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Evidence from SSU rRNA Phylogeny that *Octomitus* is a Sister Lineage to *Giardia*

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Octomitus intestinalis **is a diplomonad flagellate inhabiting the digestive tract of rodents and amphibians.** ***Octomitus*** **is of evolutionary interest because, based on ultrastructural characteristics, it is thought to be closely related to the morphologically derived genus** ***Giardia*****, and together they have been proposed to make up the Giardiinae. In molecular trees of diplomonads,** ***Giardia*** **is the deepest branching lineage, so identifying a sister group to** ***Giardia*** **that is less derived would be informative.** ***Octomitus*** **is a logical candidate for this position, but unfortunately there are no molecular data from it, and it is not available in culture. To determine the position of** ***Octomitus*****, and specifically test whether it is more closely related to** ***Giardia*** **than other diplomonads, we have isolated it directly from the caecum of wild mice and characterized its small subunit ribosomal RNA (SSU rRNA) gene. Phylogenetic analysis showed** ***Octomitus*** **to be the sister to** ***Giardia*** **with strong support, together occupying one side of the deepest split in the diplomonad tree.**

Key words: diplomonad; *Giardia*; *Octomitus*; phylogeny; SSU rRNA.

Introduction

Diplomonads are a group of flagellated protists that are mostly found in anaerobic or low oxygen environments. The majority of diplomonads that have been characterized are parasites or commensals of animals, largely in the digestive tract (Brugerolle 2000). Parasitic diplomonads are responsible for a number of medically and commercially important diseases, most notably *Giardia* and *Spironucleus*. In addition to their importance as pathogens, diplomonads have attracted attention because of their evolutionary history and their unique cell biology. For many years they were considered a candidate for the earliest lineage of eukaryotes, in part due to their apparent lack of otherwise typical eukaryotic organelles such as the mitochondrion (Cavalier-Smith 1983), and in part because molecular phylogenies of several genes placed them at the base of the eukaryotic tree (Hashimoto et al. 1994, 1995; Sogin et al. 1989). It is now known that *Giardia*, and probably all diplomonads, possesses a relic mitochondrion (Tovar et al. 2003) and the phylogenetic evidence putting them basal to other eukaryotes has been called into question (Embley

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and Hirt 1998). Currently they are considered to be excavate eukaryotes, a group united in part by molecular phylogenies and in part by ultrastructural evidence (Simpson 2003). Within the excavates, there is clear evidence from both molecular phylogeny and ultrastructure for a close relationship between diplomonads and retortamonads (Brugerolle 1977; Silberman et al. 2002), and molecular data suggest that both are sisters to the enigmatic genus, *Carpediemonas* (Simpson et al. 2002).

The relationships among diplomonads have also been studied extensively to better understand the evolution of the many unique morphological and molecular characteristics, and life strategies found in the group. The evolution of parasitism in diplomonads is of particular interest because there are many parasitic forms and a only a few free-living ones known, leading to debate as to whether parasitism evolved many times independently or if free-living species are derived from parasites (Siddall et al. 1992, 1993). Several morphological traits linked to parasitism are of particular interest in this debate because of their complexity, for example the presence or absence of a cytostome or cytopharynx associated with the recurrent flagella, the reduction or loss of which is associated with parasitic forms. However, the most obvious morphological trait within the group is the pairing of karyomastigont systems found in most diplomonads. The karyomastigont is the nucleus associated with (usually) four basal bodies and several conserved cytoskeletal elements (Brugerolle 1975; Vickerman 1990). With the exception of enteromonads, these structures have been symmetrically duplicated in all diplomonads along with associated morphological features, like the cytostome. Taking these features and other morphological characters into account, early schemes of diplomonad phylogeny converged on a view where the enteromonads were basal to those with paired karyomastigonts, and the subsequent evolutionary trend was from free-living to progressively parasitic (i.e., *Trepomonas* diverged first, followed by *Hexamita*, *Spironucleus*, *Octomitus*, and finally *Giardia*) (Brugerolle 1975; Siddall et al. 1992). Of all diplomonads, *Giardia* is the most highly adapted to its parasitic mode of life, having a unique karyomastigont formation and a complex suction cup-like organelle for attachment to the epithelium.

The view that diplomonad evolution followed a progression from free-living-to-parasitic was challenged by molecular data in two ways. First, the most reliable molecular phylogenies that included a diversity of diplomonads conflicted with the intuitive and cladistic schemes: specifically, the unrooted topology of the most robust molecular trees were essentially identical to morphology-based trees, but the root was such that *Giardia* was the first branch of diplomonads rather than being at the tip of the tree (Cavalier-Smith and Chao 1996; Keeling and Doolittle 1997; Rozario et al. 1996). Additional compelling molecular evidence also came from the distribution of a very rare non-canonical genetic code. Nearly all life uses the same genetic code, but diplomonad nuclear genomes were found to use a slight variant where canonical stop codons TAA and TAG encode glutamine (Keeling and Doolittle 1996). This trait is only found in a handful of genomes, and within diplomonads was shown to be present in *Spironucleus*, *Hexamita*, and *Trepomonas*, but not in *Giardia* (Keeling and Doolittle 1997). This distribution is very difficult to explain unless the molecular trees placing *Giardia* at the base of diplomonads are correct.

The molecular-based tree of diplomonads leaves us in a difficult position with respect to character evolution in the early history of diplomonads, because one side of the deepest divide, *Giardia*, is the single diplomonad lineage most highly adapted to parasitism and, based on the nature of the outgroup retortamonads, non-representative of the group as a whole. To make matters more confusing, molecular data from the asymmetrical enteromonads have recently shown that they are not basal to symmetrical diplomonads as expected, but instead branch within the *Hexamita*/*Spironucleus*/*Trepomonas* clade (Kolisko et al. 2005). This suggests that these species most likely reverted to a asymmetrical form by a "halving" of the cell — a rare case of true reversion in cell morphology (Kolisko et al. 2005). Overall, this situation is similar to that found in the parabasalian flagellates, where the most basal group in molecular trees is the highly adapted and very complex hypermastigotes, making it hard to reconstruct specific details about the ancestral state of the group (Dacks and Redfield 1998; Keeling et al. 1998). In the diplomonads, however, there is a potential source of ancestral state information in the genus *Octomitus*. Based on morphology (Brugerolle et al. 1974), *Octomitus* is thought to be closely related to *Giardia* because both lack a cytostome and the intracytoplasmic portion of the posterior flagella are within the cytoplasm rather than being bound by a membrane. In the intuitive and cladistic trees, *Octomitus* is accordingly sister to *Giardia* at the tip of the

tree (Brugerolle 1975; Siddall et al. 1992). These characteristics, together with the general correspondence between the unrooted morphological and molecular trees, suggest that *Octomitus* branches near the base of the diplomonad tree, but on which side of the root? *Octomitus* is far less derived and adapted to parasitism than is *Giardia*, so if they are sisters *Octomitus* is a more informative representative when evaluating ancestral states of diplomonad characters. Conversely, if *Octomitus* falls on the other side of the root, then its similarities with *Giardia* can be inferred to be ancestral. Either way, the position of *Octomitus* can help mitigate difficulties in interpreting the highly derived state of *Giardia*. To this end, we have isolated *Octomitus intestinalis* from mouse and sequenced its SSU rRNA gene, the molecule with the best sampling of close outgroups and currently best sampled and most robust molecule for diplomonad phylogeny.

Results and Discussion

Identification of *Octomitus intestinalis* in Mouse

The protist fauna of the intestinal track of wild mice was inspected by light microscopy and found to comprise the diplomonad *O. intestinalis* and two parabasalids *Tritrichomonas muris* and *T. minuta*. The gut content of a mouse containing about 30% of *O. intestinalis* was selected. Two sample were collected: one was preserved for molecular analysis in 50% ethanol, the second was fixed for light and electron microscopy. By light microscopy *O. intestinalis* was identified by the shape of the two anterior nuclei and the axial position of the two recurrent flagella that arise at the posterior end on each side of a terminal spike (Fig. 1 A). This primary identification was confirmed by electron microscopy which revealed out the cytological features of the genus (Brugerolle et al. 1974). The two anterior nuclei are invaginated, the pocket containing the basal bodies of

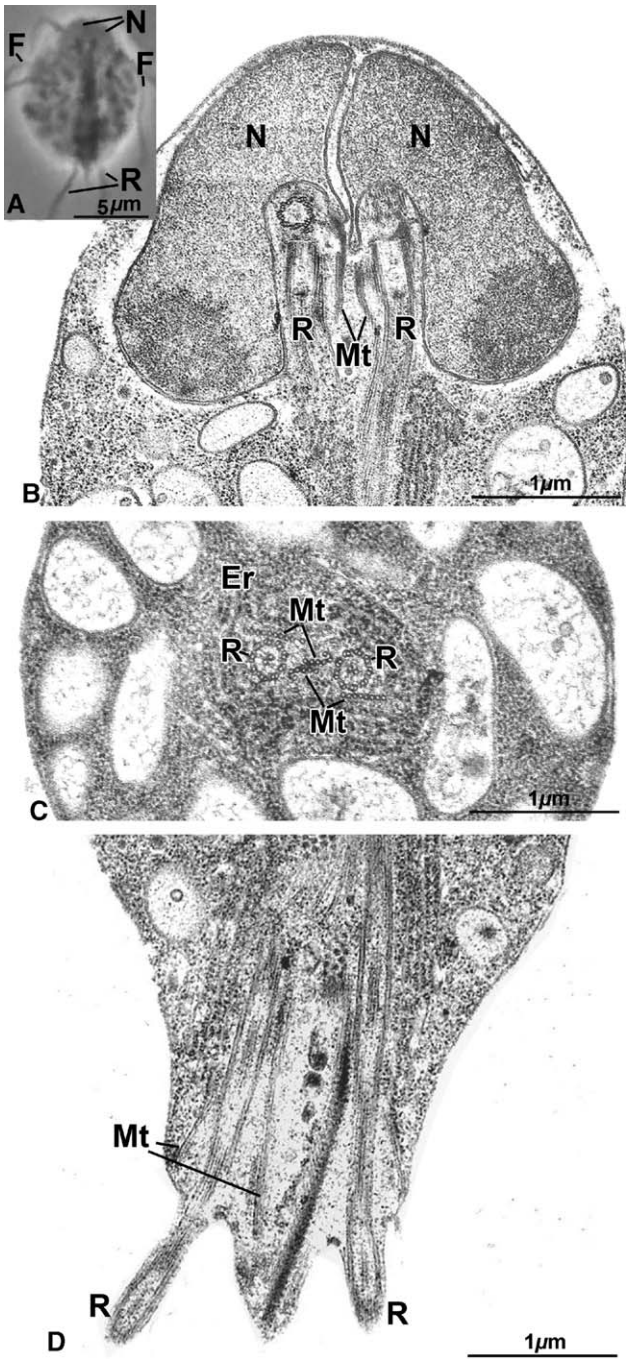

Figure 1. Morphology and relevant ultrastructural characteristics of *Octomitus*. **A:** Light microscopy view showing overall morphology for identification, including two nuclei (N) associated with two axial recurrent flagella (R) and anterior flagella (F). **B:** Longitudinal section showing the two anterior nuclei (N) with associated basal bodies and recurrent flagella (R) emerging from nuclear concavities. **C:** Transverse section showing the two axonemes of the recurrent flagella (R) within the cytoplasm accompanied by two microtubular fibres (Mt) and endoplasmic reticulum (Er). **D:** Longitudinal section showing recurrent flagella (R) emerging posteriorly in the absence of a cytostome, in contrast to *Hexamita* or *Spironucleus*.

the flagella of which two are oriented backward and lined by microtubular fibres (Fig. 1 B). A median transverse section shows the intracytoplasmic axonemes of the two recurrent flagella accompanied by two microtubular fibres and surrounded by rough endoplasmic reticulum (Fig. 1 C). A longitudinal section at the posterior end shows the two recurrent flagella arising from the recurrent axonemes. The accompanying microtubular fibres do not support any posterior cytostomal openings (Fig. 1 D). Intracytoplasmic axonemes of the recurrent flagella and accompanying microtubular fibres resemble those of the caudal flagella in *Giardia* (Brugerolle 1975).

Characterization of SSU rRNA and Phylogenetic Position of *Octomitus*

Amplification using eukaryote-specific SSU rRNA primers yielded a single band, which was cloned and the sequence of six clones determined to encode nearly identical SSU rRNA genes (heterogeneity was observed at only six positions). The amplified product (not including primers) was 1511 bp in length, in close agreement with the size from other diplomonads and parabasalids (e.g., *G. intestinalis* at 1406 bp and *T. vaginalis* at 1533 bp, for the corresponding region), but smaller than the average for a eukaryotic SSU rRNA.

The sequence of one clone was selected as representative and added to an alignment with representatives of eukaryotic diversity. Global eukaryotic phylogenetic analysis showed the sequence to branch with diplomonads with 100% bootstrap support (not shown). Retortamonads and *Carpediemonas* were found to be the closest relatives of diplomonads, as has been described previously (Silberman et al. 2002; Simpson et al. 2002), so detailed analyses were carried out using *Octomitus*, the 13 other available full-length diplomonad SSU rRNA sequences, and Retortamonads and *Carpediemonas* outgroups. Overall, the SSU rRNA tree of diplomonads is well supported statistically, with most major nodes at 100% bootstrap support by both ML and distance methods (Fig. 2) and posterior probabilities from Bayesian analyses at 0.99 or 1.00 (not shown). The relatively low support for the diplomonads as a whole was due to the retortamonads

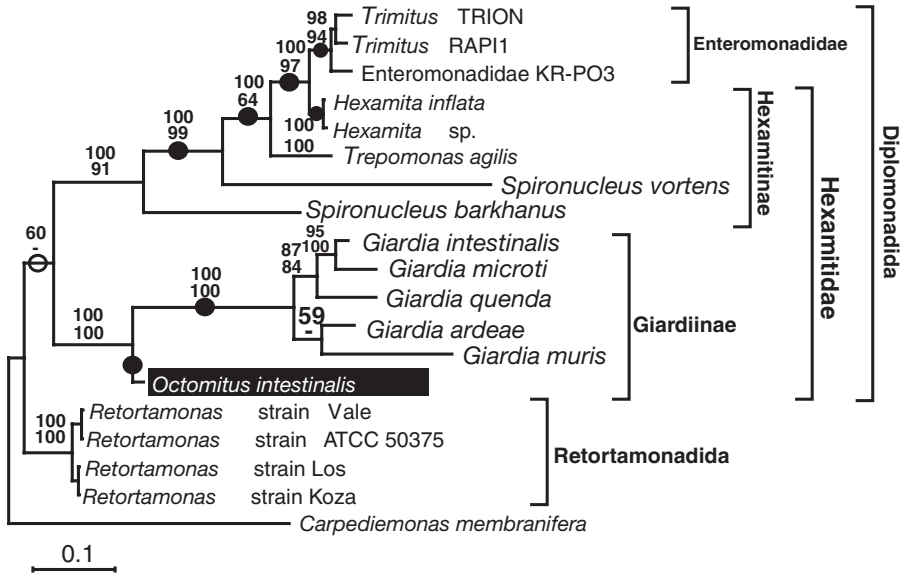

Figure 2. Maximum-likelihood phylogeny of diplomonad SSU rRNA with *Retortamonas* and *Carpediemonas* outgroups. Numbers at nodes correspond to bootstrap support from ML (above) and distance (below). Bayesian and parsimony analyses were also done and gave the same tree with strong support for the position of *Octomitus* (not shown). The position of the outgroup (*Retortamonas* plus *Carpediemonas*) was tested at 9 positions by AU tests. The open circle was the favoured position (as recovered by phylogenies using all methods) and all 8 alternatives (filled circles) were rejected at the 1% confidence level. Taxonomic divisions discussed in the text are bracketed and named to the right according to Kulda and Nohynkova (1978).

sometimes branching at the base of the *Octomitus*/*Giardia* clade. This has been observed in analyses without *Octomitus*, and the inclusion of *Octomitus* reduced the support for this relationship, consistent with its interpretation as an artifact (Silberman et al. 2002). In all analyses, *Octomitus* fell within the diplomonad group and was specifically sister group to the genus *Giardia* (Fig. 2). This relationship was strongly supported by all methods, as was the reciprocal grouping of the hexamitid and enteromonad lineages, as has been demonstrated recently (Kolisko et al. 2005). This tree provides the first molecular evidence for the position of *Octomitus* and the monophyly of the Giardiinae. It is also worth noting that the SSU rRNA gene sequence from *Octomitus* is by far the least divergent of all known diplomonad sequences. One major obstacle in determining the evolutionary place of diplomonads has been the high level of sequence divergence in many of their genes drawing them to the base of the tree. One strategy to mitigate this is to analyse only sequences with low levels of divergence (e.g., Simpson et al. 2002), and *Octomitus* is a logical candidate to represent diplomonads when more data are available.

The Root of the Diplomonad Tree

Phylogenetic analyses show strong support for *Octomitus* being more closely related to *Giardia* than to hexamitid diplomonads, but the question really being asked here is the position of the root of diplomonads, which is often the hardest part of a tree to determine. Figure 2 shows the root between Giardiinae and Hexamitinae/Enteromonadidae. There is some support for this position from ultrastructure and also from the imperfectly known distribution of a non-canonical genetic code in all Hexamitinae represented on this tree. However, these data do not rule out the possibility that *Octomitus* branches on the other side of the root and that some of its characteristics (e.g., the lack of membrane surrounding intracytoplasmic recurrent axonemes) are ancestral to all diplomonads. To examine the position of the root more thoroughly, we used approximately unbiased (AU) tests to compare the root inferred from all phylogenetic methods (open circle in Fig. 2) with eight alternative positions at major nodes within diplomonads (closed circles). All eight alternatives were rejected within a confidence interval of 1%. Overall, the root of the diplomonad tree inferred from SSU rRNA phylogeny is contested by the phylogeny of some protein coding genes where artifacts have been demonstrated (Keeling and Doolittle 1997) and cladistic reconstructions based on morphology and ultrastructure (Brugerolle 1975; Siddall et al. 1992), but is supported by the phylogeny of SSU rRNA and GAPDH (Rozario et al. 1996), the distribution of the non-canonical genetic code (Keeling and Doolittle 1996, 1997), and the ultrastructural characters that unite Giardiinae (Brugerolle 1975; Brugerolle et al. 1974; Kulda and Nohynkova 1978), making this by far the most likely position for the root based on available evidence.

Concluding Remarks

Molecular phylogenies and other molecular data have suggested that *Giardia* diverged early from other diplomonads. This, compounded with the surprising discovery that enteromonads evolved relatively recently, probably by reversion to the single karyomastigont state, have made the early evolution of diplomonads more complex to resolve. In the cladistic analysis of Siddall et al. (1992), 9 out of 23 analysed character states change in the branch leading to *Giardia*, and many of these are ambiguous in the reconstructed ancestor of diplomonads given the molecular tree. Identifying *Octomitus* as sister genus to *Giardia* in SSU rRNA helps this situation to some extent because several characters that distinguish *Giardia* from all other diplomonads can now be polarized. For example, the position of basal bodies in deep nuclear pockets is shared between *Octomitus* and *Spironucleus* and may be ancestral. Conversely, characters specifically uniting *Giardia* and *Octomitus* are probably derived in their common ancestor; for example, the absence of a cytosome or membrane surrounding the intracytoplasmic recurrent flagella. Since molecular trees and those based on cladistic character analysis are virtually identical with the exception of the root, many of the character state inferences from earlier work (e.g., Brugerolle 1975; Siddall et al. 1992) are still very informative in the context of the molecular tree.

The pattern of many other characters in the diplomonad tree remains difficult to explain, in particular the overall pattern of parasitic versus free-living species. Molecular trees consistently show the free-living diplomonads (e.g., *Trepomonas*) emerging from a paraphyletic assemblage of parasitic forms. Moreover, the apparent sister relationship between enteromonads and *Hexamita* further complicates our attempts to reconstruct the history of parasitism in diplomonads since

Hexamita is sometimes free living, or at least can survive outside its host for extended periods. It is clear that the evolution of such characters is not so straightforward as they once appeared, and it is probable that our view of some of these will change once again when data from several other diplomonads of note are available. In particular, molecular data from *Caviomonas* would be of interest since it is structurally the simplest of all diplomonads (lacking even the basic tetrakont pattern of basal body distribution). *Caviomonas* is thought to be related to enteromonads (Brugerolle and Regnault 2001) and therefore morphologically simple by degeneration, but the surprising history of entermonads may open this to question. *Brugerolleia* is also of interest as it is proposed to be closely related to *Octomitus*, and hypothesized to perhaps branch between *Octomitus* and *Giardia* (Desser et al. 1993). Data from this genus could provide valuable information on the evolutionary path that resulted in the highly derived state of *Giardia*.

Methods

Isolation and microscopy of *Octomitus intestinalis:* Intestinal flagellates were collected from the caecum of *Mus musculus* trapped in nature. Mice were killed by chloroform, and the caecal content were collected in a small dish and covered with a PBS solution at 37 °C. After 30 min, the flagellates that had migrated into the liquid phase were aspirated with a micro-pipette and washed and concentrated by centrifugation. After examination under a phase contrast microscope, a first sample of cells was mixed with 50% ethanol for molecular analyses, and a second sample was fixed for light and electron microscopy. The cells were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7 for 1 h and after washing with the buffer they were post-fixed with 1% osmium tetroxide in the buffer for 1 h. Resin embedding, sectioning and staining of the sections were performed with the classic techniques for electron microscopy. Sections were observed under a JEOL 1200 EX electron microscope at 80 kV. The sample of fixed cells was also used to identify and to photograph the species of protozoa under a Leica DMRH microscope equipped with a Station Q-Fish Light.

Sequencing SSU rRNA: Intestinal contents were pelleted by centrifugation and resuspended in 100 μl of TE buffer. An equal volume of Tris-buffered phenol was added and the suspension was ground using a 1.5 ml disposable mortar and pestle with periodic vortexing. DNA was purified by two phenol extractions and one chloroform extraction, precipitated in isopropanol and washed in 95% ethanol at room temperature. SSU rRNA was amplified by PCR using primers GCGCTACCTGGTTGATCCTGCC and TGATCCTTCTGCAGGTTCACCTAC with an annealing temperature of 50 °C and an extension time of 1.5 min, resulting in a single product of approximately 1600 bp. This product was cloned and six individual colonies were picked and sequenced. Sequence of one clone used in the phylogeny has been deposited in GenBank as accession DQ366277.

Phylogenetic analyses: The new sequence was added to an existing alignment representing a broad range of eukaryotic SSU rRNA sequences (Keeling and Leander 2003) and a second alignment was constructing including all known diplomonad, retortamonad and *Carpediemonas* sequences (alignments available upon request). Global eukaryotic trees included 48 taxa and 987 alignable sites, while the diplomonad analysis included 19 taxa and 1015 alignable sites. Some nearly identical *Retortamonas* sequences were removed and a partial sequence from *Spironucleus muris* that includes ambiguous sites was also removed. Trees were inferred by maximum likelihood using PHYML 2.4.4 (Guindon and Gascuel 2003) using the GTR substitution model and rate between sites modeled on a gamma distribution with 8 variable rate categories and invariable sites, with the proportion of invariable sites and the alpha shape parameter estimated from the data (for global and diplomonad trees, alpha parameters were 0.725 and 0.692 respectively, whereas invariable sites were 0.186 and 0.125 respectively). Bayesian analyses was carried out using MrBayes 3.0B4 (Ronquist and Huelsenbeck 2003) with 4 variable rate categories and invariable sites estimated from the data. A total of 1,000,000 generations were run with three hot chains and 1 cold chain and sampling every 100,000 generations. Log likelihoods leveled after only 2 samples and a consensus tree was generated from the remainder. This tree was identical in topology to the ML tree. Distances were calculated using TREE-PUZZLE 5.2 (Schmidt et al. 2002) with the settings described for ML analysis and distance bootstraps calculating using the shell script puzzleboot (by M. Holder and A. Roger: www.tree-puzzle.de). Trees were inferred from distances using WEIGHBOR 1.2 (Bruno et al. 2000).

Alternate positions for the root of the diplomonad subtree were tested using AU tests. The ML tree and 9 alternative trees were combined with 100 ML bootstrap trees and site likelihoods calculated by TREE-PUZZLE 5.1 using the -wsl option with the parameters used for the ML tree. Site likelihoods were converted to PAML format using the python script Puzz2Lnf (courtesy of J. Leigh, Dalhousie University) and AU tests performed using consel (Shimodaira and Hasegawa 2001).

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