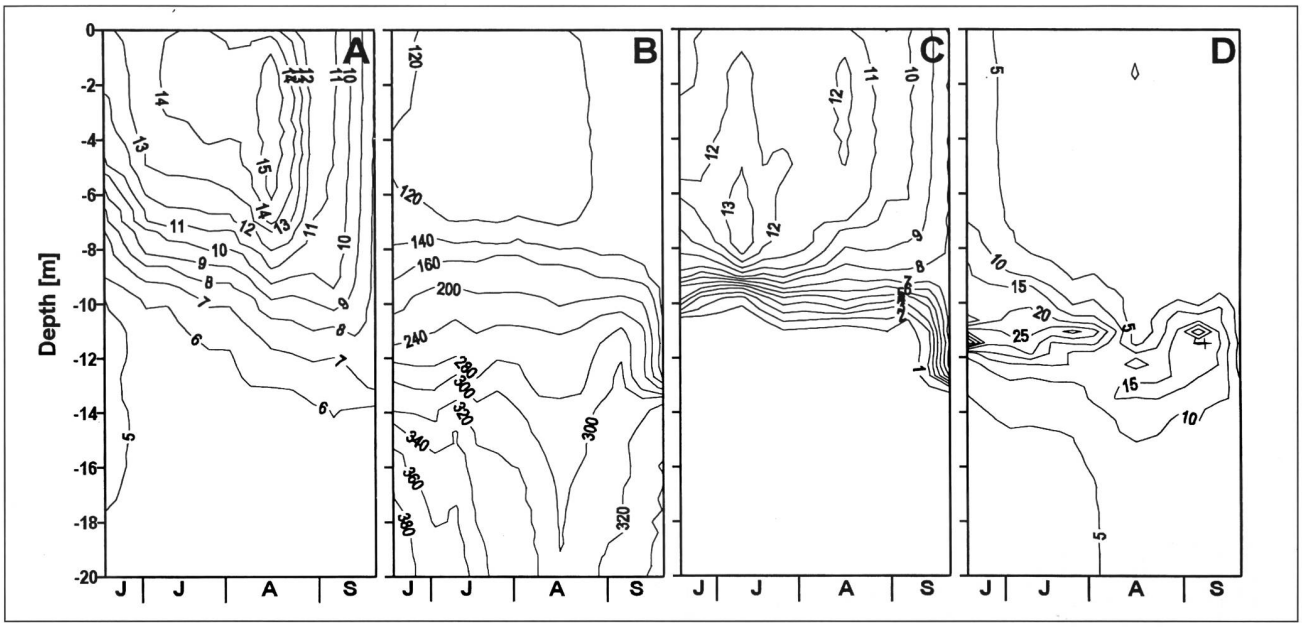

Fig. 1 - Physico-chemical characteristics of Lake Cadagno from June 8 to September 30, 1993: A: temperature (°C); B: Conductivity (µS cm^{-1}); C: oxygen (mg l^{-1}); D: turbidity (FTU).

Cells resembling *A. purpureus* showed a broader vertical distribution than *Cm. okenii* and relatively high cell concentrations not only in the chemocline, but also in the lower part of the monimolimnion. They were generally present in higher numbers than *Cm. okenii* reaching a maximum cell density in the chemocline of 9.0 (± 2.0) x 10^5 cells ml^{-1} which corresponded to approx. 30 % of the total number of bacteria. The average cell volumes were 54 (± 14) and 3 (± 0.8) µm³ $cell^{-1}$ for *Cm. okenii* and *A. purpureus*, respectively. The biovolumes of 2.7 ± (3.0) and of 0.5 ± (0.4) x 10^3 mm^3m^{-3} for *Cm. okenii* and *A. purpureus*, respectively, accounted for 40 and 9% of the total bacterial biovolume.

In addition to the phototrophic sulfur bacteria *Cm. okenii* and *A. purpureus*, two other morphotypes that contained gas-vacuoles were distinguished in the chemocline and in the monimolimnion from other bacteria by using morphological criteria. The first morphotype, named morphotype R, was a rod with round ends (0.4-0.5 µm wide and 1-4 µm long) similar to Procaryote T5 described by Caldwell & Tiedje (1975). The distribution of this morphotype correlated to the profiles of conductivity and hydrogen sulfide. The highest cell densities of this morphotype with 44.0 (± 0.6) x 10^6 cells ml^{-1} were obtained in the monimolimnion at a depth of 19 m in June 1993 and decreased slightly towards the end of the year. The second morphotype had an ovoid shape and was similar to morphotype H1 discovered in the lakes Blake Mere, Kettle Mere and Crose Mere (CLARK & WALSBY, 1978). The highest cell densities of this morphotype of 6.8 (± 3.0) x 10^3 cells ml^{-1}, representing 0.1% of total bacteria, were observed in August 1993 at a depth of 13.3 m depth.

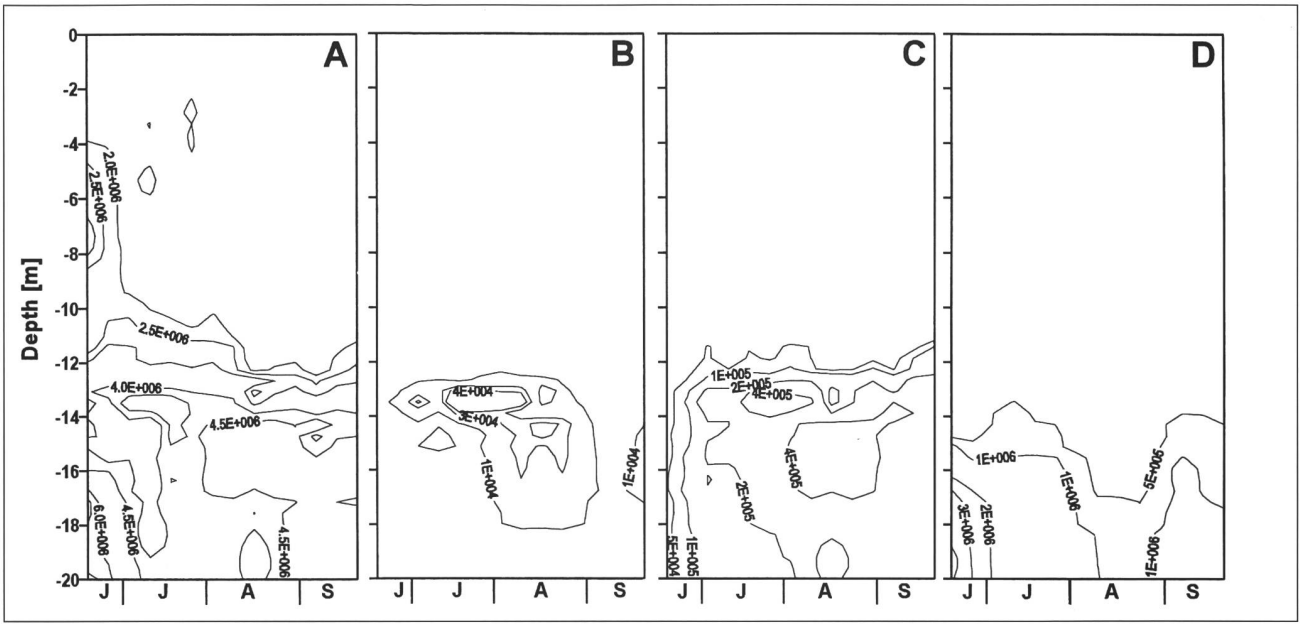

Fig. 2 - Vertical distribution of bacteria in Lake Cadagno from June 8 to September 30, 1993: A: total DAPI-stained bacteria (cells ml^{-1}); B: morphotypes resembling *Chromatium okenii* (cells ml^{-1}); C: morphotypes resembling *Amoebobacter purpureus* (cells ml^{-1}); D: morphotype R like cells (cells ml^{-1}).

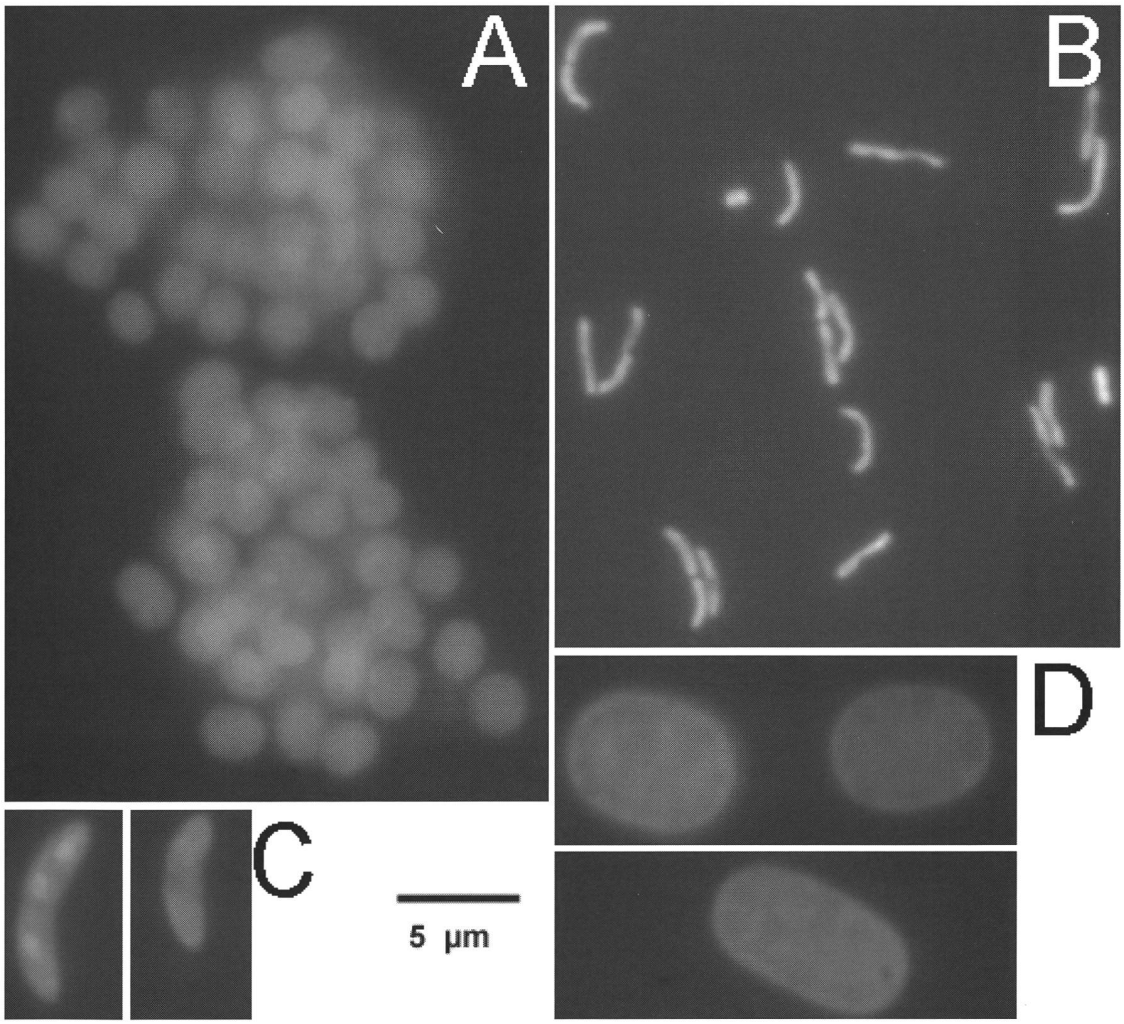

Fig. 3 - Fluorescence microscopy after acridine orange stain showing bacterial morphotypes resembling (A) *Amoebobacter purpureus*, (B) morphotype R, (C) morphotype H1-like and (D) *Chromatium okenii*.

ANALYSIS OF BACTERIOPLANKTON BY *IN SITU* HYBRIDIZATION

From October 1994, the *in situ* hybridization technique was applied to study water samples. Autofluorescence of phototrophic sulfur bacteria did not interfer with probe-conferred signals when the high quality filter system HQ-Cy3 (HQ535/50, Q565LP, HQ610/50, F41 AHF Analysentechnik, Tübingen Germany) was used.

Between 1.1 ± (0.6) x 10^4 and 1.8 ± (0.5) x 10^6 cells ml^{-1} were detectable in the chemocline by *in situ* hybridization with probe Eub338, targeting all members of the domain *Bacteria*. These cell numbers corresponded to percentages between 38 and 90% of the DAPI-stained cells. In the anoxic monimolimnion up to 70% of the DAPI-stained bacteria were detected with the same probe. With probe nonEub338, which should not bind to any rRNA, less than 1% of the total number of DAPI-stained bacteria were detectable, indicating a low non-specific binding of the probe to cell components.

Averaged over the whole chemocline, 49 (± 18)% of the DAPI-stained bacteria hybridized to probe Gam42a, targeting bacteria of the γ subdivision of Proteobacteria, whereas cells hybridizing to probes Alf1b, Bet42a, and SRB385, targeting bacteria of the α, β, and δ subdivisions of Proteobacteria respectively, accounted for 23 (± 3), 15 (± 7), and 16 (± 4)% of the DAPI-stained bacteria. In the monimolimnion, bacteria hybridizing to probe Alf1b were the most abundant organisms with 32 (± 9)% of the DAPI total count.

Cells hybridizing to probes Alf1b, Bet42a, Gam42a, and SRB385 were further differentiated into classes of morphotypes. Bacteria detected with probes Bet42a and SRB385 showed a higher number of different morphotypes than bacteria detected with probes Alf1b and Gam42a throughout the whole water column, although the latter were on average more abundant. Probes Bet42a and SRB385 generally detected the broadest range of morphotypes from small coccoid cells, with a volume of 0.1 μm^3, to large elongated cells, with a volume of up to 7 μm^3, whereas probe Gam42a generally detected large morphotypes that resembled phototrophic sulfur bacteria of the genera *Chromatium* and *Amoebobacter*.

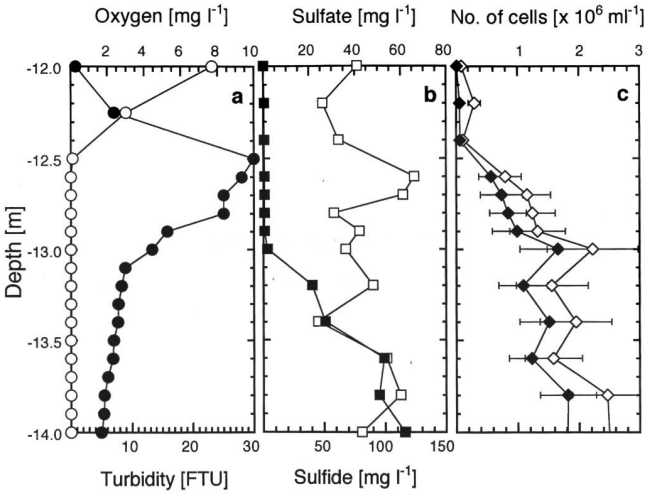

Fig. 4 - Vertical distribution of physico-chemical parameters and bacteria in the chemocline of Lake Cadagno at a depth between 12 m and 14 m (October 13, 1994);

a, oxygen (○), and turbidity (●) determined with a Hydropolytester HPT-C profiler;

b, sulfate (□), and sulfide (■);

c, numbers of DAPI-stained bacteria (◇), and cells detectable after *in situ* hybridization with probe Eub338 (◆).

Sulfate, sulfide and numbers of bacteria were determined in water samples obtained with a pneumatic multi-syringe sampler.

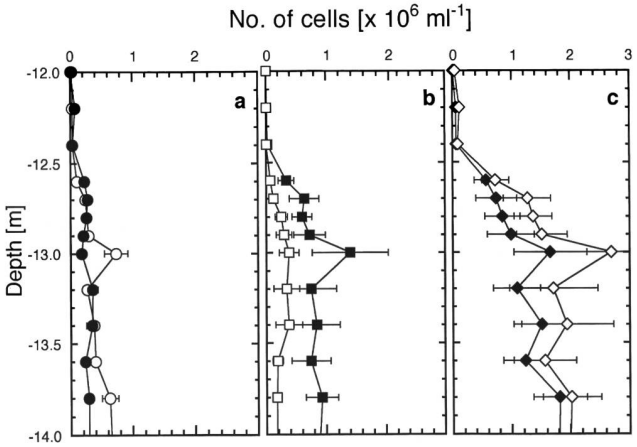

Fig. 5 - Vertical distribution of bacterial populations in the chemocline of Lake Cadagno at a depth between 12 m and 14 m (October 13, 1994);

a, cells detectable after *in situ* hybridization with probe Alf1b (○), and Bet42a (●);

b, cells detectable after *in situ* hybridization with probe Gam42a (■), and SRB385 (□);

c, cells detectable after *in situ* hybridization with probe Eub338 (◆), and the sum of cells detectable after *in situ* hybridization with probes Alf1b, Bet42a, Gam42a, and SRB385 (◇).

Probe	Morphotype[1]	Area (μm²)	Length (μm)	Width (μm)	Volume (μm³)	Cell description[2]
Alf1b	A1	1.1	1.6	0.9	0.7	Large elongated
	A2	0.9	1.6	0.7	0.5	Large elongated
	A3	2.8	3.8	0.7	1	Large elongated
	A4	4	4.6	0.7	1.2	Large elongated
	A5	4.1	5.6	0.7	1.4	Large elongated
Bet42a	B1	11.5	4.9	1.7	7.4	Large elongated
	B2	5.6	3.8	1.8	6.6	Large elongated
	B3	5.9	6	1	3.1	Large elongated
	B4	4	4.5	1.7	6.4	Large elongated
	B5	3.5	4.2	1.1	2.4	Large elongated
	B6	1.4	2.9	0.8	1	Large elongated
	B7	0.7	1.2	0.9	0.5	Large coccoid
	B8	0.8	1.6	0.7	0.4	Large elongated
	B9	1.1	1.6	0.7	0.4	Large elongated
	B10	1	2	0.4	0.2	Large elongated
	B11	0.3	0.7	0.6	0.1	Small coccoid
	B12	0.3	0.7	0.6	0.1	Small coccoid
	B13	0.7	1.6	0.7	0.4	Large elongated
	B14	0.3	0.7	0.6	0.1	Small coccoid
Gam42a	G1	25.6	7	4.6	81.1	Large coccoid
	G2	3.7	2.4	2	5.8	Large coccoid
	G3	3.9	2.3	2.2	5.6	Large coccoid
	G4	3.3	2.3	1.9	4.4	Large coccoid
	G5	2.5	2.5	1.2	2	Large elongated
	G6	0.3	0.8	0.5	0.1	Small coccoid
SRB385	S1	1.9	2.2	1.2	1.8	Large coccoid
	S2	4.4	3.3	1.3	3	Large elongated
	S3	2	3.7	0.7	1	Large elongated
	S4	0.8	1.3	1	0.7	Large coccoid
	S4	1.2	1.5	1.1	1	Large coccoid
	S5	0.3	0.9	0.4	0.1	Small elongated
	S6	0.7	1.9	0.5	0.2	Large elongated
	S7	0.9	1.8	0.7	0.5	Large elongated
	S8	0.6	1.3	0.6	0.2	Large elongated
	S9	0.9	1.6	0.8	0.5	Large elongated
	S10	1.1	1.3	1.1	0.8	Large coccoid
	S11	0.3	0.7	0.6	0.1	Small coccoid

[1] morphotype description in Figure 6

[2] according to the morphologic criteria described by Ramsing *et al.* (1996)

Tab. 1 - Morphotype description of bacteria detected by *in situ* hybridization with probes Alf1b, Bet42a, Gam42a, and SRB385.

SEQUENCES ANALYSIS, PROBES DESIGN AND ANALYSIS OF PHOTOTROPHIC SULFUR BACTERIA

To resolve the nature of the bacterial populations observed by microscopy in the anoxic layer of the meromictic Lake Cadagno, we isolated the genes encoding the small subunit ribosomal RNA from a water sample taken at 17 meters depth, using PCR with universal primers for the domain *Bacteria* followed by cloning and sequencing of the obtained fragments.

The cloned inserts were reamplified to confirm the presence of the 1.4 kb full-length insert, which was detected in 276 clones over a total of 293 putative transformants.

To identify unique clone types, ARDRA (amplified rDNA restriction analysis) was performed on all of the 276 reamplified inserts, and the profiles obtained were grouped in similar clusters using the GelCompare software. The analysis identified 69 distinct groups of restriction profiles.

Some chosen clones were further analyzed by sequencing (ABI PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit and ABI Prism 310 automated sequencer -Perkin-Elmer, Rotkreutz, Switzerland).

16S rRNA-targeted oligonucleotide probes were designed, and their specificity with reference to available 16S rRNA sequences, was checked with the ARB program (STRUNK & LUDWIG 1996) and in the EMBL/GenBank databases using FASTA (PEARSON & LIPMAN 1988).

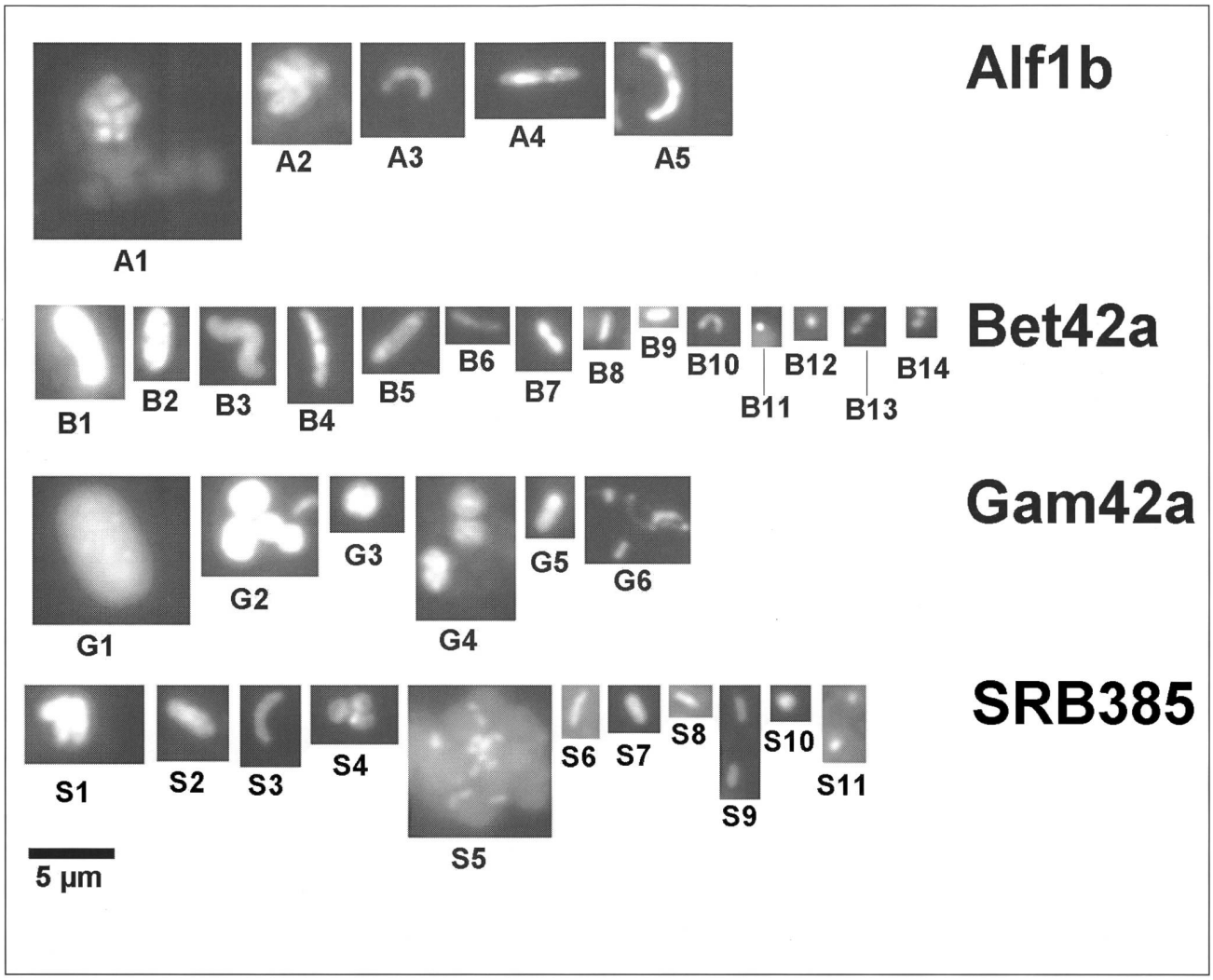

Fig. 6 - Typical morphotypes of bacteria hybridizing to Cy3-labeled oligonucleotide probes Alf1b, Bet42a, Gam42a and SRB385.

Probes Cmok453 (5'AGC CGA TGG GTA TTA ACC ACC AGG TT, pos. 453-478 according to the *E. coli* numbering of Brosius et al. 1981) and Apur453 (5'TCG CCC AGG GTA TTA TCC CAA ACG AC, pos. 453-479) were designed to detect *Cm. okenii* type strain DSM169 and *A. purpureus* type strain DSM4197, respectively.

Probe Apur453 hybridized to only a minor portion of the *A. purpureus* population identified by autofluorescence and morphological criteria in the Lake Cadagno. Even though *in situ* hybridization with probe Gam42a demonstrated the permeability to the probe of all the *Amoebobacter*-like cells in our samples, only approx. 20% (2.2 ± 0.8 x 10^5 cells ml^{-1}) could be detected with this specific probe Apur453. This result showed that the type strain *A. purpureus* DSM 4197 did not represent the major population of *Amoebobacter*-like cells in the chemocline of Lake Cadagno and that at least two different populations might be present.

A further characterization of these populations was thus based on *in situ* hybridization with probes designed after the analysis of the 16S rDNA gene sequences of the library and of the phototrophic sulfur bacteria *Lamprocystis roseopersicina* DSM229 and *Amoebobacter roseus* DSM235. All these probes were targeting 16S rRNA at the position 453 to 478, according to the *E. coli* numbering, which is a highly variable region revealing at least five differences between sequences of the phototrophic sulfur bacteria investigated.

Probe Apur453 targeted *A. purpureus* DSM 4197, but also the rRNA of bacteria harboring the sequence of our clone 345. Probe Laro453 targeted *L. roseopersicina* DSM229 as well as the sequence of clone 136, while probe S453D targeted the 16S rRNA of bacteria harboring the sequence of clone 261, and probe S453F this of clones 335 and 371.

The specificity of the probes was checked with pure cultures of phototrophic sulfur bacteria as well as with those of bacteria from other phyla.

In situ hybridization with probes Apur453, Laro453, S453D, and S453F, targeting a phylogenetically and morphologically very tight cluster of different small-celled phototrophic sulfur bacteria, resulted in the detection of distinct populations in the chemocline of Lake Cadagno. Probe Apur453 detected up to 7 x 10^5 cells ml^{-1}, whereas probes Laro453, S453D, and S453F detected 1 x 10^5, 7 x

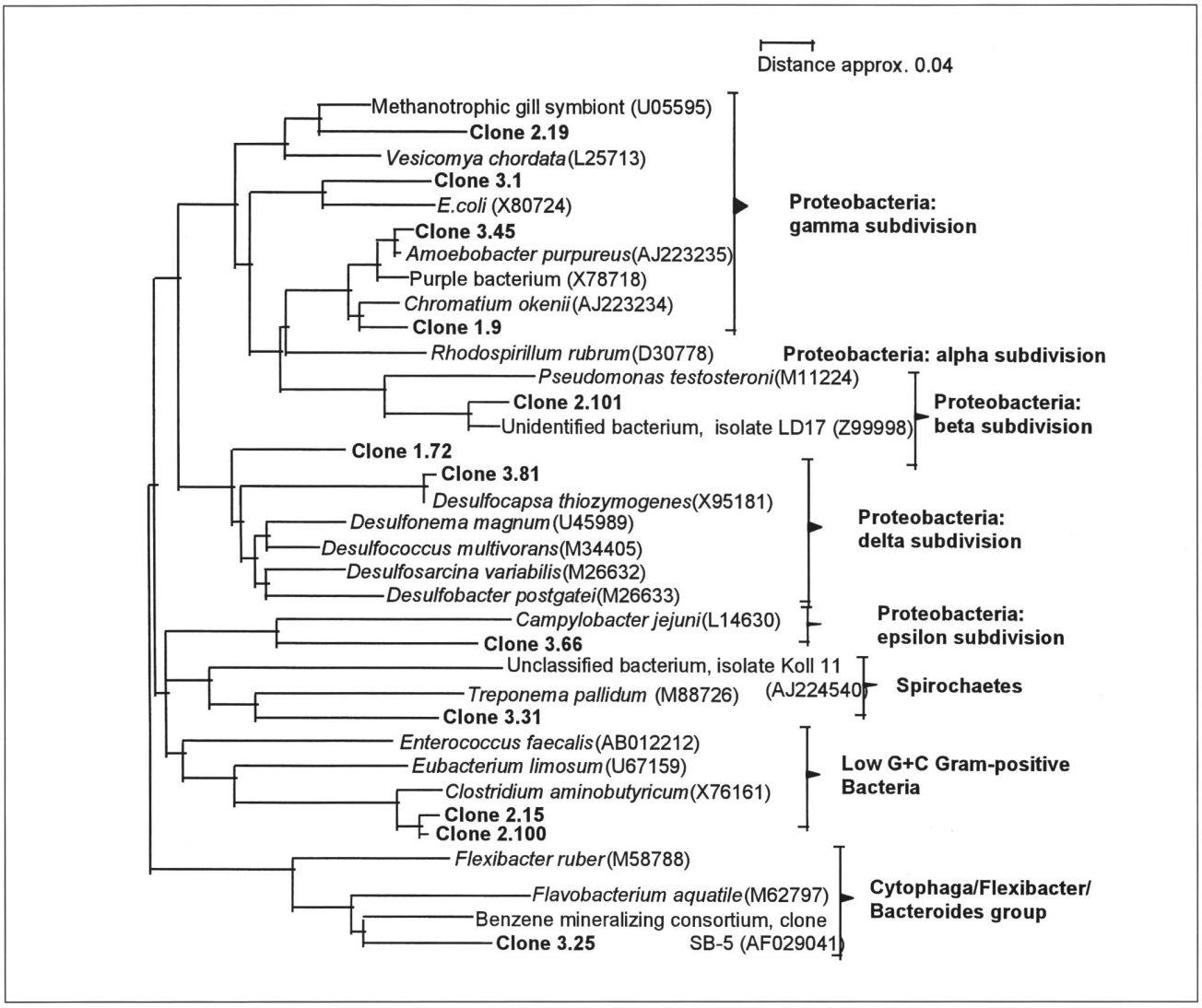

Fig. 7 - Phylogenetic tree showing the relationship of the clones partially sequenced to other members of the domain *Bacteria*. 334 nucleotide positions were included in the analysis (Jukes-Cantor distance and Neighbor-joining method). Between brackets the RDP accession numbers of each organism retrieved from this database are indicated. The scale bar indicates 4 substitutions per 100 nucleotide positions (Demarta A. et al., 1998)

10^5, and 4 x 10^5 cells ml^{-1} at their maximum occurrence, respectively.

The analysis revealed that the *Amoebobacter*-like populations showed a broader distribution than *Cm. okenii,* with high cell densities in the lower part of the chemocline which might be due to their high sulfide tolerance.

Populations of small-celled phototrophic sulfur bacteria not only differed with respect to total population sizes but also differed with respect to population profiles in depth. While populations detected with probe S453D only revealed a distinct maximum occurrence at depth of 12.5 m (sampling of the 3rd of October 1997), populations detected with probe S453E showed a second maximum occurrence at a depth of 13.1 m, and those detected with probe S453F showed an evenly high distribution at a depth of between 12 and 13.1 m.

Between 96 and 100% of autofluorescent cells were detected through the whole chemocline when a combination of all probes was used.

The type strain of *A.purpureus* DSM4197 did thus not represent the major population of *Amoebobacter*-like cells in the chemocline of Lake Cadagno and different populations were present. This result confirmed earlier studies reporting some size differences between an isolate obtained from Lake Cadagno (LcCAD1) and the type strain, which was isolated from Lake Schleinsee in Germany,.were already reported (EICHLER & PFENNIG, 1988).

Further studies on the populations of small-celled phototrophic sulfur bacteria in the chemocline of Lake Cadagno will be focused on the analysis of temporal and spatial distributions of specific populations in relation to the environmental factors such as light, electron donors, oxygen and carbon sources.

The 16S rDNA sequences determined have been depostited in the EMBL/GenBank databases under accession no. AJ223234, AJ223235, AJ006221, and AJ006057 to AJ006063.

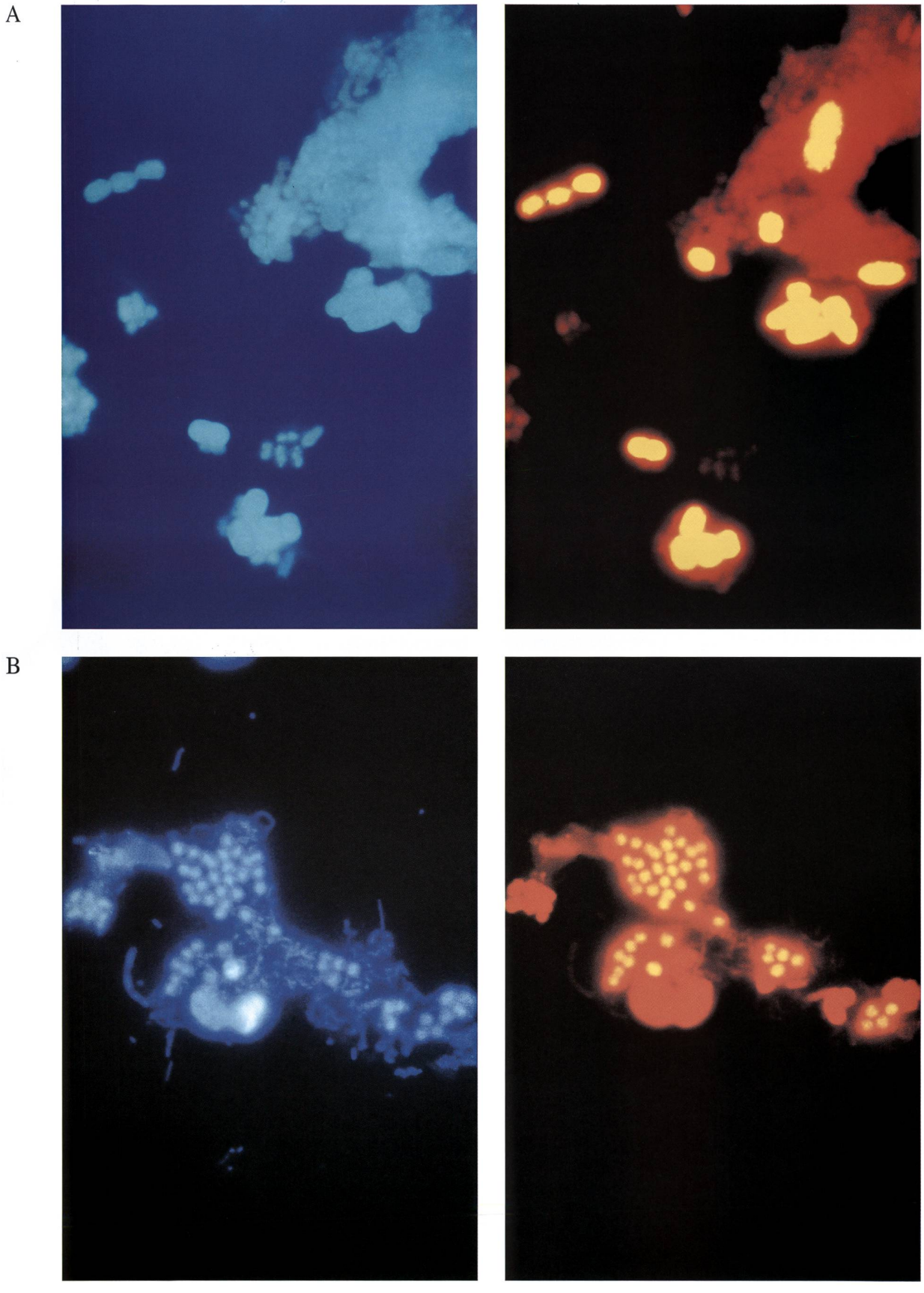

Fig. 8 - Total bacterial stains with DAPI (**left**) and *in situ* detection of phototrophic sulfur bacteria with Cy3-labeled probes (**right**). **Panel A**: Probe Cmok453 targeting *Cm. okenii*. **Panel B**: Probe Apur453 targeting *A. purpureus*.
Bar represents 10 µm.

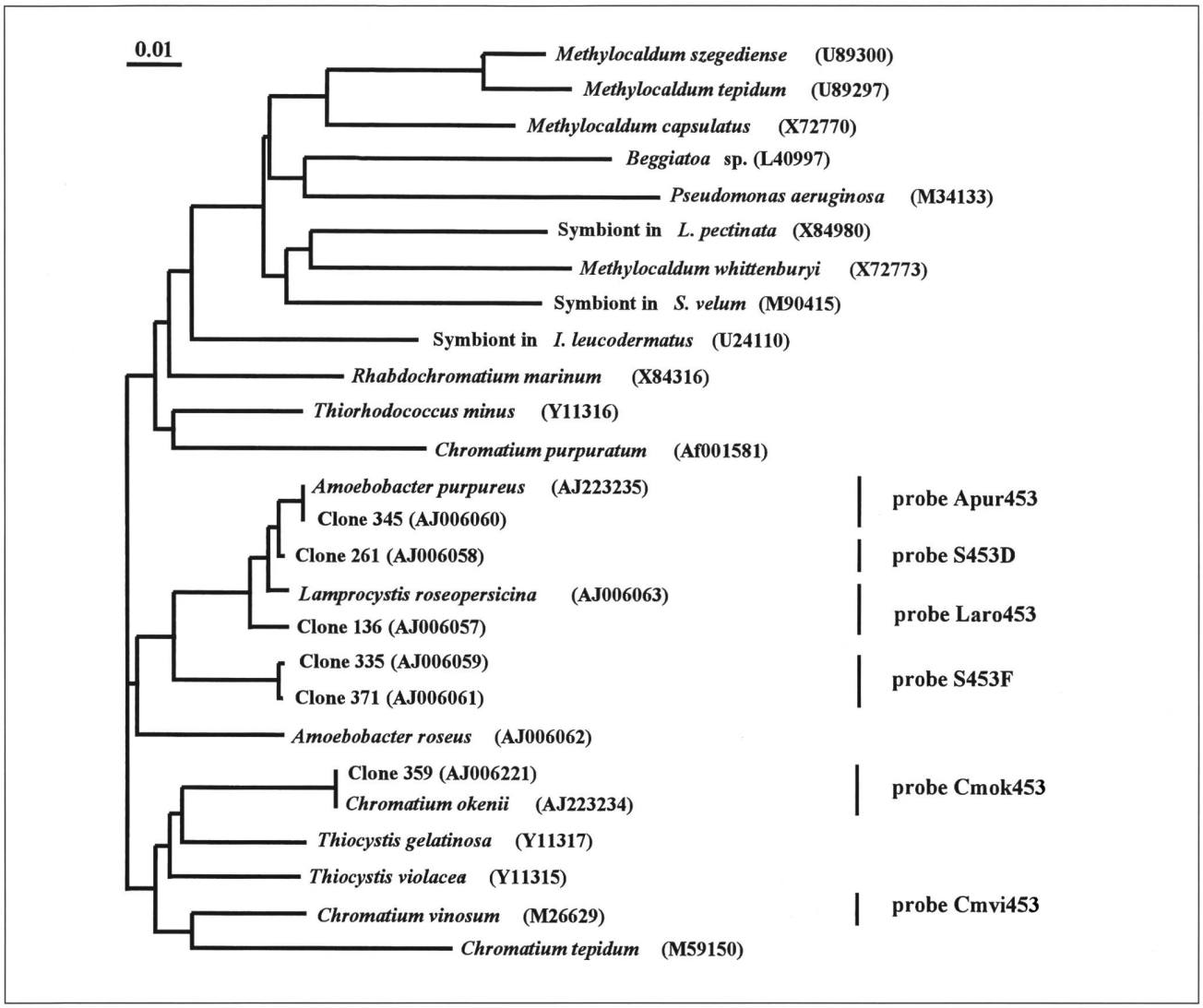

Fig. 9 - Neighbor-Joining tree based on the aligned sequences of selected clones from the 16S rRNA gene library of the chemocline of Lake Cadagno and of selected bacteria searched from the EMBL/GenBank databases using FASTA through the GCG package. The distance scale indicates the expected number of changes per sequence position. Bars and probe designations indicate target groups of phototrophic sulfur bacteria for specific oligonucleotide probes (Tonolla M. et al. 1999).

DISTRIBUTION OF SULFATE-REDUCING BACTERIA IN THE CHEMOCLINE.

In situ hybridization with probe SRB385 targeting bacteria of the d-subdivision of Proteobacteria (presumably mainly sulfate-reducing bacteria) showed the presence of sulfate-reducing bacteria which number went together with some environmental parameters. The maximum of bacteria detected with probe SRB385 of $3.8 \pm 1.7 \times 10^5$ cells ml^{-1} was obtained in the chemocline, where also the phototrophic sulfur bacteria reached their maxima. At the same depth a minimum concentration of sulfate and increasing concentrations of sulfide where present.

Sulfate-reducing bacteria and sulfur bacteria such as purple and green sulfur bacteria have been shown to co-occur in the same habitat (CAUMETTE *et al.* 1994, OVERMANN *et al.* 1996, ØVREÅS *et al.* 1997). It was therefore speculated that sulfate-reducing bacteria may complement sulfur- and sulfide-oxidizing bacteria ecologically (MUYZER *et al.* 1995). Our results on the association of both sulfate-reducing and phototrophic sulfur bacteria in the chemocline of Lake Cadagno add some more evidence to this speculation thought the character of a possible interaction, competitive or commensalistic, remains uncertain. A deeper analysis of these co-occurence as been undertaken by the use of new and specific 16S rRNA targeting probes.

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REFERENCES

BROSIUS J., T.J. DULL, D. SLEETER, and H.F. NOLLER. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. 148: 107-127.

BENSADOUN J.-C., M. TONOLLA, A. DEMARTA, F. BARJA and R. PEDUZZI. 1998. Vertical distribution and microscopic characterization of a non-cultivable micro-organism (morphotype R) of Lake Cadagno. In: PEDUZZI R., R. BACHOFEN, and. M. TONOLLA (eds) 1998. Lake Cadagno: ameromictic alpine lake. Documenta Ist. ital. Idrobiol. 63: 45-51.

CALDWELL D.E. & J.M. TIEDJE. 1975. The structure of anaerobic bacterial communities in the hypolimnia of several Michigan lakes. *Can. J. Microbiol.* 21: 377-385.

CAUMETTE P., R. MATHERON, N. RAYMOND, J.C. RELEXANS 1994. Microbial mats in the hypersaline ponds of Mediterranean salterns (Salins-de-Giraud, France). FEMS Microbiol. Ecol. 13: 273-286.

CLARK A.E. & E. WALSBY. 1978. The development and vertical distribution of populations of gas-vacuole bacteria in a eutrophic, monomictic lake. Arch. Microbiol. 118: 229-233.

DEMARTA A., M. TONOLLA, A.P. CAMINADA, N. RUGGERI and R. PEDUZZI. 1998. Phylogenetic diversity of the bacterial community from the anoxic layer of the meromictic lake Cadagno. In: PEDUZZI R., R. BACHOFEN, and. M. TONOLLA (eds) 1998. Lake Cadagno: ameromictic alpine lake. Documenta Ist. ital. Idrobiol. 63: 19-30.

EICHLER B. and N. PFENNIG. 1988. A new purple sulfur bacterium from stratified freshwater lakes, *Amoebobacter purpureus* sp. nov. Arch Microbiol 149: 395-400.

KHANNA P., B. RAJKUMAR and N. JOTHIKUMAR. 1996. Microbial recovery of sulfur from thiosulfate-bearing wastewater with phototrophic and sulfur-reducing bacteria. Curr Microbiol 32: 33-37.

MUYZER G., A. TESKE, C.O. WIRSEN, H. W. JANNASCH. 1995. Phylogenetic relationship of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164: 165-172.

OVERMANN J., J. T. BEATTY, H. R. KROUSE and K. J. HALL. 1996. The sulfur cycle in the chemocline of a meromictic salt lake. Limnol Oceanogr 41: 147-156.

ØVREÅS L., L. FORNEY, F. L. DAAE, V. TORSVIK.1997. Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63: 3367-3373.

PEARSON W.R., and D.J. LIPMAN. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85: 2444-2448.

PEDUZZI R., R. BACHOFEN, and M. TONOLLA (eds) 1998. Lake Cadagno: ameromictic alpine lake. Documenta Ist. ital. Idrobiol. 63: 152 pgg.

RAMSING N.B., H. FOSSING, T.G. FELDERMAN, F. ANDERSEN & B. THAMDRUP. 1996. Distribution of bacterial populations in a stratified fjord (Mariager Fjord, Denmark) quantified by *in situ* hybridization and related chemical gradients in water column. *Appl. Environ. Microbiol.* 62: 1391-1404.

TONOLLA M., A. DEMARTA, D. HAHN and R. PEDUZZI. 1998. Microscopic and molecular *in situ* characterization of bacterial populations in the meromictic lake Cadagno. In: PEDUZZI R., R. BACHOFEN, and. M. TONOLLA (eds) 1998. Lake Cadagno: ameromictic alpine lake. Documenta Ist. ital. Idrobiol. 63: 31-44.

TONOLLA M., A. DEMARTA, R. PEDUZZI and D. HAHN 1999. *In situ* analysis of phototrophic sulfur bacteria in the chemocline of meromictic Lake Cadagno (Switzerland), Appl. and Environ. Microbiol. 65, (3): 1325-1330.

Das Ausmass der bakteriellen Vielfalt - oder wieviele Bakterien leben in Tessiner Bergseen

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Die Mikroorganismen spielen in nahezu allen Ökosystemen der Biosphäre eine wichtige Rolle. Trotz ihrer Bedeutung auch für den Menschen werden sie in Diskussionen um die Artenvielfalt allzuoft vergessen. Die Analyse der Diversität mittels neuen, genetischen Verfahren erlaubt nun einen Einblick in eine unerwartet grosse Vielfalt von Mikroorganismen.

DAS PROBLEM DER KLEINHEIT

Heute wird von der Annahme ausgegangen, dass das Leben auf der Erde nur einmal entstanden ist. Der Begriff Biodiversität, so wie er heute Verwendung findet, beschreibt die biologische Vielfalt der Arten, wie auch die genetische Variation innerhalb einer Art. Bei höheren Organismen drückt sich die Biodiversität in einer Vielzahl von phänotypischen Merkmalen aus. Obwohl trotz intensiver Forschung das Ausmass der Diversität des Lebens nach wie vor unbekannt ist, erlaubt das unterschiedliche Erscheinungsbild höherer Organismen, beispielsweise der Säugetiere, wenigstens eine grobe Abschätzung der Artenzahl.

Tab. 1 - Anzahl der beschriebenen Arten von lebenden Organismen (E.O. Wilson und F. M. Peter, ed., Biodiversity, Washington, 1988).

Viren	1'000
Bakterien	3'000
Blaualgen	1'700
Pilze	46'983
Algen	26'900
niedere Pflanzen	28'000
höhere Pflanzen	220'500
Protozoen	30'800
Invertebraten	989'700
(davon Insekten: 751'000)	
Wirbeltiere	42'600
Total	1'392'500

Weit schwieriger gestaltet sich da eine Schätzung der Artenzahl bei Mikroorganismen. Um die Vielfalt mikroskopisch kleiner Organismen untersuchen zu können, stehen in der Regel nur wenige und unscheinbare morphologische Merkmale zur Verfügung. Die Grundlage zur Definition von Gattungen bildet daher nebst den morphologisch-anatomischen Unterschieden die bei Bakterien häufig anzutreffende ausgeprägte physiologische Differenzierung. Grundvoraussetzung dafür ist allerdings, dass es gelingt, die Organismen in Reinkultur zu züchten. Wie in Tabelle 1 aufgeführt, konnten bis heute auf diese Weise etwa 3000 Bakterienarten, neuere Untersuchungen gehen inzwischen von etwa 5000 Bakterienarten aus, beschrieben werden.

EINE NEUE KLASSE VON MERKMALE

Eine wichtige Frage bleibt aber unbeantwortet: Gibt die Anzahl der bis heute beschriebenen Bakterien das wirkliche Ausmass der mikrobiellen Vielfalt wieder oder steht die oben genannte Zahl lediglich für diejenigen Organismen, die heute kultivierbar sind? Hinweise, die für die zweite Annahme sprechen, erhält man aus Untersuchungen, die zeigen, dass nur ein Bruchteil der mikroskopisch sichtbaren Mikroorganismen auch auch als Reinkultur isoliert und demzufolge physiologisch näher beschrieben werden können.

1990 wurden in der gleichen Ausgabe der renommierten Fachzeitschrift «Nature» zwei Studien zum Thema mikrobielle Vielfalt veröffentlicht, die beide viel Beachtung erhalten haben. Dabei setzten die zwei Forschergruppen um S. Giovannoni und D. Ward ein neues Verfahren für die Fahndung nach bisher unbekannten Mikroorganismen ein, deren Idee ebenso einfach wie genial ist: Anstatt einzelne Organismen aus einer Umweltprobe zu kultivieren, haben die Wissenschafter das gesamte DNS-Material der Zellen isoliert und daraus ein bestimmtes Gen, die ribosomale rDNS, mittels Polymerase Kettenreaktion (PCR) vermehrt. Die so erhaltenen Genfragmente, die aus den verschiedenen, in der Probe vorkommenden Organismen stammte, wurden durch einen Klonierungsschritt vereinzelt und anschliessend analysiert. Durch Vergleich der Gensequenzen konnten so Rückschlüsse auf die stammesgeschichtliche Beziehungen der Organismen gemacht werden. Ein solches Vorgehen ist nur möglich, weil mit der ribosomalen rRNS ein Molekül zur Verfügung steht, das nicht nur zum Grundbestand einer jeden Zelle gehört, sondern auch in hoher Kopienzahl vorkommt. Dabei ist es von Vorteil, dass das ribosomale Gen stark konserviert ist. Un-

terschiede in der Abfolge der Sequenz geben nämlich Hinweise auf den Verwandtschaftsgrad und lassen eine Einordnung im phylogenetischen Stammbaum zu. Da alle Sequenzinformationen von den Forschern auf riesigen Datenbanken abgespeichert werden, von wo sie von jedermann über das weltweite Computernetz abgerufen werden können, ist eine immer genauere Einordnung der Organismen möglich. Molekulare Daten, z.B. Unterschiede in der DNS-Sequenz, stellen daher eine neue unabhängige Klasse von Merkmalen dar.

Bemerkenswert an den Arbeiten der zwei Forschergruppen war nun, dass sie in den untersuchten Ökosystemen zahlreiche bislang nicht kultivierte Organismen gefunden haben. Diese Resultate nährten die Spekulationen, dass die Vielfalt der Mikroorganismen weit grösser ist als bis dahin angenommen. Berichte wie diese, und mittlerweile sind es eine beachtliche Menge, haben zu revolutionären Veränderungen der Vorstellungen über das wahre Ausmass der mikrobiellen Biodiversität geführt. Die Schätzungen der tatsächlichen Artenzahlen streuen stark, es scheint aber, dass die Zahl der Bakterien bislang um Grössenordnungen unterschätzt worden ist. Nach vorsichtiger Annahme sind lediglich 1 % beschrieben worden, andere sprechen sogar von nur 0,1 % und noch weniger.

Eine Idee über die wirkliche Grösse der bakteriellen Artenvielfalt erhält man auch aus dem folgenden bekannt gewordenen Vergleich aus dem Umfeld der Entomologie: Neuere Studien sprechen heute von über einer Million verschiedenen Insektenarten auf der Erde. Geht man von der durchaus realistischen Annahme aus, dass in mindestens 10 % dieser Insekten mikrobielle Symbionten leben, welche durch eine obligate Wechselwirkung ganz spezifisch für eine bestimmte Insektenart sind, so müsste die Artenzahl der Bakterien allein durch diesen Umstand auf über 100'000 korrigiert werden. Und was für Insekten gilt, dürfte auch für andere Organismen gelten.

BIODIVERSITÄT IM LAGO DI CADAGNO

Im Val Piora im Tessin liegt auf einer Höhe von 1923 m.ü.M. der nur ca. 25 ha grosse, alpine See Lago di Cadagno. Für Wissenschaftler speziell interessant ist dieser See durch seine ausgeprägte Meromixis. Dadurch wird er zu einem interessanten Forschungsgebiet und eignet sich ganz besonders, um die Beziehungen zwischen den Organismen sowie zwischen diesen und den abiotischen Faktoren zu untersuchen. Zusammen mit einer Tessiner Arbeitsgruppe um Raffaele Peduzzi vom kantonalen Institut für Mikrobiologie in Lugano verfolgen wir in Zürich das Ziel, mehr über die bakterielle Vielfalt in diesem meromiktischen See sowie in den angrenzenden Feuchtgebieten in Erfahrung zu bringen.

Verfahren sind wir in unserer Gruppe ähnlich wie die beiden erwähnten Forschungsgruppen um Stephen Giovannoni und David Ward. In einem ersten Ansatz haben wir 16S rDNS-Fragmente aus See und Mikrobenmatten kloniert und davon 47 Klone genauer analysiert. Was die Vielfalt der untersuchten Sequenzen anbelangt, stimmt unsere Untersuchung weitgehend mit Resultaten anderer Forschungsgruppen überein, die sie von verschiedensten Habitaten erhalten haben. Nur eine einzige Sequenz ist völlig identisch zu bekannten Sequenzen in den Datenbanken - die restlichen weisen teilweise so geringe Ähnlichkeiten auf, dass eine genaue Zuordnung sogar problematisch ist. Extrapoliert man diese Untersuchungsresultate, so kommt man zu dem Ergebnis, dass der Cadagnosee einen Artenreichtum aufweist, das selbst kühne Schätzungen übertrifft.

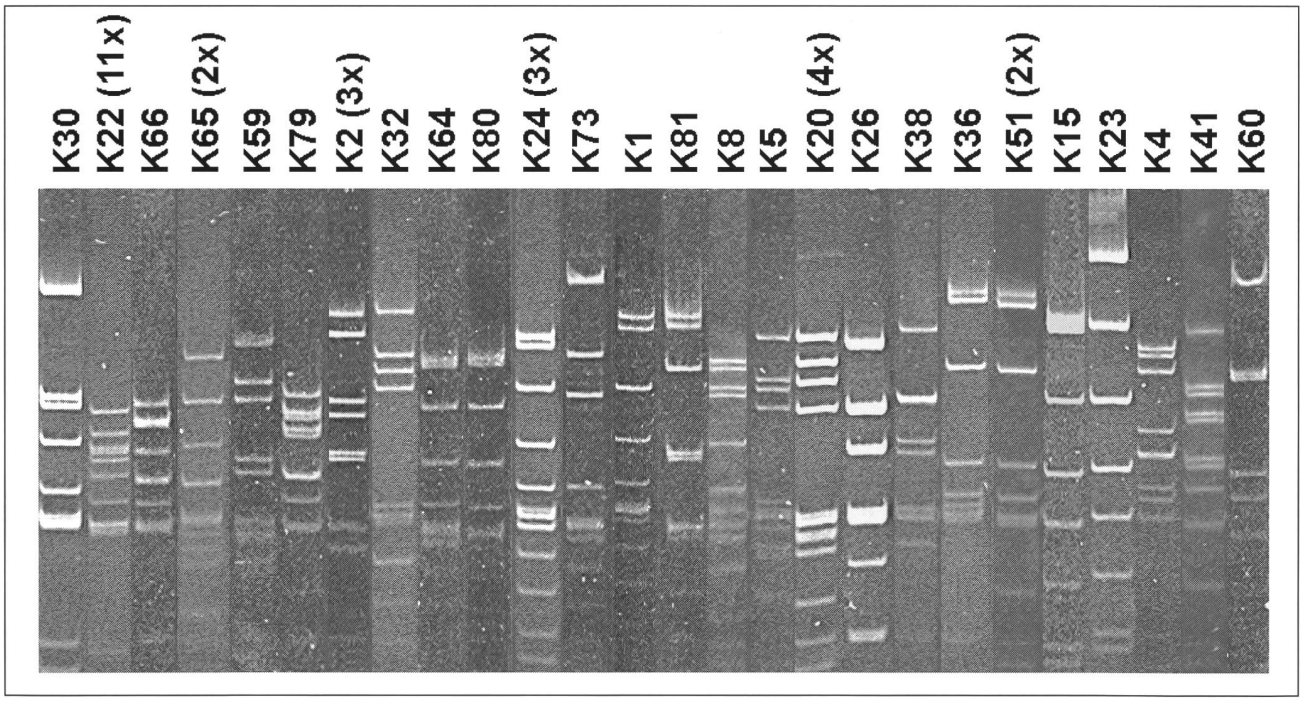

Abbildung 1: ARDRA-Muster von 45 zufällig ausgewählte 16S rDNA-Klone.

Die Vielfalt von rRNS-Genen in einer Bakteriengemeinschaft kann auch ohne vorhergehende klonale Seperation erhalten werden. Allerdings ist auch hier der erste Schritt eine Polymerase Kettenreaktion. Da die so vermehrten Genabschnitte alle ungefähr die gleiche Länge aufweisen, können sie mit einer konventionellen Agarose-Gelelektrophorese nicht aufgetrennt werden. In der «temporären Temperatur-Gradienten Gelelektrophorese» (TTGE) werden hingegen die PCR-Produkte abhängig von ihren verschiedenen Schmelzpunkten aufgetrennt. Besonders zur Bestimmung der Zusammensetzung der häufigsten Arten einer mikrobiellen Gemeinschaft wird das TTGE von 16S rDNS-Fragmenten verwendet. Mittels dieser Methode konnten wir die Resultate der Klonbiblothek weitgehend bestätigen. Zusätzlich haben wir aber auch Informationen über die mengenmässig wichtigen Organismen erhalten. Für uns speziell auffallend war in diesem Zusammenhang, dass einzelne Gruppen von Bakterien, wie beispielsweise Vertreter der Gattung Chromatium, untereinander eine beachtliche Vielfalt aufweisen.

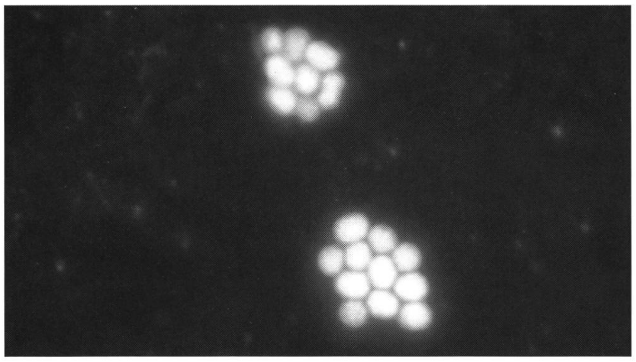

Abbildung 2: *In situ*-Detektion mit einer Probe aus dem Cadagnosee. Hybridisiert wurde mit einer Oligonukleotidsonde für Proteobakterien der g-Unterklasse.

Ergänzend zu diesen Untersuchungen haben wir auch Ganzzellhybridisierungen mit fluoreszenzmarkierten, rRNS-gerichteten Oligonukleotidsonden durchgeführt. Durch dieses Verfahren können Gruppen von Mikroorganismen *in situ* identifiziert und ihre Häufigkeit sowie Verteilung in der Umwelt direkt bestimmt werden. Damit wird es möglich, die Diversität der Mikroorganismen in ihrem natürlichen Habitat zu untersuchen.

WARUM EINE MIKROBIELLE BIODIVERSITÄTSFORSCHUNG?

Die Bestimmung des Ausmasses der Biodiversität auf der Erde, deren Entstehung und Erhaltung, ist einer der grossen Problemkreise in der Biologie. Vor allem in der Mikrobiologie ist unser gegenwärtiges Verständnis der Vielfalt sehr oberflächlich. Fragen beispielsweise, wieviele Arten in einem bestimmten Ökosystem existieren und warum gerade soviele und nicht mehr oder weniger, oder warum gerade eine bestimmte Art in dem Biotop zu finden ist, während eine verwandte Art ein ganz anderes Umfeld vorzieht, werden die Mikrobiologen auch in Zukunft beschäftigen.

Gerade weil wir die Bedeutung der mikrobiellen Vielfalt noch nicht exakt einschätzen können, dürfen sich Debatten über Biodiversität nicht nur auf die gut sichtbaren Tier- und Pflanzenarten beschränken. Nur wenn realistische Erhebungen über Artenzahl von Mikroorganismen und deren (genetisch bestimmten) Stoffwechselfähigkeiten vorliegen, können überhaupt erst Aussagen gemacht werden, wieweit die vom Menschen verursachten Veränderungen einen Einfluss auf die mikrobiellen Gemeinschaften und die durch Mikroorganismen katalysierten Prozesse in unserer Umwelt haben.

Peculiarità del ciclo del carbonio organico nel lago di Cadagno

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Il Lago di Cadagno è un lago alpino situato sul massiccio del San Gottardo (Canton Ticino, Svizzera) ad una quota di 1923 m s.l.m. Ha una superficie di 26 ettari ed una profondità massima di 21 metri.

Questo lago, apparso peculiare già al primo ricercatore che lo studiò agli inizi del secolo (BOURCART, 1906), presenta un raro fenomeno di meromissi crenogenica (PEDUZZI, 1990). Esso è, in altre parole, costituito da due strati d'acqua immiscibili a causa della loro diversa densità. La meromissi del Cadagno è naturale, conseguenza all'apporto di soluti da sorgenti site sul fondo della cuvetta lacustre, e non legata a fenomeni di eutrofizzazione antropogenica com'è il caso, per esempio dei laghi di Lugano e di Iseo. Una ulteriore particolarità del Cadagno sta nel fatto che il chemoclinio, ossia la zona di transizione tra strato profondo, anossico e con alta concentrazione di soluti, e strato superficiale, ben ossigenato e troficamente simile ad un lago alpino, ospita un ingente popolamento di solfobatteri autotrofi. Questi producono rilevanti quantità di sostanza organica utilizzando la radiazione luminosa che attraversa le acque sovrastanti e l'idrogeno solforato ipolimnetico come accettore di protoni.

In questa ricerca si è voluto valutare, misurando le variazioni spazio-temporali del carbonio organico particellato (POC) e disciolto (DOC), dei pigmenti fotosintetici in toto nonché delle biomasse batteriche, se ed in che misura l'origine e lo standing crop della sostanza organica del Lago di Cadagno fossero dipendenti dallo strato di batteri fotosintetici presenti alla profondità di 11-14 m, in coincidenza col chemoclinio. Le raccolte dei campioni necessari alle analisi sono state effettuate nel 1994, 95, 96 durante il periodo estivo con frequenza mensile.

Dalla ricerca è emerso che nel Lago di Cadagno DOC, è presente in concentrazioni assai elevate per un lago alpino (1-4 mg l^{-1}). La sua distribuzione verticale, caratterizzata da valori più elevati in prossimità della superficie e alla profondità del chemoclinio, suggerisce che tali strati siano punti di produzione della sostanza organica disciolta.

Il POC presenta generalmente un marcato picco di concentrazione al chemoclino che, quindi, risulta essere lo strato di maggior produzione del POC stesso. La concentrazione del particellato organico ha raggiunto, nel corso di questa ricerca, i 6 mgC l^{-1}.

La sovrapponibilità dei profili verticali di concentrazione dei pigmenti fotosintetici e del POC indica l'origine autoctona di quest'ultimo. Inoltre la stretta relazione tra distribuzione verticale del POC e del biovolume dei batteri autotrofi *Chromatium okenii* e *Amoemobacter* evidenzia che nel Lago di Cadagno la principale sorgente di carbonio sono i popolamenti batterici del chemoclinio. Quindi questi popolamenti, di indubbio interesse dal punto di vista autoecologico per le loro peculiarità metaboliche e per la loro capacità di condizionare la biochimica dello strato da essi occupato e degli strati limitrofi, sono importanti anche nell'ecologia generale del Lago di Cadagno perché essi sono, probabilmente, anche i principali fornitori di energia per i livelli trofici superiori insediati nella porzione ossigenata del lago.

I meccanismi del trasferimento di energia dal monimolimnio e dal chemoclino verso il mixolimnio non sono noti. Tale trasferimento può avvenire per diffusione soltanto per la frazione disciolta. Data la permanente stratificazione del lago, potrebbe essere poco rilevante un trasporto promosso dalla turbolenza indotta dal vento. Si può supporre che il trasporto di materia organica sia mediato da organismi capaci di attività di grazing in condizioni di anossia o ipossia e in grado di spostarsi lungo la colonna d'acqua.

È perciò da ritenere che nel Lago di Cadagno esista una catena alimentare microbica diversa da quelle convenzionali.

Rimane comunque il fatto che nel Lago di Cadagno dal 63 al 83% del carbonio organico totale è confinato al monimolimnio.

Anche il mixolimnio del Lago di Cadagno è però sede di intensa produzione ad opera del fitoplancton. L'attività e l'efficienza di produzione dei popolamenti algali sono tali da collocare il Cadagno epilimnico in una condizione trofica vicina alla mesotrofia. La produzione picoplanctonica in particolare ha rappresentato il 13% della produzione totale. Questo contributo non è trascurabile se si considera nei laghi situati alla stessa altitudine i picocianobattteri sono generalmente molto rari o assenti. La loro presenza e attività nel Lago di Cadagno è spiegabile considerando che i primi metri d'acqua di questo lago hanno un contenuto di DOC e materiale particellato tale da schermare le alte intensità di radiazione PAR e UV.

La conseguente limitata penetrazione della radiazione

solare, ed in particolare della componente UV, è probabilmente la ragione dell'assenza di una evidente inibizione delle attività microbiche autotrofe ed eterotrofe imputabile alla radiazione UV-B nonostante essa sia, a questa quota, più intensa che nei laghi di pianura.

BIBLIOGRAFIA

BOUCART F., 1906 - *Les lacs alpins suisses, étude chimique et physique.* Thèse présentée à l'Université de Genève. Georg & Co. Editeurs, Genève.

PEDUZZI R., 1990 - Etude d'un filtre bactérien retenant les composés toxiques ed trophogènes dans un lac alpin (Lac de Cadagno, Massif du St. Gothard). *Cahier de la Faculté des Sciences*, Univ. Genève, 20: 121-133.