

Incomplete Co-cladogenesis Between *Zootermopsis* Termites and Their Associated Protists

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Subject Editor: Andy Michel

Received 29 August 2017; Editorial decision 14 November 2017

Abstract

Coevolution is a major driver of speciation in many host-associated symbionts. In the termite-protist digestive symbiosis, the protists are vertically inherited by anal feeding among nest mates. Lower termites (all termite families except Termitidae) and their symbionts have broadly co-diversified over ~170 million yr. However, this inference is based mainly on the restricted distribution of certain protist genera to certain termite families. With the exception of one study, which demonstrated congruent phylogenies for the protist *Pseudotrichonympha* and its Rhinotermitidae hosts, coevolution in this symbiosis has not been investigated with molecular methods. Here we have characterized the hindgut symbiotic protists (Phylum Parabasalia) across the genus *Zootermopsis* (Archotermopsidae) using single cell isolation, molecular phylogenetics, and high-throughput amplicon sequencing. We report that the deepest divergence in the *Zootermopsis* phylogeny (*Zootermopsis laticeps* [Banks; Isoptera: Termopsidae]) corresponds with a divergence in three of the hindgut protist species. However, the crown *Zootermopsis* taxa (*Zootermopsis angusticollis* [Hagen; Isoptera: Termopsidae], *Z. nevadensis nevadensis* [Hagen; Isoptera: Termopsidae], and *Z. nevadensis nuttingi* [Haverty & Thorne; Isoptera: Termopsidae]) share the same protist species, with no evidence of co-speciation under our methods. We interpret this pattern as incomplete co-cladogenesis, though the possibility of symbiont exchange cannot be entirely ruled out. This is the first molecular evidence that identical communities of termite-associated protist species can inhabit multiple distinct host species.

Key words: coevolution, phylogenetics, protists, symbiosis, termite

Symbiotic mutualisms are ubiquitous in nature, and can dramatically impact the evolution of all species involved in such an association (Rosenberg and Rosenberg 2016). This is especially apparent with mutualisms between multicellular hosts and their microbial symbionts. A common prediction in the field of symbiology is that the divergence of a host should result in the reciprocal divergence of any vertically transmitted mutualistic symbionts (i.e., symbionts that are transmitted from parents to offspring), eventually leading to co-cladogenesis between the hosts and symbionts (Bright and Bulgheresi 2010). Co-cladogenesis has been observed as predicted in some symbiotic mutualisms, such as a variety of associations between insects and bacteria (e.g., Moran and Baumann 1994, Lo et al. 2003, Kikuchi et al. 2009, Kleinschmidt and Kölsch 2011), flatworms and bacteria (Gruber-Vodicka et al. 2011), plants and endophytic fungi (Clay and Schardl 2002), *Azolla* water ferns and cyanobacteria (Zheng et al. 1999), flagellated protists and endosymbiotic bacteria (Noda et al. 2007, Ikeda-Ohtsubo and Brune 2009), and lower termites and flagellated protists (Noda et al. 2007).

Despite theoretical expectations, co-cladogenesis is not a given evolutionary outcome. Incongruencies can arise between host and symbiont phylogenies because of occasional horizontal transmission

of mutualistic microbes among hosts or from environmental sources (Bright and Bulgheresi 2010). Co-cladogenesis is therefore not always apparent on species or genus levels, and congruent patterns often emerge only when examining taxa at deeper phylogenetic levels (Aanen et al. 2007, Arnold et al. 2010). Clearly, symbioses need to be examined on a case-by-case basis, to better understand the coevolutionary processes that occur between hosts and mutualistic symbionts.

The associations between lower termites (i.e., all termites that do not belong to the phylogenetically derived family Termitidae) and their gut microbes provide ideal systems with which to study coevolution and co-cladogenesis between hosts and mutualistic microbes. Lower termites are a diverse and globally widespread group of insects that consume recalcitrant plant materials as a source of carbohydrates. In order to digest cellulose, termites depend on a diverse assemblage of bacteria and archaea, as well as two groups of protists from the supergroup Excavata (parabasalids, which belong to the Phylum Parabasalia, and oxymonads, which belong to the Class Oxymonadea, Phylum Preaxostyla), that are present in termite hindguts (Ohkuma and Brune 2010). In addition to facilitating the digestion of cellulose, the microbial symbionts benefit their hosts in other ways, e.g., by suppressing spores of entomopathogenic fungi that are ingested by the

insects (Peterson and Scharf 2016). In turn, the termites provide food and suitable environments for the microbes to live and reproduce.

The association between the lower termites and protists is ancient, as the termites and protists have coevolved since the divergence between wood roaches (*Cryptocercus* [Scudder; Blattodea: Cryptocercidae]) and lower termites ~170 million yr (Bourguignon et al. 2015). The protists are transferred intergenerationally via procotodeal trophallaxis (i.e., the transmission of hindgut fluids containing the microbiota from a donor termite to a recipient; Nalepa et al. 2001). As expected for vertically transmitted microbes, there is evidence that the community assembly of protists within a termite hindgut depends on the termite host's phylogenetic placement. Early surveys of the gut microbiomes of termites, which were based solely on cell morphology, suggested that some closely related termite species harbor similar sets of protist species (Kirby 1934, 1937; Yamin 1979). However, molecular tools led to the discovery of higher than expected protist diversities in many termite guts, including numerous cryptic species (Stingl and Brune 2003, Harper et al. 2009, Tai et al. 2013). The discovery of the previously unknown diversity, combined with evidence of strict co-cladogenesis in many other vertically-inherited symbiotic associations (Nieberding and Olivieri 2007, de Vienne et al. 2013), led to the assumption that each individual species of lower termite contains its own unique assemblage of hindgut protists, while individual protist species are associated with only one termite species (Tai et al. 2015). In addition, it is generally assumed that protist communities are consistent across different populations of individual termite species (Kirby 1934). However, these paradigms have been largely untested.

Although vertical transmission is the primary means by which the gut fauna are transmitted, there is some evidence that horizontal transmission has occurred at least once in the evolutionary history of lower termites. Termites in the genus *Reticulitermes* (Holmgren; Isoptera: Rhinotermitidae) carry a symbiont assemblage that is dramatically different from the other termites in their family, Rhinotermitidae, suggesting that *Reticulitermes* may have replaced their gut communities via an ancient horizontal transmission event (Kitade and Matsumoto 1998, Kitade 2004, Tai et al. 2015). In addition, termites in the family Serritermitidae harbor a dramatically different flagellate community from their close relatives in the Rhinotermitidae, suggesting that the gut community in the Serritermitidae may have been shaped by horizontal transfer (Radek et al. 2017). However, it is unknown whether horizontal transfers of gut protists have occurred in lower termites outside of these examples. The distribution of protist taxa across termite taxa is broadly consistent with vertical transmission, e.g., the genera *Pseudotriconympha* (Grassi & Foá; Trichonymphida: Teranymphidae) and *Holomastigotoides* (Grassi & Foá; Trichonymphida: Teranymphidae) are restricted to the Rhinotermitidae, while the protist families Calonymphidae and Devescovichidae are restricted to the Kalotermitidae (Yamin 1979). Furthermore, an 18S ribosomal RNA amplicon survey of hindgut communities across lower termites and *Cryptocercus* revealed that the communities are structured by host phylogeny, not biogeography (Tai et al. 2015). Therefore, despite the theoretical possibility of horizontal transmission, vertical inheritance is much more important to the evolution of protist communities (Yamin 1979, Kitade 2004, Rahman et al. 2015, Tai et al. 2015).

Termites in the genus *Zootermopsis* (Emerson; Isoptera: Termopsidae) provide a tractable system with which to test for co-cladogenesis. This genus contains only three species, each of which is present only in western North America: *Zootermopsis angusticollis* (Hagen; Isoptera: Termopsidae), *Zootermopsis nevadensis* (Hagen; Isoptera: Termopsidae), and *Zootermopsis laticeps* (Banks; Isoptera: Termopsidae) (Thorne et al. 1993). *Z. nevadensis* is subdivided into two subspecies (*Z. nevadensis nevadensis* and *Z. nevadensis nuttingi*)

based on differences in cuticular hydrocarbons (Haverty et al. 1988) as well as aggressive behavior exhibited between members of the two subspecies (Haverty and Thorne 1989). *Z. angusticollis* and *Z. nevadensis nuttingi* have nearly identical ranges along the west coast of the United States (Fig. 1), while *Z. nevadensis nevadensis* has a range that is further inland and partially overlaps with the ranges of *Z. angusticollis* and *Z. nevadensis nuttingi* (Thorne et al. 1993). *Z. laticeps* is geographically isolated from the other species, with a range in central and southern Arizona (Thorne et al. 1993).

Currently, *Z. angusticollis* is the only species within the genus *Zootermopsis* with a well-documented protist community based on both morphological and molecular data, although all four taxa have been investigated by morphological methods (Yamin 1979). Tai et al. (2013) identified seven species of parabasalids associated with *Z. angusticollis* in Vancouver, British Columbia, based on morphology and molecular phylogenetics: *Trichomitopsis minor* (Tai & Keeling; Trichomonadida: Trichomonadidae), *Trichomitopsis parvus* (Tai & Keeling; Trichomonadida: Trichomonadidae), *Trichomitopsis termopsidis* (Cleveland; Trichomonadida: Trichomonadidae), *Trichonympha campanula* (Kofoed & Swezy; Trichonymphida: Trichonymphidae), *Trichonympha collaris* (Kirby; Trichonymphida: Trichonymphidae), *Trichonympha postcylindrica* (Tai & Keeling; Trichonymphida: Trichonymphidae), and *Trichonympha sphaerica* (Kofoed & Swezy; Trichonymphida: Trichonymphidae). Two additional parabasalid species, *Hexamastix termopsidis* (Kirby; Honigbergiellida: Honigbergiellidae) and *Tricercomitus termopsidis* (Kirby; Honigbergiella: Tricercomitidae), and an oxymonad species, *Streblomastix strix* (Kofoed & Swezy; Oxymonadida: Streblomastigidae), are also known to inhabit this termite (Kofoed and Swezy 1919, Kirby 1930). The same four species of *Trichonympha* were also found in *Z. nevadensis* (most likely *Z. nevadensis nevadensis* based on the collection locations of the termite, Mount Pinos in the Los Padres National Forest, California, and the Chilao Flats Campground in the Angeles National Forest, California; Ikeda-Ohtsubo and Brune 2009, Zheng et al. 2015). However, the other protists in the *Z. nevadensis nevadensis* community were not investigated, and to date no other *Zootermopsis* taxa have been examined using molecular methods.

In this study, we characterized the community of parabasalid protists associated with termites in the genus *Zootermopsis*. We used microscopy, single cell isolation, and Sanger sequencing to identify the species of *Trichomitopsis* and *Trichonympha* present in the guts of each *Zootermopsis* species. In addition, we used high-throughput amplicon sequencing to determine the overall composition of the parabasalid communities, and to test for the presence of cryptic species that were potentially missed using Sanger sequencing. Finally, we compared the phylogeny of the termites with those of the associated protists to test the hypothesis that vertical transmission of obligate protist symbionts should have resulted in co-cladogenesis.

Materials and Methods

Termite and Parabasalid Collections

Z. angusticollis and *Z. nevadensis nuttingi* individuals were obtained from colonies maintained by one of the authors (J.R.L.) at Arizona State University. The *Z. angusticollis* colonies were originally collected in Monterey, California, on 24 July 2016, and San Bernardino, California, on 31 January 2009 (Fig. 1), while the *Z. nevadensis nuttingi* colonies were originally collected in Monterey on 27 May 2014. *Z. nevadensis nevadensis* individuals were collected from the rotting log of a ponderosa pine (*Pinus ponderosa* [Douglas; Pinaceae]) in El Dorado National Forest in California on 18 November 2016.

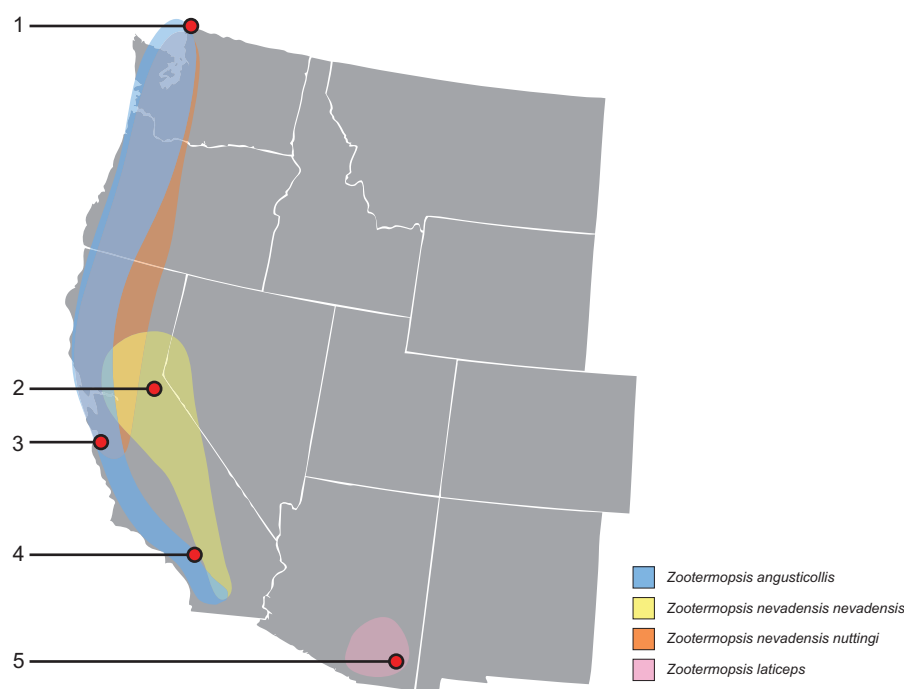


Fig. 1. Approximate ranges of the four *Zootermopsis* taxa based on Thorne et al. (1993). Red dots indicate the locations from which the samples were collected for the study by Tai et al. (2013): 1 – Vancouver; and the present study: 2 – El Dorado National Forest, 3 – Monterey, 4 – San Bernardino, 5 – Gray Hawk Nature Center.

Z. laticeps individuals were collected from dead branches of live Fremont's cottonwoods (*Populus fremontii* [Watson; Malpighiales: Salicaceae]) along the San Pedro river in the Gray Hawk Nature Center in Arizona on 22 September 2016. Termite identities were confirmed based on morphology and cuticular hydrocarbon profiles which were assessed using gas chromatography (except for *Z. laticeps*, which was identified based on morphology). Cuticular hydrocarbons from individual termite workers were extracted with hexane of which 1 μ l was injected into an Agilent 6890N GC (Agilent, Santa Clara, CA) coupled with an Agilent 5975 mass selective detector, operated in splitless injection mode with helium as carrier gas at 1 ml min⁻¹ flow rate. The GC was fitted with a 30-m $\times$ 0.25-mm (ID) $\times$ 0.1- μ m DB-1MS non-polar column (Agilent). The oven temperature was programmed to rise from 60 to 200°C at 40°C min⁻¹ after an initial delay of 2 min including a splitless time of 0.5 min. Subsequently the temperature rose from 200 to 320°C at 5°C min⁻¹. Injector temperature was 260°C, MS quad 150°C, MS source 230°C, and transfer line 300°C. Compound identification was based on fragmentation patterns, retention time and comparison with previously identified compounds in the *Zootermopsis* species and subspecies (Haverty et al. 1988, Haverty and Thorne 1989). Numbers of termites processed for this study are summarized in Supplementary Table S1.

The hindguts were removed from live termites, placed in 50 μ l Ringer's solution (8.5 g NaCl, 0.20 g KCl, 0.20 g CaCl₂, NaHCO₃ per liter, HiMedia Laboratories), and crushed to form mixtures, or 'slurries'. Parabasalid cells from the slurries were visualized at 200 $\times$ magnification using a Zeiss Axio Vert A1 inverted microscope (Zeiss). Individual *Trichonympha* and *Trichomitopsis* cells were collected and manipulated using glass capillaries. After washing individual cells once or twice in Ringer's solution, the cells were photographed and placed in individual 500 μ l microcentrifuge tubes. The single cells, the remaining hindgut slurries, and the termite thoraxes were stored at -20°C until DNA extractions.

Numbers of cells collected and identified in this study are summarized in Supplementary Table S1.

DNA Extraction, Amplification, and Sequencing of Single Cells and Insects

We extracted DNA from the single cells, hindgut slurries, and termite thoraxes using the Epicentre MasterPure DNA purification kit following the manufacturer's protocol (Epicentre, Madison, WI). We then amplified a near full-length fragment of the small subunit ribosomal RNA (SSU rRNA) of the single cells using the primers PF1 (Keeling 2002) and FAD4 (Deane et al. 1998). We used SSU because it is a reliable marker for distinguishing between parabasalid species (Čepička et al. 2017). Each reaction contained 15 μ l of EconoTaq PLUS GREEN 2 $\times$ Master Mix (Lucigen Corporation, Middleton, WI), 3 μ l of each primer, 7 μ l of dH₂O, and 2 μ l of template for a total reaction volume of 30 μ l. PCR reactions consisted of an initial denaturing step of 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 53°C for 15 s, and 72°C for 90 s, and ending with a step of 72°C for 7 min. A nested PCR was then conducted on the PCR product from the initial amplification using the primers GGF and GGR (Gile et al. 2011). The nested PCR followed the same amplification protocol as the initial PCR reaction.

PCR products from the nested reaction were run in 1% agarose gels, stained using GelGreen (Biotium, Fremont, CA), and visualized under a blue light. Visible bands of ~1,500 base pairs were cut from the gel, and DNA was extracted from the excised gel pieces using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Because individual parabasalid cells often contain multiple distinct SSU copies, the gel-extracted DNA was cloned using a TOPO TA cloning kit (Invitrogen, Paisley, UK). Between two and five clones per cell were sequenced on an Applied Biosystems 3730 capillary sequencer by the DNA Laboratory at Arizona State University. Sequences of the two most phylogenetically divergent clones from

a single cell of each parabasalid-host species combination (the sequences used to generate the *Trichonympha* and *Trichomitopsis* phylograms; see Results) were deposited in GenBank (accession numbers MF477204-MF477235).

For the termites, we amplified a fragment of the COI gene (using the primers LepF1 and LepR1; Hebert et al. 2004) and the COII gene (using the primers A-tLeu and B-tLys; Wirth et al. 1999). Reactions contained the same reagent volumes as the single cell reactions. PCR reactions consisted of an initial denaturing step of 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 43°C for 15 s, and 72°C for 90 s, and ending with a step of 72°C for 7 min. PCR products were sequenced directly on both strands as above. Sequences of each unique termite haplotype were deposited in GenBank (accession numbers MF477188-MF477203).

Phylogenetic Analyses—Parabasalids

We separated the parabasalid sequences into two datasets: one for species of *Trichonympha* and one for species of *Trichomitopsis*. For the *Trichonympha* dataset, we compared the sequences from *Zootermopsis* with eight previously published sequences of *Trichonympha* species (two sequences per *Trichonympha* species) isolated from *Z. angusticollis* in Vancouver (Tai et al. 2013), as well as ten sequences of *Trichonympha* species isolated from termite species in the genera *Hodotermopsis* (Holmgren; Isoptera: Termopsidae) and *Reticulitermes* (Ohkuma et al. 1998, 2000; Ikeda-Ohtsubo et al. 2007; Ikeda-Ohtsubo and Brune 2009, Supplementary Table S2). The *Trichonympha* sequences from these termite species form a sister clade to the *Trichonympha* present in *Zootermopsis* species (James et al. 2013b). In addition, the *Trichonympha* dataset contained three sequences of *Staurojoenina* (Grassi; Trichonymphida: Staurojoeninidae) species (Ohkuma et al. 2005, Gile et al. 2013) which were included as an outgroup. For the *Trichomitopsis* dataset, we compared the sequences from *Zootermopsis* with six previously published sequences of *Trichomitopsis* species (two per species), as well as five sequences of *Pseudotrypanosoma giganteum* (Grassi; Trichomonadida: Trichomonadidae) (Supplementary Table S2; Keeling et al. 1998). Both datasets were separately aligned using MAFFT v. 7 (Katoh and Standley 2013) and ambiguously aligned regions were removed by eye.

We conducted both maximum likelihood (ML) and Bayesian inference (BI) analyses to determine the phylogenetic relationships among the parabasalids within each dataset. ML analyses were conducted using RAxML v. 8.2.9 (Stamatakis 2014), while BI analyses were conducted using Mr. Bayes v. 3.2 (Ronquist et al. 2012). ML and BI analyses both followed a GTR+ Γ substitution model. Statistical support for the nodes in the best ML tree was generated using 1,000 bootstrap replicates. For the BI analyses, four Markov chain Monte Carlo chains were run with 5,000,000 generations. Posterior probabilities were then generated by sampling a tree every 100 generations. The program Tracer v. 1.6 (Rambaut et al. 2014) was used to estimate the burn-in value, and trees generated in the burn-in phase (the first 25% of trees in each dataset) were discarded.

After the individual cells were identified, mean p-distances were calculated using MEGA 7 (Kumar et al. 2015) to determine the diversity of SSU sequences within individual cells. In addition, p-distances were calculated to compare the diversities of sequences of individual parabasalid species within and among the host termite taxa.

Phylogenetic Analyses—Termites

We first analyzed the COI and COII regions separately to determine if the phylogenies of the two gene regions were congruent. We compared the COI dataset with five previously published

sequences of COI from *Z. nevadensis nuttingi* and *Z. angusticollis* (Booth et al. 2012), as well as a sequence of COI from *Archotermopsis wroughtoni* (Desneux; Isoptera: Termopsidae) (Legendre et al. 2008) which was included as an outgroup. In addition, we aligned the COII dataset with a sequence of COII from *Archotermopsis wroughtoni* (Legendre et al. 2008). The datasets were aligned using MAFFT. Phylogenetic analyses were conducted following the same protocol as with the parabasalid datasets. After determining that the two single gene phylogenies are congruent (data not shown), we conducted the above analyses on the concatenated dataset.

High-Throughput Sequencing and Bioinformatics Analyses

The protist hindgut communities of *Zootermopsis* taxa listed above were also investigated by high-throughput amplicon sequencing. The V4-V5 regions of the protist SSU genes were amplified using the primers ParaF and ParaR (Jasso-Selles et al. 2017). These primers yield an approximately 450 bp amplicon, depending on species, which is roughly 30% of the total SSU length. Amplicons were sequenced using 2 × 300 paired end chemistry on the Illumina MiSeq platform at MR DNA (Shallowater, TX).

We assembled the forward and reverse reads with Pandaseq (Masella et al. 2012) using a minimum overlap of 45 bp. The reconstructed sequences were then demultiplexed using QIIME v.1.9 (Caporaso et al. 2010), while barcodes and primers were trimmed with Cutadapt (Martin 2011). For each sample, the reads were dereplicated with vsearch v. 2.0.2 (Rognes et al. 2016) and clustered in operational taxonomical units (OTUs) with SWARM 2 (Mahé et al. 2015). SWARM builds clusters using a local clustering threshold (d) instead of an arbitrary global clustering threshold. Different clustering thresholds were tested for each sample with a range between $d = 1$ and $d = 15$, in order to find the most accurate characterization of the parabasalid community for each sample. Chimeras were then detected de novo with vsearch (Rognes et al. 2016).

Only OTUs that represented at least 0.5% of the total clean reads were considered for taxonomic assignment and phylogenetic analysis. Taxonomy was assigned using a custom parabasalid SSU sequence reference file using RDP Classifier 2.2 (Wang et al. 2007) as implemented in QIIME 1.9. Unclassified OTUs were manually blasted against the NCBI nucleotide database (GenBank). The most abundant sequences (OTU representatives) from each cluster (OTU) were aligned with all the Sanger sequences obtained in this study, as well as sequences from closely related species that were available on GenBank (Hampl et al. 2004, Čepička et al. 2010, James et al. 2013a, Smejkalová et al. 2014), using MAFFT (Katoh and Standley 2013). The ends of the alignments were trimmed manually using MEGA. Based on the taxon assignments, four different phylogenetic trees were built: one for *Trichonympha* OTUs, one for *Trichomitopsis*, one for *Hexamastix*, and one for *Tricercomitus*. ML trees were constructed for each dataset in RAXML, using the GTRGAMMA model, with statistical support for nodes given by percentage of 1,000 total bootstrap replicates.

Results

Molecular Diversity and Phylogeny of *Zootermopsis*

Cuticular hydrocarbon analyses matched previously published hydrocarbon profiles for each species and subspecies tested (Supplementary Fig. S1, Supplementary Table S3; Haverty et al. 1988, Haverty and Thorne 1989). Based on phylogenetic analyses the *Zootermopsis* individuals that we collected branched where expected (Fig. 2; Broughton

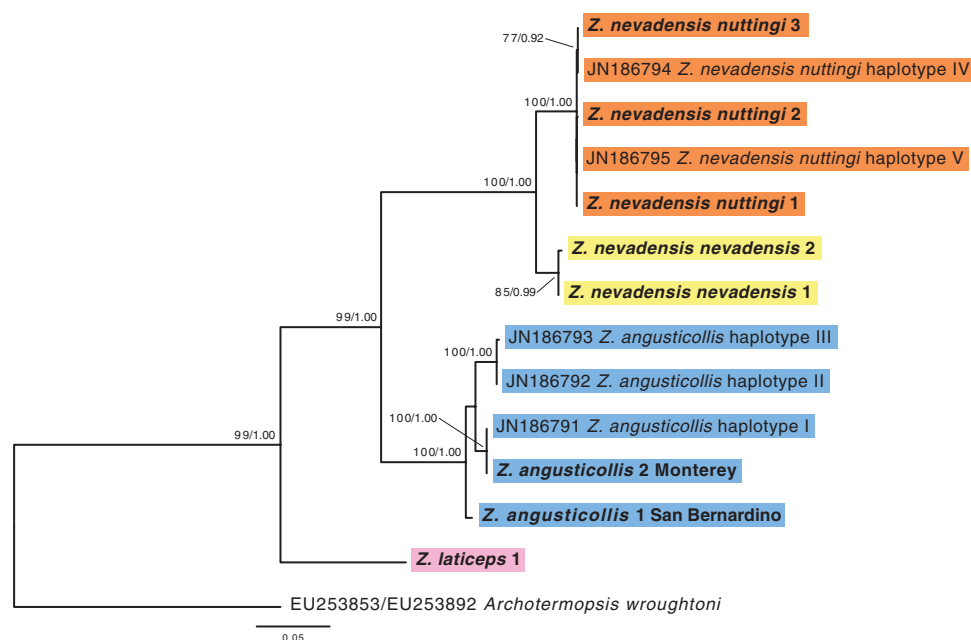


Fig. 2. Maximum Likelihood (ML) phylogram of *Zootermopsis* termites based on concatenated sequences of COI and COII. *Archotermopsis wroughtoni* is included as an outgroup. Bolded taxon labels represent sequences that were generated in this study. Genbank accession numbers are included for previously published sequences. Values at nodes represent ML bootstrap values and Bayesian Inference (BI) posterior probabilities. *Zootermopsis* taxa are color coded as for Fig. 1. The final, trimmed, concatenated alignment of the *Zootermopsis* COI and COII genes had 1,326 sites of which 358 were variable.

and Kistner 1991, Thorne et al. 1993), supporting their identification based on morphology, cuticular hydrocarbon profiles, and collection location. *Z. nevadensis nevadensis* and *Z. nevadensis nuttingi* branched together with strong support, while *Z. angusticollis* was closely related (with strong support) to the *Z. nevadensis* subspecies. *Z. laticeps* formed the deepest branch in the *Zootermopsis* phylogeny. These nodes were also well supported in the individual COI and COII phylogenies, although the ML support was lower in the COI phylogeny relative to the COII and concatenated phylogenies.

Hindgut Protist Community of *Zootermopsis*

The symbionts associated with *Z. angusticollis* collected in Monterey and San Bernardino closely resembled those described from the *Z. angusticollis* collected in Vancouver (Tai et al. 2013). Based on morphology, Sanger sequencing of single cells and high-throughput amplicon sequencing of whole termite hindguts (Figs. 3 and 4; Supplementary Figs. S1 and S2), we detected all four described *Trichonympha* species and all three described *Trichomitopsis* species. The only exception was the Monterey population lacked *T. campanula* according to both single cell isolation and high-throughput amplicon sequencing data. In addition, we observed *Tricercomitus termopsidis* and *Hexamastix termopsidis* in both populations of *Z. angusticollis*. Altogether this indicates a nearly identical symbiont community between the three geographically distinct collection locations of *Z. angusticollis*.

We additionally inspected the hindgut communities of both subspecies of *Z. nevadensis*, *Z. nevadensis nuttingi* and *Z. nevadensis nevadensis*. For *Z. nevadensis nuttingi* and *Z. nevadensis nevadensis*, we observed the same range of *Trichonympha* and *Trichomitopsis* morphotypes as for *Z. angusticollis* (Figs. 3 and 4), with the exception of *T. termopsidis* in *Z. nevadensis nuttingi*, which was present in termites from San Bernardino but not from Monterey. We examined the guts of three separately collected colonies of *Z. nevadensis nuttingi* from the same area using high-throughput sequencing to test whether the missing *T. termopsidis* cells were a colony-level or population-level discrepancy. The parabasalid species was absent from

all three colonies, suggesting that *T. termopsidis* was absent from the Monterey population of *Z. nevadensis nuttingi*.

Surprisingly, sequences obtained from single isolated cells of each parabasalid morphotype in the two *Z. nevadensis* subspecies were nearly identical to those from their counterparts in *Z. angusticollis* (Figs. 3 and 4), strongly suggesting that the same parabasalid species are present across these taxa. However, we also observed significant variability among SSU sequences from each parabasalid species (Fig. 5), even retrieving distinct sequences from a single isolated cell (Figs. 3 and 4). This is consistent with previous reports of intraspecific and even intragenomic SSU sequence variation in parabasalids, though some of this variation is likely due to amplification errors (Saldarriaga et al. 2011, Tai et al. 2013).

In order to determine whether the intraspecific SSU sequence variability was equal within and across hosts (indicating the same species present in each host) or greater across hosts than within hosts (suggesting incipient cladogenesis), we computed uncorrected pairwise distances (p-distances) for all clones sequenced in this study (Fig. 5; Supplementary Table S1). The p-distances, which correspond to the number of nucleotide differences in a pairwise comparison divided by the total number of nucleotides (in our case, a p-distance of 0.003 corresponds to five nucleotides difference), ranged from 0 to 0.041 within individual cells and from 0 to 0.053 among cells of a given species from a single host. The greatest sequence variability (i.e., highest observed within-cell and within-species p-distances) was observed in *T. collaris* followed by *T. sphaerica* (Fig. 5; Supplementary Table S1). The other parabasalid species showed similar lower levels of sequence variability. Meanwhile, the p-distances measured from a single protist species across host species ranged from only 0 to 0.058, nearly identical to the maximum within-host p-distances. We accordingly conclude that the same *Trichonympha* and *Trichomitopsis* species are present in *Z. angusticollis* and both subspecies of *Z. nevadensis*.

The symbionts present in *Z. laticeps* differed from those present in the other *Zootermopsis* species and subspecies (hereafter referred to collectively as the ‘west coast taxa’) based on molecular data

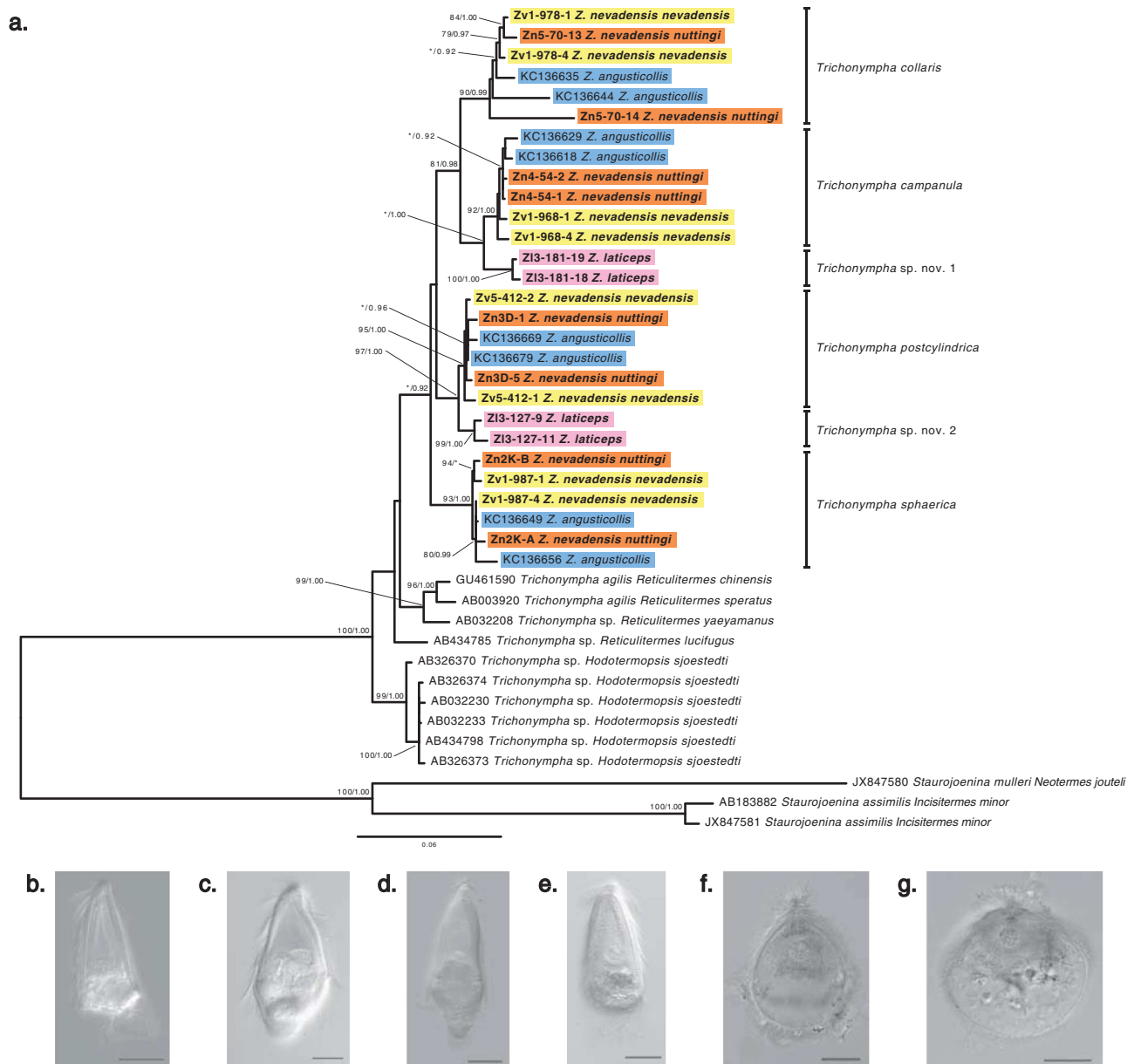


Fig. 3. (a) ML phylogram of *Trichonympha* species based on near full-length SSU rRNA gene sequences from individually isolated cells. Of the sequences obtained in this study, only two clones from one cell of each parabasalid species-host species combination were included in this tree (shown in bold). The sequences are color coded according to host species, following the color scheme in Fig. 1. *Staurojoenina* sequences were included as an outgroup. Values at nodes represent ML bootstrap values (when greater than 75) and BI posterior probabilities (when greater than 0.9). The final, trimmed alignment had 41 taxa and 1,492 sites (635 were variable). (b–g) Differential interference contrast micrographs of *Trichonympha* cells collected from *Z. nevadensis nevadensis* (b–e) and *Z. laticeps* (f–g): (b) *T. campanula*, (c) *T. collaris*, (d) *T. postcylindrica*, (e) *T. sphaerica*, (f) *Trichonympha* sp. 1, (g) *Trichonympha* sp. 2. Scale bars represent 50 μ m.

(Figs. 3 and 4). Previous morphology-based studies of the *Z. laticeps* hindgut community identified one species of *Trichonympha* (*T. campanula*) and one species of *Trichomitopsis* (*T. termopsidis*) along with *Tricercomitus termopsidis* and *Hexamastix laticeps* (Kirby 1930, 1931, 1932). We found two species of *Trichonympha* and one species of *Trichomitopsis*, none of which belonged to the previously identified species according to molecular data. These distinct species are referred to here as *Trichonympha* sp. 1, which branches sister to *T. campanula*, *Trichonympha* sp. 2, sister to *T. postcylindrica* (Fig. 3), and *Trichomitopsis* sp. 1, sister to *T. parvus* (Fig. 4). The observed species had comparably high intraspecific SSU sequence diversity to the *Trichonympha* and *Trichomitopsis* species from the west coast taxa (Supplementary Table S1). None of the parabasalid symbionts of the west coast taxa were found in *Z. laticeps*.

High-Throughput SSU rRNA Gene Amplicon Sequencing

The *Zootermopsis* protist symbiont identities revealed by deep amplicon sequencing were consistent with the Sanger sequences from isolated cells (Supplementary Figs. S2 and S3). The most abundant sequence (OTU representative) from each sequence cluster (OTU) matched the sequenced clones from all four species of *Trichonympha* and all three species of *Trichomitopsis* in *Z. angusticollis* and *Z. nevadensis*, and no additional distinct sequence types were found. Also consistent with the single cell data, we found no OTUs matching *T. campanula* in *Z. angusticollis* collected from Monterey, nor did we find any OTUs matching *T. termopsidis* in *Z. nevadensis nuttingi*. In addition, we recovered OTUs matching

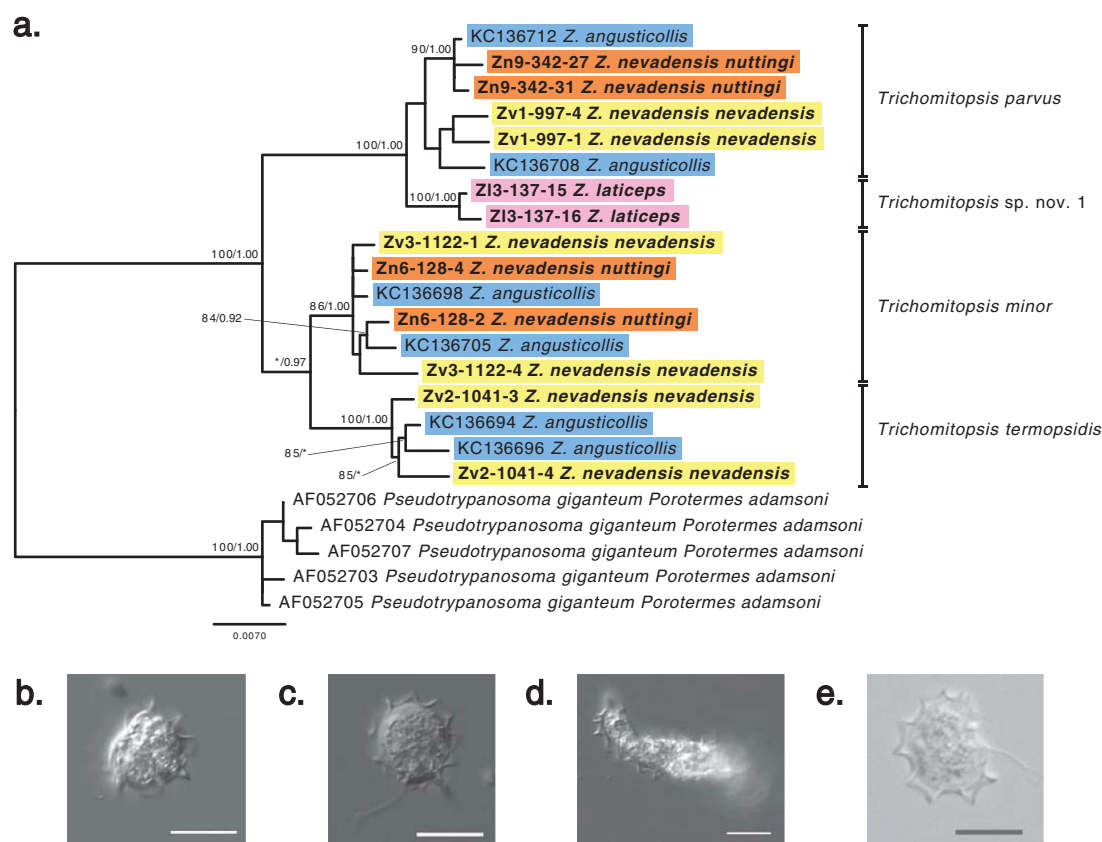


Fig. 4. (a) ML phylogram of *Trichomitopsis* species based on near full-length SSU rRNA gene sequences from individually isolated cells. The tree was generated and labeled following the same criteria as Fig. 3, except that sequences of *Pseudotrypanosoma giganteum* were included as an outgroup. The final trimmed alignment had 23 taxa and 1,483 sites (160 were variable). (b–e) Differential interference contrast micrographs of cells collected from *Z. nevadensis nevadensis* (b–d) and *Z. laticeps* (e): (b) *Trichomitopsis minor*, (c) *Trichomitopsis parvus*, (d) *Trichomitopsis termopsidis*, (e) *Trichomitopsis* sp. 1. Scale bars represent 20 μm .

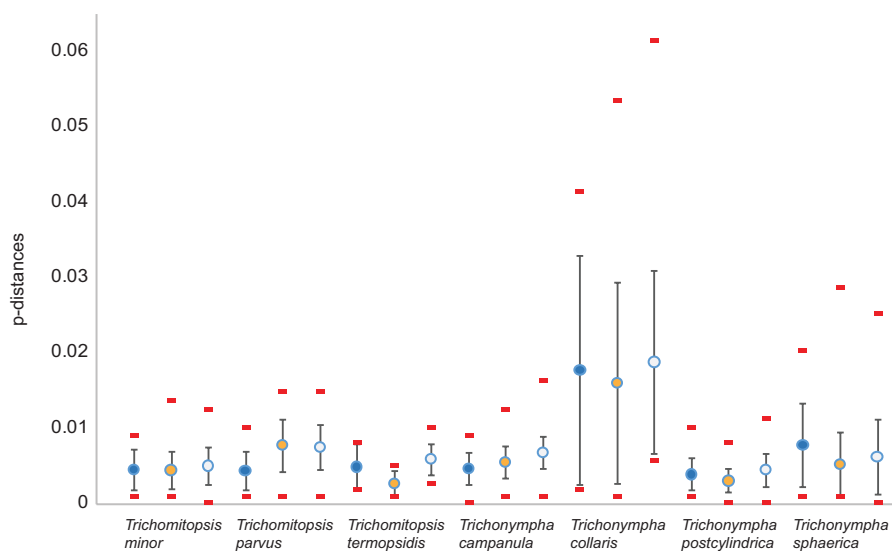


Fig. 5. Intraspecific and intragenomic SSU rRNA gene sequence variability in parabasalid symbionts of *Zootermopsis* according to uncorrected pairwise distances (p-distances). Minimum and maximum p-distances in each category are given by red dashes. Means and standard deviations are given by circles and error bars, respectively. For each parabasalid species, circle color indicates category of pairwise comparison: blue represents p-distances between clones from the same cells, orange represents p-distances between clones from different cells within the same host species, and white represents p-distances between clones from different host species.

clones of *Trichonympha* sp. 1 and 2 as well as *Trichomitopsis* sp. 1 from *Z. laticeps*.

In addition to the OTUs from *Trichonympha* and *Trichomitopsis*, which all matched isolated cell clones, our amplicon sequencing data

revealed two additional parabasalid OTUs. One of the OTU representatives branches with *Simplicimonas* (Trichomonadida) and the other branches with *Cthulhu*, *Cthylla*, *Hexamastix*, and related unidentified termite symbionts (Honigbergiellida). Although we did

not attempt to isolate the very small single cells of *Tricercomitus* or *Hexamastix*, it is reasonable to conclude that the Honigbergiellida-related sequence belongs to the *Hexamastix* morphotype, and that the *Simplicimonas*-related sequence belongs to the *Tricercomitus* morphotype (pictured in Fig. 6). *Z. angusticollis* and both subspecies of *Z. nevadensis* all contained *Hexamastix termopsidis*, as well as a species designated here as *Tricercomitus termopsidis* var. 1, while *Z. angusticollis* and *Z. nevadensis nevadensis* also contained a distinct sequence type designated as *Tricercomitus termopsidis* var.

3. *Z. laticeps* contained *Hexamastix laticeps* and its own distinct sequence type of *Tricercomitus*, here designated as *Tricercomitus termopsidis* var. 2 (Fig. 6).

Discussion

There was partial support for the hypothesis that divergence in *Zootermopsis* termites resulted in subsequent divergence of their gut symbionts, as *Z. laticeps* contained parabasalid species that

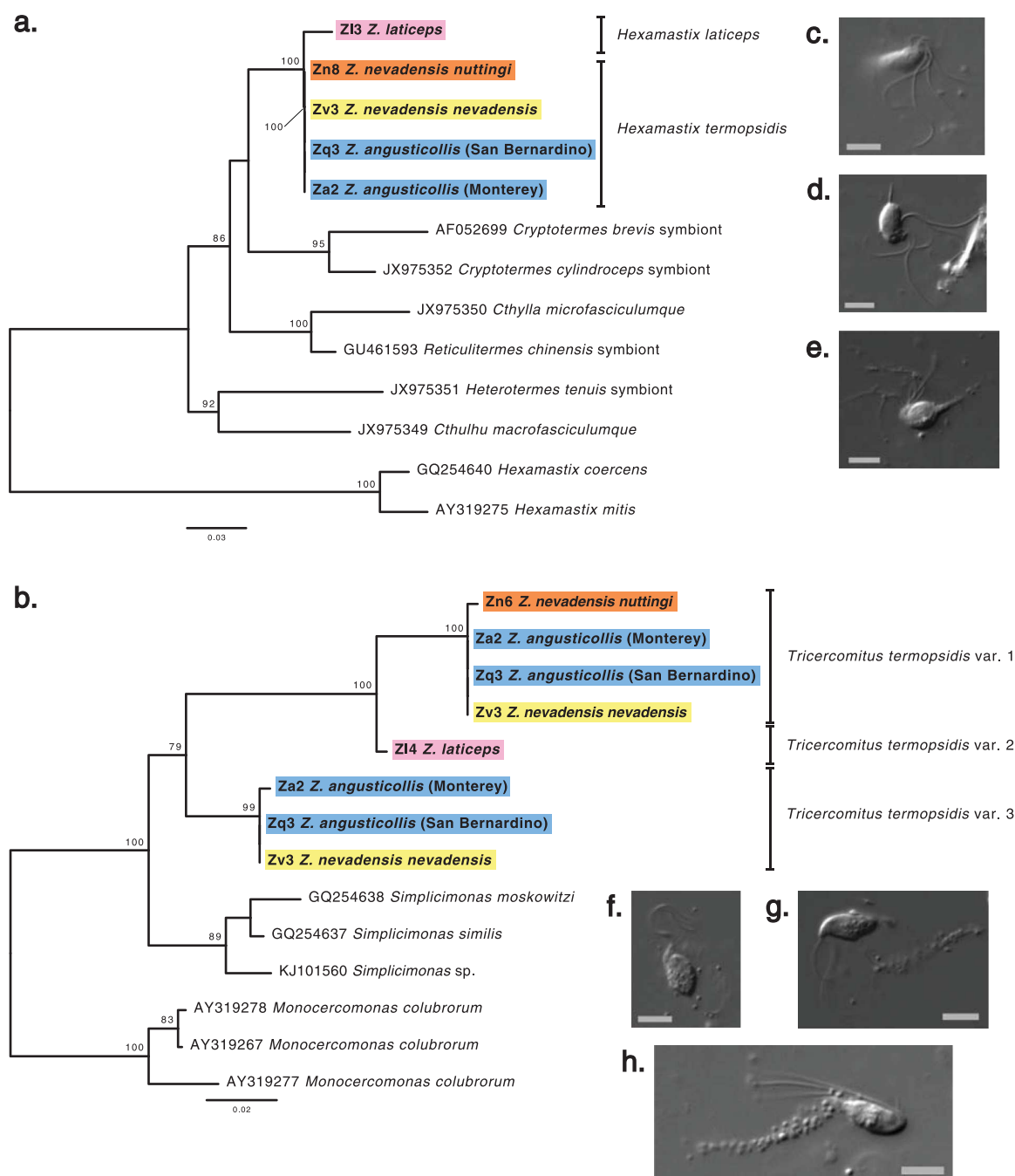


Fig. 6. Phylogenetic positions of additional parabasalid OTU representatives from *Zootermopsis* species. (a) ML phylogram of Honigbergiellida-related SSU rRNA gene amplicon sequences, likely derived from *Hexamastix termopsidis* and *Hexamastix laticeps*. Only one OTU representative for each parabasalid species was included per host taxon/population. Values at nodes represent ML bootstrap values. (b) ML phylogram of *Simplicimonas*-related SSU rRNA gene amplicon sequences, likely derived from *Tricercomitus* spp., generated and labeled the same as (a). (c–e) Differential interference contrast images of *Hexamastix termopsidis* cells from *Z. nevadensis nevadensis*. (f–g) Differential interference contrast images of *Tricercomitus termopsidis* cells from *Z. nevadensis nevadensis*.

were genetically distinct from (although closely related to) the protists present in the west coast taxa. The divergence between *Z. laticeps* and the west coast taxa (most likely as a result of vicariance) is considered to be the most ancient divergence event within the genus (Thorne et al. 1993). This hypothesis was supported by the basal placement of *Z. laticeps* in our termite COI and COII phylogenetic tree. However, there was no evidence of divergence among the parabasalids found in the west coast taxa, as all hosts contained the same parabasalid species in nearly identical communities (except that *Z. angusticollis* from Monterey was missing *T. campanula* and *Z. nevadensis nevadensis* was missing *T. termopsidis*). For this reason, we are describing the observed pattern as incomplete co-cladogenesis.

The lack of divergence among the parabasalids associated with the west coast taxa was surprising, as there is clear genetic differentiation between *Z. angusticollis* and *Z. nevadensis*, as well as between *Z. nevadensis nevadensis* and *Z. nevadensis nuttingi*. Broughton and Kistner (1991) estimated that *Z. laticeps* diverged from the west coast taxa 13 million years ago (mya), *Z. angusticollis* diverged from *Z. nevadensis* 9 mya, and the two *Z. nevadensis* subspecies diverged 2 mya. These estimates should be taken with caution as they were based on a strict molecular clock generated from DNA hybridization experiments, and the clock was not calibrated based on geological events or fossil records. However, even if the estimates are incorrect, it is likely that the divergence events occurred several million years ago, considering that the genus *Zootermopsis* is likely quite ancient (the common ancestor of *Zootermopsis* and the closely related genus *Microhodotermes* [Sjöstedt; Isoptera: Hodotermitidae] is estimated to have diverged ~90 mya, CI ~70 to 110 mya; Bourguignon et al. 2015). In addition, the species and subspecies are believed to have diverged in part because of the formation of geological barriers and changes in the distributions of their host species due to repeated glaciation events (Thorne et al. 1993), suggesting that sufficient time should have passed for the associated parabasalids to have diverged.

Horizontal transmission among the west coast taxa might explain their identical symbiont species. The ranges of *Z. angusticollis* and *Z. nevadensis nuttingi* almost entirely overlap (Thorne et al. 1993), and the two species have been observed co-occurring within the same log (J. R. Liebig, unpublished data). Symbiont transmission might occur if workers or soldiers from the two species eat each other during antagonistic interactions (Haverty and Thorne 1989, Thorne 1990). Hybridization might also allow symbiont transmission although it has not been demonstrated that *Z. angusticollis* and *Z. nevadensis nuttingi* can form hybrids. Similarly, the northern and southern edges of the *Z. nevadensis nevadensis* range overlap with the ranges of *Z. angusticollis* and *Z. nevadensis nuttingi*, and in this case it has been demonstrated that the subspecies can hybridize (although the gene flow is primarily from *Z. nevadensis nuttingi* to *Z. nevadensis nevadensis*; Aldrich and Kambhampati 2009). The tendency of *Zootermopsis* termites to outcross via colony fusion (Aldrich and Kambhampati 2007, Howard et al. 2013) could also help to maintain homogeneity of hindgut communities across species and subspecies, in a manner analogous to gene flow. However, it is unclear whether any of these processes could be efficient enough to maintain identical species composition across the hosts' continent-scale ranges, especially because there tends to be little gene flow between *Zootermopsis* populations (Booth et al. 2012). In addition, the slightly different symbiont communities maintained by sympatric populations of *Z. nevadensis nuttingi* and *Z. angusticollis* in Monterey (i.e., *T. campanula* was absent in *Z. angusticollis*, while *T. termopsidis* was absent in *Z. nevadensis nuttingi*) provides evidence against frequent horizontal transfer of symbionts.

A more plausible explanation is that there has been insufficient time for the parabasalids to speciate since the divergence of their host species. It has been widely assumed that parabasalids associated with termites evolve rapidly, in large part because of the high intraspecific and even intragenomic diversity of rRNA sequences (Saldarriaga et al. 2011, Tai et al. 2013). Rapid evolution has been linked to rapid diversification and speciation in plants (e.g., Barraclough and Savolainen 2001) and animals (e.g., Lanfear et al. 2010), and we expected a similar pattern for the protists. However, very little is known about genome evolution in parabasalids, and it may be that a large amount of standing sequence diversity in a population of parabasalids or within an individual cell does not correlate with rapid evolution. Furthermore, morphological evolution in parabasalids is known to be quite slow. For example, the genus *Trichonympha* is older than termites themselves, as it is present in *Cryptocercus* and most lower termite families (Kirby 1930, Yamin 1979).

Additional support for the slower evolution view is that the phylogenetic branch lengths separating the *Z. laticeps* symbionts from those in the other *Zootermopsis* species were not very long, indicating little sequence divergence relative to the divergence between *Trichonympha* from *Zootermopsis* and *Reticulitermes* (Fig. 3), or between *Trichomyces* and its sister genus *Pseudotrypanosoma* (Fig. 4). Considered from this perspective, the evolutionary divergence of the four *Zootermopsis* *Trichonympha* species would have occurred in the stem lineage of extant *Zootermopsis*, over the tens of millions of years since it diverged from *Hodotermopsis*. Note that the *Trichonympha* species associated with *Reticulitermes* are most likely descended from *Trichonympha* species associated with *Hodotermopsis*, via lateral symbiont transfer; *Hodotermopsis* is a close relative of *Zootermopsis* (Kitade 2004, Bourguignon et al. 2015). Losses of *T. collaris* and *T. sphaerica* would therefore have occurred in the ancestor of *Z. laticeps* (Fig. 3), just as we observed losses of *T. campanula* and *T. termopsidis* in the Monterey populations of *Z. angusticollis* and *Z. nevadensis nuttingi*, respectively.

It should also be noted in this context that mitochondrial COI and COII genes (as used for the *Zootermopsis* phylogenetic analysis) typically evolve much faster than nuclear SSU genes (as used for parabasalid phylogenies). Perhaps a faster-evolving gene in the parabasalids would reveal some sequence divergence to mirror that of the hosts. The SSU rRNA gene in parabasalids evolves rapidly enough that molecular phylogenies have successfully delimited all recently described parabasalids from distinct host species (e.g., Harper et al. 2009; Gile et al. 2011, 2013) so its lack of divergence here is nevertheless surprising.

Despite the paradigm of co-cladogenesis between termites and their gut symbionts (Lo and Eggleton 2011, Cruaud and Rasplus 2016), there have been surprisingly few demonstrations of clear co-cladogenesis between lower termites and associated protists. The only published example of congruent termite and protist phylogenies was for select termite species in the family Rhinotermitidae and their *Pseudotrichonympha* symbionts (Noda et al. 2007). However, in that study the *Pseudotrichonympha* species were isolated from termite species that were distantly related. For example, although the termite species *Coptotermes testaceus* (Linnaeus; Isoptera: Rhinotermitidae) and *Coptotermes formosanus* (Shiraki; Isoptera: Rhinotermitidae) are members of the same genus, the species are separated by a divergence event that occurred ~22 mya, CI ~18 to 25 mya (Bourguignon et al. 2016), and no *Pseudotrichonympha* from any termite species that diverged between *C. testaceus* and *C. formosanus* were included in the analyses. It is therefore unclear whether the entire Rhinotermitidae phylogeny is congruent with the phylogeny of associated *Pseudotrichonympha*, or whether the co-cladogenesis observed

by Noda et al. (2007) can only be observed with sufficiently long divergence times between termite species. Indeed, until the current study, no surveys had been conducted on parabasalid associates within an entire termite genus to test for coevolutionary patterns at the species level. Additional surveys across other lower termite genera are required to test whether our findings are typical for lower termite-parabasalid associations, or if *Zootermopsis* is an exception.

An interesting finding from this study was that some of the *Zootermopsis* species and populations appeared to have lost parabasalid species (*T. campanula* from *Z. angusticollis* in Monterey, and *T. termopsidis* in *Z. nevadensis nuttingi*). As the missing symbionts were consistent within colonies (and in the case of *Z. nevadensis nuttingi*, across geographically close colonies), it is likely that the termite reproductives that founded the colonies or populations lacked the symbionts. This is the first report of absent parabasalids species in *Zootermopsis* termites. However, the absence of certain protist species from termite individuals, colonies and populations has been reported in various lower termite species in Asia (Kitade and Matsumoto 1993, Kitade et al. 2012, 2013). In these studies, the authors thoroughly examined the protist community assemblages of different termite colonies of *Reticulitermes* species (Kitade and Matsumoto 1993), *Hodotermopsis sjoestedti* (Holmgren; Isoptera: Termopsidae) (Kitade et al. 2012) and *Coptotermes formosanus* (Kitade et al. 2013) across the termite species' ranges. With each host, the authors observed instances where one or two protist species were absent, either from individuals within a colony, or from the entire colony. However, protist species that were reported as missing may have simply been overlooked, as the identifications in each study were based solely on morphology. Our study confirms the absence of protist species using molecular identification, including high-throughput sequencing of entire hindguts.

Supplementary Material

Supplementary data are available at *Environmental Entomology* online.

Acknowledgments

We would like to thank Mikaela Garcia, Daniel Jasso-Selles, Trevor Merrell, and Katalina Freeman for assistance with cell isolations and molecular work. In addition, we would like to thank Kevin Haight for maintaining the termite colonies at Arizona State University and helping with termite collection. We would also like to thank Barbara Thorne, Michael Haverly, and Bill Donahue for termite collection advice. Finally, we are grateful to Sandy Anderson for allowing us to collect *Z. laticeps* on the Gray Hawk Nature Center and the administrators of the Pebble Beach Company for permission to collect termites in their forest. This work was supported by the College of Liberal Arts and Sciences and the School of Life Sciences, Arizona State University (startup grant to GHG) and by the National Science Foundation (Award #1644328).

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