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The Phylogenetic Position of the Oxymonad *Saccinobaculus* Based on SSU rRNA

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The oxymonads are a group of structurally complex anaerobic flagellates about which we know very little. They are found in association with complex microbial communities in the guts of animals. There are five recognized families of oxymonads; molecular data have been acquired for four of these. Here, we describe the first molecular data from the last remaining group, represented by *Saccinobaculus*, an organism that is found exclusively in the hindgut of the wood-eating cockroach *Cryptocercus*. We sequenced small subunit ribosomal RNA (SSU rRNA) from total gut DNA to describe *Saccinobaculus* SSU rRNA diversity. We also sequenced SSU rRNA from manually isolated cells of the two most abundant and readily identifiable species: the type species *Saccinobaculus ambloaxostylus* and the taxonomically contentious *Saccinobaculus doroaxostylus*. We inferred phylogenetic trees including all five known oxymonad subgroups in order to elucidate the internal phylogeny of this poorly studied group, to resolve some outstanding issues of the taxonomy and identification of certain *Saccinobaculus* species, and to investigate the evolution of character states within it. Our analysis recovered strong support for the existence of the five subgroups of oxymonads, and consistently united the subgroups containing *Monocercomonoides* and *Streblomastix*, but was unable to resolve any further higher-order branching patterns.

Key words: oxymonad; phylogeny; SSU rRNA; symbiont.

Introduction

Oxymonads are a group of anaerobic eukaryotic flagellates that constitute one of the more poorly understood groups of protists, in terms of both their overall biology and their evolutionary history. They are nonetheless of great interest in both regards. In terms of their basic biology, they possess a number of highly complex structural characteristics and behaviours, but at the same time lack typical eukaryotic characters such as discernable mitochondria and Golgi apparatus (Brugerolle and Lee 2000). In terms of their evolutionary history, their position on the tree of eukaryotes has been varied and disputed (Cavalier-Smith 1998; Dacks et al. 2001; Hampl et al. 2005; Moriya et al. 1998, 2001; Patterson 1994; Simpson 2003). They are now generally regarded to be members of the excavate eukaryotes, a diverse collection of flagellates and amoeboflagellates that includes many parasites and anaerobes (Simpson 2003). Molecular data support a close relationship between oxymonads and the excavate flagellate *Trimastix* (Dacks et al. 2001),

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but there is no definitive evidence for either their higher-order position or their relationship to other excavate protists (Hampl et al. 2005; Simpson 2003). Many of the unique characteristics of oxymonads may be explained by their ecology: they are obligate symbionts, found only in the gut lumen of animals. A few inhabit the guts of vertebrates, but most of the known diversity of oxymonads is restricted to the hindguts of termites and related xylophagous insects. In the insect environments, oxymonads are members of complex microbial communities. It is likely because of the complexity of these communities that only one strain, *Monocercomonoides* strain PA203 (a vertebrate symbiont), has been cultivated (Hampl et al. 2005). This inability to cultivate most oxymonads has meant there are few gene sequences from any given species, and a general lack of molecular data from the group as a whole.

In addition to their uncertain placement amongst eukaryotes, the lack of molecular data contributes to a poor understanding of the interrelationships among oxymonads. Molecular data have been slow in coming, but in recent years such data have been obtained for four of the five groups of oxymonads (traditionally regarded as families): the Pyrsonymphidae (Dacks et al. 2001; Moriya et al. 2003), Oxymonadidae (Moriya et al. 2003), Streblomastigidae (Keeling and Leander 2003), and Polymastigidae (Hampl et al. 2005). Resolving the internal phylogeny of this group will require the molecular characterization of the remaining sub-group, the Saccinobaculidae, for which there have so far been no molecular data.

The members of this last group can be quite spectacular. They are found in a single natural community within the hindgut of the wood-eating cockroach *Cryptocercus*, although in that community they are abundant and diverse. Members of the family are morphologically so plastic that they defy ready description (Cleveland et al. 1934): like all oxymonads, they have a prominent axostyle running along the length of the cell, which in the case of *Saccinobaculus* twists, coils, writhes, and straightens in a variety of changing patterns within the cell. This movement creates the appearance of a snake (the axostyle) in a bag (the plasma membrane), from which the name is derived (Cleveland et al. 1934).

All members of the Saccinobaculidae were originally described by Cleveland et al. (1934), who at first classified all of the oxymonads in *C. punctulatus* in the genus *Saccinobaculus*, with the single exception of the small flagellate *Monocercomonoides globus*. Three distinct species were described, *S. ambloaxostylus* (the type species, and the most abundant), *S. doroaxostylus* (the largest species, which is in addition morphologically and behaviourally distinct from *S. ambloaxostylus*), and *S. minor*, a looser collection of small flagellates. Beyond these three species, Cleveland et al. (1934) initially suggested that as many as six more might exist, but the extent of the diversity was difficult to determine at the time. In subsequent years, subtle details of staining properties, observations of granules on or within the axostyle, and studies of reproductive life histories led to a whittling away of *Saccinobaculus* diversity as species were reassigned or new groups created. Most significantly, the type species *S. ambloaxostylus* was split to create the new genus *Notila* (Cleveland 1950c), *S. doroaxostylus* was transferred to *Oxymonas* as *Oxymonas doroaxostylus* (Cleveland 1950a), and the smaller *Saccinobaculus* species were transferred to *Oxymonas* as *O. nana* (Cleveland 1950a).

Here, we provide the first molecular data, small subunit rRNA (SSU rRNA), from the Saccinobaculidae, and by doing so allow the first molecular-based analysis of global oxymonad phylogeny. We combined an environmental-PCR strategy (sequencing SSU rRNA from the whole gut) to examine overall diversity of the Saccinobaculidae, with the manual isolation and characterization of two easily identifiable and key species: the type species *S. ambloaxostylus* and the taxonomically ambiguous *S. doroaxostylus* (*O. doroaxostylus*).

Results and Discussion

Surveying Phylogenetic Diversity of Oxymonads in *Cryptocercus* by Environmental PCR

We surveyed SSU rRNA sequence diversity from the *Cryptocercus* gut in order to assess the phylogenetic breadth of oxymonads in this environment. Amplification with oxymonad-specific primers yielded a single detectable band of approximately 2100 bp. This fragment was cloned and sequenced, resulting in 14 unique sequences, all most similar to other oxymonad rRNAs. Phylogenetic analysis with the global eukaryotic alignment confirmed this by placing all new sequences within the oxymonad/*Trimastix* clade with 96% support (not shown). Oxymonads generally have larger-than-average SSU rRNA sequences, ranging from about 2000—2900 bp

(by contrast, the average eukaryotic sequence is 1800 bp). The oxymonad genes all have insertions at various points, many of which are shared amongst the groups of oxymonads (Hampl et al. 2005).

We narrowed our focus to oxymonads and *Trimastix* to allow a greater number of alignable sites to be used, a phylogeny of which is shown in Figure 1. Here, as in the global analysis, the environmental sequences (our *Saccinobaculus* spp. E1—E14) represent an appreciable degree of sequence diversity, and yet form a single clade with 93—100% support to the exclusion of all other oxymonad groups. Overall, the environmental sequences are all derived from a single oxymonad lineage, and there is no evidence of close relatives of other sampled oxymonad groups that have been proposed to exist in

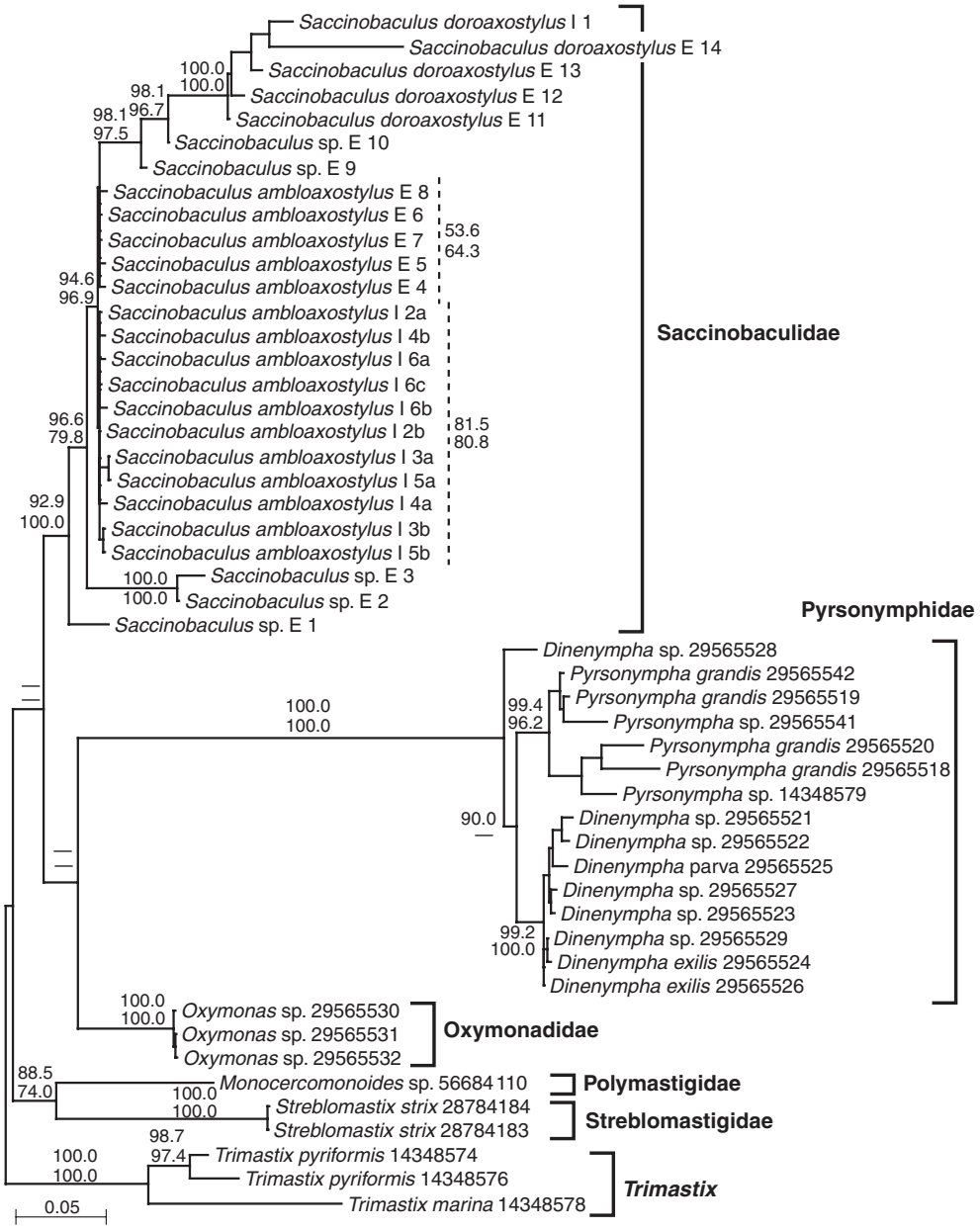

Figure 1. Phylogeny of oxymonad SSU rRNA using *Trimastix* as an outgroup. The tree shown is from Bayesian analysis with maximum likelihood branch lengths. Numbers at nodes correspond to percentage of bootstrap support from 1000 replicates using ML (above) and distance (below).

Cryptocercus, such as *Monocercomonoides* and *Oxymonas*.

Single Cell Isolation of *S. ambloaxostylus* and *S. doroaxostylus*

To identify the source of the oxymonad rRNA genes amplified in environmental sample, we manually isolated samples of the two must abundant and conspicuous species of oxymonad in the *Cryptocercus* gut: *S. ambloaxostylus* and *S. doroaxostylus*.

Saccinobaculus ambloaxostylus is the most abundant oxymonad in *Cryptocercus* and is the type species for the genus *Saccinobaculus.* It is intermediate in size (65—110 μm) amongst *Cryptocercus* symbionts and extremely plastic in form (Cleveland 1950b). This is demonstrated in Figure 2 E—J, which shows a variety of the major features that allowed us to identify the species, and also shows the plasticity of the cell due to the motile axostyle, resulting in several distinct appearances. Because of this great degree of plasticity, we repeated our isolations five times from different samples (four times from Bear Trap Gap cockroaches and once from Black Rock Mountain cockroaches) to confirm that they all correspond to a single sequence type. Phylogenetic analysis shows that all isolated specimens (sequences *S. ambloaxostylus* I2—I6) are closely related, and indeed form a group within the environmental sequences (Fig. 1). As expected, the isolated *S. ambloaxostylus* sequences were nearly identical to one of the environmental sequence groups (*S. ambloaxostylus* E4—E8), but interestingly the sequences obtained from single-cell isolations formed distinct subgroups within the tree generated from environmental sequences. The cockroaches collected from Mountain Lake Biological Station, from which all environmental data were generated, are of a different karyotype ($2n = 43$) from those of Bear Trap Gap ($2n = 45$) and Black Rock Mountain ($2n = 39$), suggesting that distinct populations of cockroaches also contain distinct populations of symbionts. Whereas the Black Rock Mountain group (I2—I5) branched within the Bear Trap Gap group (I6), all three of its sequences formed a clade within that group (Fig. 1). This is the first evidence for such population variation in these protist endosymbiont communities, suggesting the symbionts and hosts may be co-evolving.

The other conspicuous oxymonad in *Cryptocercus* is *S. doroaxostylus*, which is larger than *S. ambloaxostylus* (its size range is reported to be 150—170 μm), and is also distinguished by cytoplasmic granules appearing brown in colour, which we infer to be a carbohydrate storage product. The cell is less plastic than *S. ambloaxostylus* but does have a motile axostyle with a characteristic protruding tip and a distinctive pyriform nucleus (Fig. 2 A—D). Originally described as a member of *Saccinobaculus* (Cleveland et al. 1934), this species was subsequently reassigned to the genus *Oxymonas* (as *O. doroaxostylus*), primarily based on characteristics of its nuclear division (Cleveland 1950a). However, in most respects the species does not closely resemble *Oxymonas*, and we never saw it presenting anything that could be taken to be a rostellum, one of the distinguishing features of the family Oxymonadidae (Brugerolle and Lee 2000). The SSU rRNA sequence from manually isolated *S. doroaxostylus* (*S. doroaxostylus* I1 in Fig. 1) strongly supports its original placement in the genus *Saccinobaculus*, as it branched within the *Saccinobaculus* clade and formed a group with 100% support in the more-divergent clade of environmental sequences (*S. doroaxostylus* E11—E14 in Fig. 1).

The third described species of *Saccinobaculus* is *S. minor*, which was also present in all cockroaches examined (e.g. See Fig. 2 F, lower right). This taxon was erected to collect all *Saccinobaculus* cells too small to permit reliable identification, and Cleveland suggested that they represent several distinct species that could not easily be distinguished (Cleveland et al. 1934). After additional study, Cleveland subsequently renamed this species *Oxymonas nana* (Cleveland 1950a). Since only about half of the environmental sequences are unambiguously attributable to either *S. ambloaxostylus* or *S. doroaxostylus*, we hypothesize that at least some of the remaining sequences (*Saccinobaculus* sp. E1—E3 and E9—E10) are derived from these smaller oxymonads. The high degree of variability in these sequences, together with the fact that they do not form a single cluster in the phylogeny, lead us to propose that these indeed represent several species. Their inclusion in the clade including *S. ambloaxostylus*, the type species of *Saccinobaculus*, also leads us to propose that their original generic classification was correct, although they are not all one species of *Saccinobaculus*, but rather several.

Relationships between Oxymonad Subgroups

Our characterization of molecular data from *Saccinobaculus* allows a molecular phylogeny

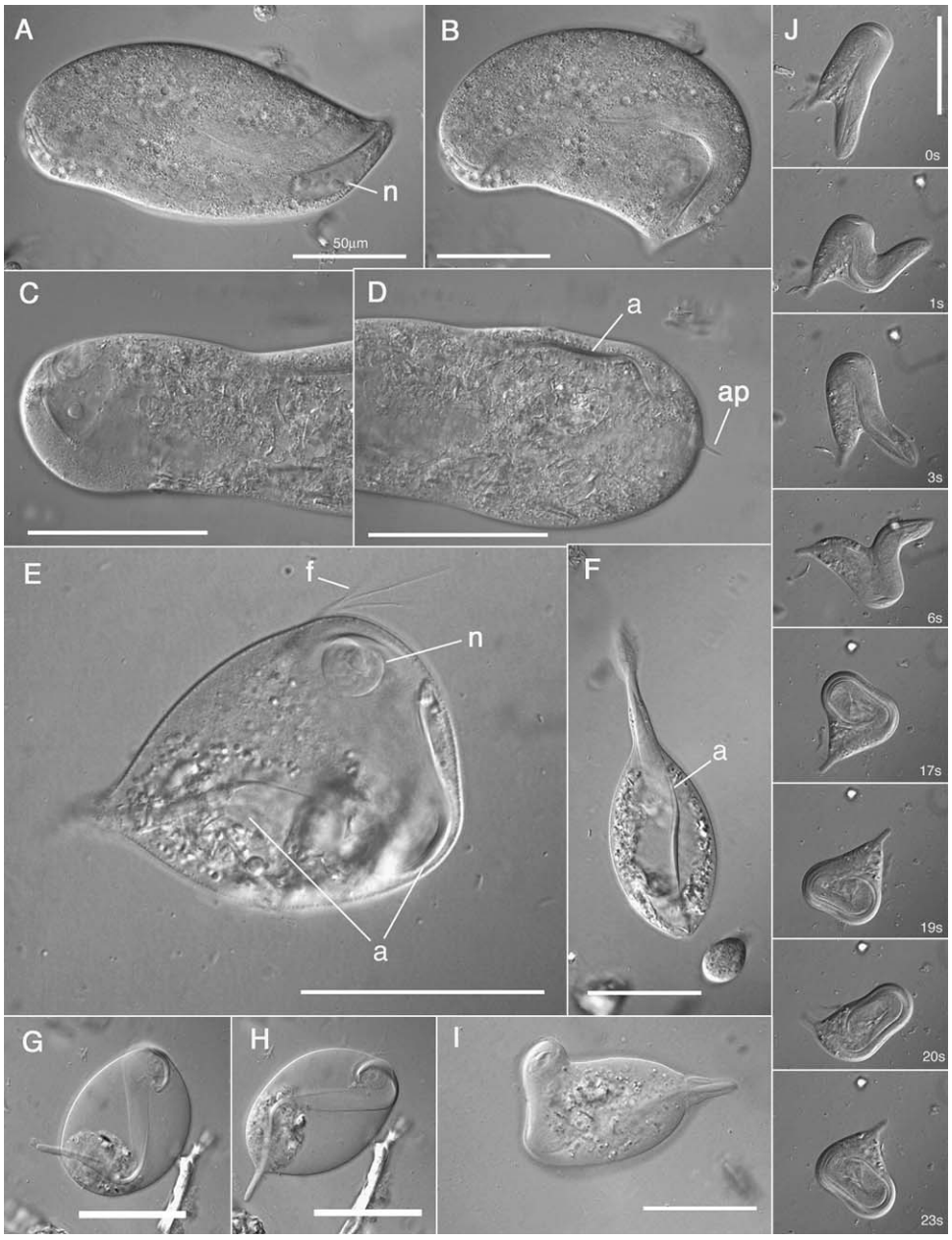

Figure 2. Light micrographs showing overall morphology of *Saccinobaculus ambloaxostylus* and *Saccinobaculus doroaxostylus*. These are representative cells that show the characteristics used in the identification of cells for single-cell isolations. (A—D): *Saccinobaculus doroaxostylus*. Two cells are shown (A, B; C, D), both about 160 μm in size. The cytoplasm is granular, with a brown colour and wood particles visible in all individuals. The axostyle (a) is relatively thin and extends through the length of the cell. The nucleus (n) is typically pyriform and associated with the anterior end of the axostyle. In detail of the posterior end, a pointed axostylar projection (ap) can be seen in many individuals. The axostyle is motile, but moves slowly, and the cells maintain a roughly ovoid shape in our observations. (E—J): *Saccinobaculus ambloaxostylus*. Five individual cells are shown, all between 65-100 μm in size. Cells were mostly rounded (e.g., E, G, H) and changed shape rapidly due to axostylar motion. Highly elongated forms (F), in which the axostyle contracted and extended, were also abundant. The nucleus (n) is circular and associated with the anterior end of the axostyle (a) where the flagella (f) emerge. The axostyle is visibly a flexible flattened band (see G, H), which sometimes projects from the posterior end (I). (J): Time-lapse sequence of a single cell of *Saccinobaculus ambloaxostylus*, showing the remarkable plasticity of this cell, characteristic of all members of the genus. All scale bars correspond to 50 μm.

including all five subgroups of oxymonad to be inferred for the first time. Each of the subgroups represented by more than one sequence was strongly recovered, but the relationships between the groups were not well supported by bootstrap analysis, and were different between analyses using different methods (Fig. 1). The only consistent relationship that emerged from these trees was the placement of *Monocercomonoides* and *Streblomastix* in a single clade.

We narrowed the taxon selection to short-branch representatives of each group, allowing a more thorough analysis (Fig. 3), but the support for the inter-group relationships did not improve: the only one with any support was once again that uniting *Monocercomonoides* and *Streblomastix*. We therefore tested all possible relationships among the six groups (the five oxymonad groups and *Trimastix*) using AU tests. We generated a data set where each group was represented by two sequences (except for *Monocercomonoides*, for which only a single sequence is available): this constituted the same data set as in Figure 3, but with *Saccinobaculus* sp. E7 and E14, *Dinenympha* sp. 29565528, and *Trimastix pyriformis* 14348576 removed, and *Streblomastix strix* 28784183 and *Saccinobaculus doroaxostylus* I1 added. Pairs of taxa representing known groups were constrained to branch together to avoid intra-subgroup topologies. We generated all 105 trees possible for this data set and carried out AU tests on them. Eighty trees were rejected at the 95% level, and from a consensus of the remaining 25 trees, no clear pattern emerged. The grouping of *Monocercomonoides* and *Streblomastix* was found in the three most favoured trees, but not in the majority of trees that were not rejected. No other grouping was prevalent amongst the accepted trees, and no particular grouping was clearly over-represented by a majority of the rejected trees. The highly divergent nature of the pyrsonymphid sequences is clearly a potential problem with the tests, so we repeated the analysis while excluding this subgroup. In this test, we found six trees that were not rejected, and three of these (including the two most likely trees) did indeed group *Monocercomonoides* and *Streblomastix*.

Conclusions: Phylogeny of Oxymonads

The characterization of the first molecular data from *Saccinobaculus* has brought the taxomony of several species full-circle. With the exception of *M. globus*, Cleveland originally regarded all of the oxymonads in the hindgut of *Cryptocercus* to be members of the genus *Saccinobaculus* (Cleveland et al. 1934). Sixteen years later he revisited the problem and reassigned two groups to *Oxymonas* based on their different life cycles and their behaviour under the influence of the host insect's moulting hormone (Cleveland 1950a). Molecular data support the original classification, since the only oxymonad sequences that we were able to recover from the gut of *Cryptocercus* formed a well-supported group not related to the previously published sequences from *Oxymonas*, and the molecular phylogeny of rRNA from manually isolated *S. doroaxostylus* is specifically and clearly in disagreement with its reassignment to *O. doroaxostylus*. Cleveland also reassigned

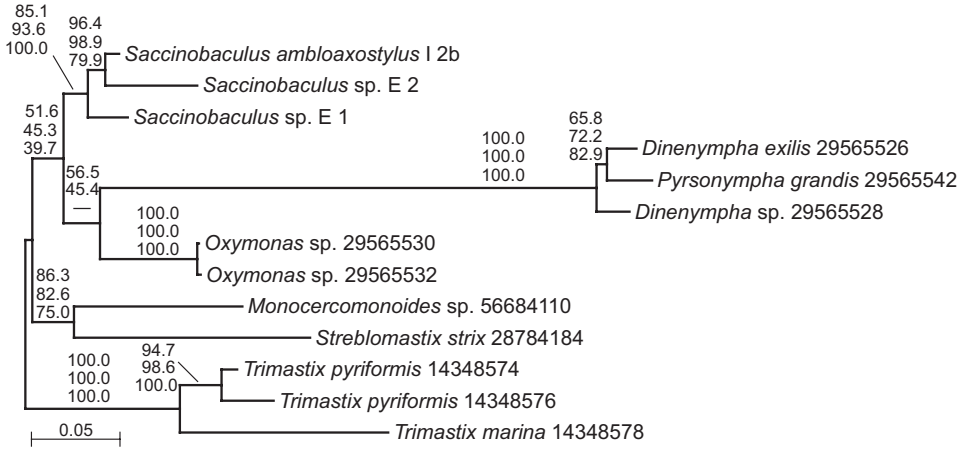

Figure 3. Phylogeny of oxymonad SSU rRNA using *Trimastix* as an outgroup. Tree generated as for Fig. 1. Numbers at nodes correspond to percentage of bootstrap support from ML trees generated with stepwise addition (top), ML trees generated with neighbour-joining starting trees (middle), and distance (bottom).

certain morphotypes of *S. ambloaxostylus* to the new genus *Notila* on the basis of granules embedded in the axostyle, and of differences in the timing of meiosis (Cleveland 1950c). We are unable to determine where *Notila* fits in our phylogeny, nor whether its recognition would make *Saccinobaculus* paraphyletic. It bears mentioning that the differences between the sexual cycles of *Saccinobaculus*, *Notila*, and *Oxymonas* (Cleveland 1950a—c) appear to be attributable to heterochrony.

Our data therefore suggest that, while *Saccinobaculus* is restricted in distribution to the gut of *Cryptocercus*, it is both abundant and diverse in that environment. Indeed, our data are most consistent with the original suggestion that most oxymonads in *Cryptocercus* belong to the genus *Saccinobaculus*. No data clearly attributable to *M. globus* was present in our survey, but this is perhaps not surprising since the sequenced *Monocercomonoides* SSU rRNA is 2900 bp long, about 800 bp longer than *Saccinobaculus*, and might therefore be selected against in environmental PCR.

The molecular tree of oxymonads, while still poorly resolved, is nevertheless beginning to shed some light on the evolution of the some characters in the group. The relationships between oxymonad families have primarily been hypothesized based on characteristics of the axostyle and on putative homologies between attachment organelles, specifically the rostellum unique to the family Oxymonadidae and the microfibrillar holdfast seen in several families. The rostellum is thought to be a highly derived trait that arose after the development of the holdfast (Moriya et al. 2003). The holdfast, on the other hand, is reported in the Pyrsonymphidae, *Streblomastix*, and some members of the Oxymonadidae (Brugerolle and König 1997). Assuming that all oxymonad holdfasts are homologous led Moriya et al. (2003) to propose a single acquisition, and to place the Polymastigidae alone at the base of the oxymonad tree, a view also held by Simpson et al. (2002). However, our molecular analysis, as well as that of others (Hampl et al. 2005), suggest that the presence of the holdfast is not a reliable character trait; indeed, there is no dispute that it has been lost in the pyrsonymphid *Dinenympha*. Resolving this issue will depend on acquiring additional molecular and morphological data, and in particular on characterizing the taxonomic diversity of additional genes.

Another feature of the molecular tree of oxymonads is the resolution of *Monocercomonoides* and *Streblomastix* as a single clade, which has been noted before in SSU rRNA analyses (Hampl et al. 2005). This was an unexpected development: previous (morphologically based) analyses of the relationships between the oxymonad groups tended not to group these two organisms together (Moriya et al. 2003). However, after being treated to remove its surface bacteria, the morphology of *Streblomastix* resembles that of *Monocercomonoides* (Leander and Keeling 2004). In addition, Hampl et al. (2005) pointed out that both *Monocercomonoides* and *Streblomastix* have unusually large small-subunit rDNA genes. We can confirm that the *Saccinobaculus* gene, while also large for a eukaryote, is within the range of already-published *Oxymonas*, *Dinenympha*, and *Pyrsonympha* genes, making the increased size of the *Monocercomonoides* and *Streblomastix* SSU a potential synapomorphy for the group. Confirmation of this hypothesis, as well as further support for the grouping of the two families as a clade, will depend on the sequencing of the SSU rRNA genes from additional members of the Polymastigidae, the group to which *Monocercomonoides* belongs.

Methods

Sample acquisition, single cell isolation, and molecular methods: Specimens of the insect host *Cryptocercus punctulatus* were collected by C.A. Nalepa from Mountain Lake Biological Station, Giles Co., Virginia; Black Rock Mountain, Rabun Co., Georgia; and Bear Trap Gap, Haywood Co., North Carolina (Nalepa et al. 2002). Gut contents of one cockroach (from Mountain Lake Biological Station) were obtained by massaging fluid out of the insect's abdomen and suspending it in Trager's medium U (Trager 1934), and whole gut DNA was extracted from this suspension using the DNEasy kit (QIAGen). For single-cell isolations, gut contents of several cockroaches (from Black Rock Mountain and Bear Trap Gap) were obtained either as above or by dissecting the cockroach and suspending the gut contents in either Trager's medium U or 0.4% sodium chloride. Cells were isolated and DNA was extracted as described (Keeling and Leander 2003).

Oxymonad SSU rRNA was amplified from environmental samples using an oxymonad-specific forward primer 5′-GCCACTACTCATATGCTTGTCTC-3′ and a eukaryote-specific reverse primer 5′-AACCTTGTTACGACTTCTCCTTCC-3′ with an annealing temperature of 52° and an

extension time of 2 min. SSU rRNA was amplified from manually isolated cells using eukaryote-specific primers 5′-GCGCTACCTGGTTGATCC-TGCC-3′ and 5′-TGATCCTTCTGCAGGTTCACC-TAC-3′ with an annealing temperature of 45°C and an extension time of 1.5 min. In all cases products of approximately 2100 bp were obtained, which were cloned using TOPO TA cloning (Invitrogen), and several clones were sequenced on both strands.

The environmental samples yielded 14 sequences, here called E1—E14. The single-cell isolates yielded a total of 12 sequences: one sequence (here called I1) from 40 large pigmented cells of *Saccinobaculus doroaxostylus* from the Black Rock Mountain population; two sequences each from isolates of 27 (I2a and I2b), 10 (I3a and I3b), approximately 12 (I4a and I4b), and four (I5a and I5b) cells of *Saccinobaculus ambloaxostylus*, all taken from the Bear Trap Gap population; and three sequences (I6a, I6b, and I6c) from eight cells of *Saccinobaculus ambloaxostylus* taken from the Black Rock Mountain population. New sequences were deposited in GenBank as accessions DQ525690—DQ525715.

Phylogenetic analyses: We generated an alignment of the small subunit ribosomal RNA (SSU rRNA) from inspection of secondary structure of a number of eukaryotes for which such data are available. To this, we included SSU sequences from selected other taxa, all previously published oxymonad sequences, and our experimentally generated sequences (alignment available upon request). To ensure that all new sequences were derived from oxymonads, this alignment included 83 taxa representing all major groups of eukaryotes, including the host insect *Cryptocercus* and parabasalian relatives of other symbionts known to be present. Global analysis of eukaryotes and two oxymonad-specific analyses were performed using 1089 positions and 83 taxa. Oxymonad-specific analyses allowed the use of 1479 alignable sites: one including 50 taxa with all publicly available full-length oxymonad and *Trimastix* sequences, and a second including 13 taxa (two to three representatives of each of the six groups, except for *Monocercomonoides*, for which only one sequence was available).

We generated trees from these data sets using PHYML 2.4.4 (Guindon and Gascuel 2003) using the GTR substitution model (except for the 83-taxon data set, for which we used the HKY model). Site-to-site rate variation was modelled on a gamma distribution with 8 variable rate categories and invariable sites, with the shape parameter alpha and proportion of invariable sites estimated form the data. Alpha was estimated at 0.538101, 0.378790, and 0.282216 for the 83-, 50-, and 13-taxon data sets, respectively, and proportions of invariants were calculated to be zero for the first two and 0.067 for the last. We also carried out Bayesian analyses on the two smaller data sets using MrBayes 3.0B4 (Huelsenbeck and Ronquist 2001) with these same parameters. A total 1,000,000 generations with one cold and three hot chains were sampled every 1,000 generations; the first 33 and 2 trees, respectively, from the 50- and 13-taxon data sets were discarded as burn-in, and consensus trees were generated from the remainder. The branch lengths of the consensus topology were calculated by maximum likelihood using PHYML. Bootstrap resampling of the 83-taxon data set was based on 100 replicates using both maximum likelihood and the BioNJ distance methods provided by PAUP* 4.0b10 (Swofford 2002). We analysed 1000 bootstrap replicates of both the 50- and 13-taxon datasets with both maximum likelihood (using PHYML) and neighbour-joining (using the BioNJ method as implemented by PAUP*). Additionally, we calculated 1000 bootstrap replicates for the 13-taxon data set using maximum likelihood with stepwise addition (using PAUP*).

The relations between the various groups of oxymonads were investigated using Approximately Unbiased (AU) tests (Shimodaira 2002). Eleven taxa were selected such that each group of oxymonads was represented by two taxa, except for *Monocercomonoides* (for which, again, only a single sequence is known); these were constrained to provide six groups. All 105 possible topologies for these six groups were generated using PAUP*, and site likelihoods for each calculated using TREE-PUZZLE 5.1 (Schmidt et al. 2002) with the parameters described above. AU tests were performed using CONSEL 1.19 (Shimodaira and Hasegawa 2001). Parallel tests were also carried out with the nine-taxon data set obtained by excluding the divergent pyrsonymphid group.

Light microscopy: Cockroaches were dissected and protist fauna suspended in Trager's medium U as described for DNA isolation. Live cells were observed by Nomarski differential interference contrast microscopy using a Zeiss Axioplan 2 microscope with 63 × and 100 × Plan-Apochromat lenses. Digital images were taken using a QImaging MicroImager II monochrome camera. Behaviour was observed on live cells soon after dissection, while some structural features were observed on cells once they had

ceased to move, so that structural features could be observed more clearly.

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